Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction

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Abstract

An important goal in cardiology is to minimize myocardial necrosis and to support a discrete but resilient scar formation after myocardial infarction (MI). Macrophages are a type of cells that influence cardiac remodelling during MI. Therefore, the goal of the present study was to investigate their transcriptional profile and to identify the type of activation during scar tissue formation. Ligature of the left anterior descending coronary artery was performed in mice. Macrophages were isolated from infarcted tissue using magnetic cell sorting after 5 days. The total RNA of macrophages was subjected to microarray analysis and compared with RNA from MI and LV-control. mRNA abundance of relevant targets was validated by quantitative real-time PCR 2, 5 and 10 days after MI (qRT-PCR). Immunohistochemistry was performed to localize activation type-specific proteins. The genome scan revealed 68 targets predominantly expressed by macrophages after MI. Among these targets, an increased mRNA abundance of genes, involved in both the classically (tumour necrosis factor α, interleukin 6, interleukin 1β) and the alternatively (arginase 1 and 2, mannose receptor C type 1, chitinase 3-like 3) activated phenotype of macrophages, was found 5 days after MI. This observation was confirmed by qRT-PCR. Using immunohistochemistry, we confirmed that tumour necrosis factor α, representing the classical activation, is strongly transcribed early after ligature (2 days). It was decreased after 5 and 10 days. Five days after MI, we found a fundamental change towards alternative activation of macrophages with up-regulation of arginase 1. Our results demonstrate that macrophages are differentially activated during different phases of scar tissue formation after MI. During the early inflammatory phase, macrophages are predominantly classically activated, whereas their phenotype changes during the important transition from inflammation to scar tissue formation into an alternatively activated type.

Keywords: myocardial infarction • macrophages • tissue remodelling • scar formation

Introduction

Myocardial infarction (MI) and the resulting complications, due to cardiac remodelling and scar formation, are a major cause of death and morbidity in the Western countries [1]. Therefore, influencing myocardial remodelling, resulting in the reduction of necrosis and the advancement of discrete but resilient scar formation, might be a major therapeutic strategy [2].

The mechanisms of post-infarction remodelling have been studied extensively [3, 4]. It is well understood that with the beginning of the infarction a strong inflammatory response is initiated, which later ceases until the infarction area is remodelled into a thin and largely acellular collagenous scar. Although the mechanisms of the onset of inflammation have already been elucidated, little is known on how this inflammatory response is primarily maintained and, later on, terminated [5].

In myocardial infarction as well as in other inflammatory processes, macrophages play a pivotal role. Their main functions include phagocytosis of cellular debris from the site of myocardial damage, paracrine stimulation by secretion of cytokines and the reorganization of the tissue matrix by production of metalloproteinases (MMPs) and their inhibitors (TIMPs) [6, 7].

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Macrophages are known to induce a profound inflammatory response, depending on their distinct activation type [8]. The 'classical' (inflammatory) activation of macrophages, which comprises the production of nitric oxide (NO) and the induction of the pro-inflammatory cytokines interleukin 1 β (IL1β), interleukin 6 (IL6) and tumour necrosis factor α (TNFα), results in a strong inflammatory reaction in the host tissue. However, a non-inflammatory expressional phenotype has also been described for macrophages [9]. This 'alternatively' activated phenotype features the induction of arginase 1 and 2 (ARG1, 2) instead of inducible nitric oxide synthase (iNOS). Arginases compete with iNOS and deplete arginine stores of macrophages and produce polyamines and proline instead of NO, products especially important for cell differentiation and collagen production, respectively [10]. Hence, classically activated macrophages are associated with inflammation, whereas alternatively activated macrophages have been found to promote fibrosis, wound healing, neovascularization and granuloma formation [11]. Recently, several populations of macrophages that do not fit into the rigid classification of classical and alternative activation have been described, and it has been proposed that these macrophages are capable of reacting to different surroundings rather flexibly [8, 11–14].

Although the existence of macrophages after myocardial infarction has been unequivocally described before, little is known about the expressional profile and the task of these cells.

Thus, we tested the hypothesis whether macrophages show a time-dependent appearance of classical and alternative activation during different phases of scar tissue formation after myocardial infarction. For this purpose, a unique cell isolation method was developed, followed by RNA isolation and microarray analysis to create a macrophage-specific transcriptional profile. A detailed expression profile of these inflammatory cells during the proliferation phase of infarct healing and their type of activation helps to understand the complex role of this cell type during myocardial remodelling.

Methods

Mouse model of myocardial infarction

Mice were subjected to coronary artery occlusion ($n = 103$, C57bl6, Charles River Laboratories, Sulzfeld, Germany) and randomized in three groups of 2, 5 or 10 days survival after myocardial infarction, respectively. Twenty-three mice were sham-operated. All surgical procedures were performed as described recently [15]. In brief, the mice were anaesthetized intraperitoneally by injection of ketamine (100 mg/kg body weight) and xylazine (6 mg/kg body weight). Mice were intubated endotracheally and ventilated with a rodent ventilator (Hugo Sachs Electronics, Mach, Germany). A thoracotomy was performed at the fourth intercostal space. All muscles overlying the intercostal space were laid open and retracted with 5–0 silk threads; the intercostal muscles were transsected. A ligature with a 7–0 prolene thread (Ethicon, Norderstedt, Germany) was placed around the left anterior descending artery just below the atrioventricular border. Discoloration of the ventricle and ECG-changes provided evidence of ischaemia. The lung was reinflated and muscle and skin layers were closed separately. The animals were weaned by the respirator and extubated. Sham-operated animals were subjected to similar surgery, except that the ligature was not tied tightly.

All investigations conform with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH publication no. 85–23, revised 1996) and were approved by the appropriate authorities (RP Darmstadt, Hessen, Germany).

Isolation of monocytes from the infarcted area and blood

Two, 5 and 10 days after surgery infarcted tissue was pooled (Fig. 1A), minced and digested 3 times in cell extraction buffer (1 mg collagenase IV, 1 mg dispase and 0.5 mg hyaluronidase per 1 ml ADS buffer [0.11 M NaCl, 5 mM KCl, 5 mM Dextrose, 0.8 mM $MgSO_4$, 12.5 mM NaH_2PO_4, 20 mM HEPES]). After each digestion, the supernatant containing the single-cell solution was removed and fresh enzyme solution was added. During cell extraction, the solution was immersed in a shaking water bath at 37°C and bubbled with carbogen gas during the whole process. The single-cell solution was cooled down to and subsequently handled at 4°C.

In preliminary experiments, density-gradient centrifugation (Percoll® gradient [Sigma, Munich, Germany] 1077 g/ml for 25 min. 600 g) was used prior to magnetic labelling in order to deplete granulocytes, natural killer cells and B cells before macrophages/monocytes enrichment was commenced. This step was abandoned after repeated FACS analyses failed to show improved purification when judged by the comparison of the forward and side scatter and fluorescence intensity pattern of the enriched cell population, regardless whether density centrifugation was used or not (data not shown). This is in line with previous findings that neutrophils loose their expression of CD11b shortly after they have invaded the infarct zone [16]. In addition, NK and B cells are not known to settle down into the infarct area and can therefore most probably be excluded as contaminants in our experimental set-up [17].

Erythrocytes were lysed using RBC lysis Buffer (50 mM NH_4Cl, 10 mM $KHCO_3$, 100 μM EDTA). Then the remaining cells were incubated with anti-CD11b microbeads and anti-CD11b-APC (Miltenyi Biotec, Bergisch-Gladbach, Germany).

To obtain blood-monocytes, blood was collected from five mice. Erythrocytes were also lysed using RBC lysis Buffer. The sample was washed in PBS (1×), EDTA (2 mM) and incubated with anti-CD11b microbeads and anti-CD11b-APC.

Labelled cells were sorted using the VariomacS magnetic separator (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. The purity of the isolated monocytes/macrophages was determined by diverting a fraction of the isolate to flow cytometry analysis (FACSCalibur; BD Biosciences, San Jose, CA). In addition, the isolated cells were spotted on a microscope slide using a cytospin protocol, followed by immunofluorescence staining with macrophage-specific antibody F4/80 (Calltag, Invitrogen, Karlsruhe, Germany).

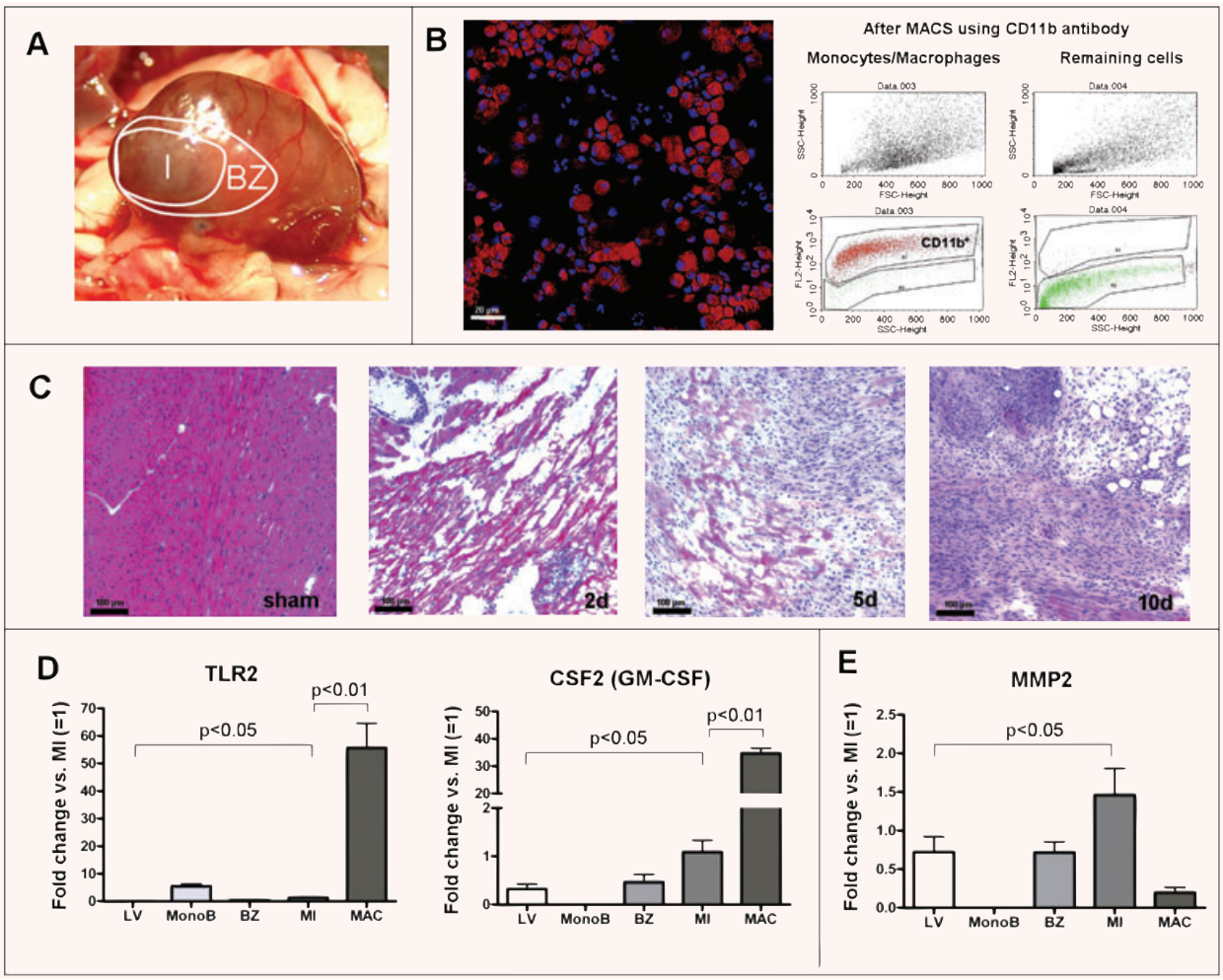

Fig. 1 Isolation of macrophages from infarcted area in mice. (**A**) Macrophages were isolated from infarcted area (I) after ligature of the left anterior descending artery. mRNA abundance of all investigated targets was also compared with the infarct border zone (BZ). (**B**) After isolation of macrophages, the cell population was spotted on slides using cytospin technique and immunostained using F4/80 antibodies (red). Note that the majority (>90%) of isolated cells are macrophages. (Nuclei: DAPI, blue). FACS analyses showed that when using CD11b, monocytes were successfully separated from the isolated cell population by using magnetic cell sorting (MACS). (**C**) Representative HE-staining of infarcted tissue, which was used for immunohistochemistry. The infarcted tissue was used to investigate the time course of activation type-specific genes. It was isolated 2, 5 and 10 days after myocardial infarction and compared with sham-operated mice. (**D**) qRT-PCR of genes known to be expressed by macrophages after MI. Fold changes (FC) are expressed relative to mRNA abundance in myocardial infarction (MI), which was set to 1. Both targets, *toll-like receptor 2* (TLR2) and *colony stimulating factor 2* (CSF2, also designated GM-CSF), are strongly expressed in macrophages compared with MI, BZ, MonoB and LV. This indicates that these genes are transcribed infarct-specific and derive predominantly from macrophages after myocardial infarction. (**E**) *Matrix metalloproteinase 2* (MMP2) is not transcribed by macrophages after myocardial infarction. Even though up-regulated in myocardial infarction *versus* LV, the FC of macrophages *versus* LV is less than 1. (FC, fold change; LV, left ventricle; MonoB, monocytes isolated from blood; BZ, border zone of infarcted area; MI, entire infarcted area; MAC, macrophages isolated from infarcted area; each $n = 6$).

Phenol-chloroform extraction of total RNA

The tissue was homogenized in a MixerMill 301 (Retsch, Haan, Germany) together with 800 μl TRIzol$^{\#®}$ reagent (Invitrogen). After addition of 400 μl chloroform, the samples were centrifuged and the aqueous phase (400 μl) was transferred to a fresh reaction tube. Nucleic acids were precipitated with 400 μl isopropanol and washed with 75% ethanol. DNA digestion was performed using the TURBO DNA-free™ (Ambion, Applied Biosystems, Darmstadt, Germany).

Microarray analysis

Microarray analysis was performed on three groups: (*i*) macrophages isolated from infarcted myocardium, (*ii*) complete tissue of infracted myocardium and (*iii*) left ventricular control tissue obtained from sham-operated mice. Macrophages were isolated 5 days after myocardial infarction and pooled from 10 animals to obtain sufficient RNA amounts per analysis. All microarrays were performed in triplicate to allow statistical analysis.

For microarray analysis, 500 ng of total RNA was reversed, transcribed and labelled, using the cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA). Microarray hybridizations were performed using the Affymetrix GeneChip® Mouse Genome 450 2.0 Array for the analyses of over 39,000 transcript variants from the mouse genome (Affymetrix Inc.). Because of the built-in redundancy in the array design, different probe sets sometimes lead to the same transcript, contributing to a total of 34,000 well-known mouse genes on the Mouse Genome 450 2.0 Array. The sequences were derived from GenBank, dbEST and RefSeq. Further information on the Gene chip system can be obtained at http://www.affymetrix.com.

The results of the microarray experiments were analysed using the following method. Among the over 39,000 targets present on the microarray, 11,101 were significantly measured (detection P value < 0.05). From these genes, 2334 showed a fold change (FC) greater than 2 of the infarcted zone compared with LV of sham-operated mice. The infarct-specific up-regulated genes were then assigned to the following categories.

Up-regulated genes were considered to be expressed by other cell types during myocardial infarction, but not by macrophages if they showed decreased mRNA abundance in macrophages compared with LV. Targets were defined as not predominated by macrophages if they did not show a strong increase (<10-fold) in macrophages compared with infarcted zone. Target genes were considered to derive predominantly from macrophages during myocardial infarction if they showed a FC greater than 10 in macrophages compared with infarcted myocardium. The latter derived from the observation of genes, which are *a priori* known to be macrophage-specific like TLR2 or CSF2 (Fig. 1D) [6]. In contrast, genes like matrix metallopeptidase 2 (MMP2), even though increased in the infarcted zone, did not fulfil these criteria and were considered as not being transcribed by macrophages during myocardial infarction (Fig. 1E).

Quantitative real-time polymerase chain reaction

Results of microarray experiments were validated by quantitative real-time polymerase chain reaction (qRT-PCR), for which a *de novo* series of mice were operated. Infarct specificity of differentially expressed genes in macrophages was verified by comparing the transcriptional FC of all genes of interest to the targets' transcriptional level of monocytes isolated from the blood of healthy mice ($n = 5$).

Therefore, gene-specific primers were synthesized using data from the PrimerBank database [18] or designed using Primer3 online tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). cDNA was synthesized for 50 min. at 50°C in a 20 μl reaction containing 1× First-Strand Buffer, 500 ng total RNA, 200 ng of random hexamer oligonucleotides, 10 mM DTT, 0.5 mM dNTPs, 40 units RNase inhibitor and 200 units Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed in a 25 μl reaction, 96-well format (1.0 μl cDNA; 200 nM each primer; 1× SYBR Green Super Mix; BioRad, München, Germany) using a 7500 real-time PCR system (Applied Biosystems). Three samples were measured in each experimental group in triplicate, with a minimum of two independent experiments. The relative amount of target mRNA normalized to hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) was calculated according to the method described by Pfaffl [19].

All FCs in the manuscript, which were obtained by qRT-PCR, are normalized to cDNA derived from total RNA of MI. The amount of mRNA detected in MI was set to 1.

Histological analysis

To address the question whether the isolated macrophages show a time-dependent expression of the investigated genes, we performed immunohistochemistry of infarcted tissue 2, 5 and 10 days after MI to localize specific targets inside the infarcted zone.

Serial cryosections (5 μm) of whole hearts were cut, starting from the apex to the base (two sections every 100 μm). Slices of the whole myocardium were used, including infarcted, periinfarct and remote areas. For the analyses of isolated macrophages cell, suspensions were spotted on silane-coated glass slides using Shandon Cytospin® 2 (Thermo Scientific, Waltham, MA). The antibodies used and their concentrations were CD11b 1:100 (ImmunoTools, Friesoythe, Germany), IL10 1:200 (Abcam, Cambridge, UK), TNFα 1:200 (Abcam), ARG1 1:200 (Santa Cruz, Santa Cruz, CA), LGALS3 1:50 (Abcam) and F4/80 1:100 (Caltag, Invitrogen).

All sections were incubated for 2 hrs at room temperature. Incubation with the first antibody was followed by treatment with biotinylate second antibody when non-directly labelled antibodies were used. The directly labelled antibodies were conjugated to Cy3. The last incubation was carried out with fluoroisothiocyanate-linked streptavidin-Cy2 (FITC, Rockland, NY). Nuclei were stained with DRAQ-5 (Axxora, Loerrach, Germany). Pictures were taken with a Leica TCS SP laser scanning confocal microscope (Leica, Wetzlar, Germany) equipped with appropriate filter blocks using a Silicon Graphics Octane workstation (Silicon Graphics, Sunnyvale, CA) and three-dimensional multi-channel image processing software (Bitplane, Zürich, Switzerland).

HE-staining was performed according to the standard protocol. To quantify the number of macrophages expressing specific targets, 10 representative images were taken. Cells co-localizing the murine macrophage-specific F4/80, also known as Emr1 (EGF-like module containing, mucin-like, hormone receptor-like sequence 1), and the targets of interest were counted and compared with the total number of F4/80-positive cells.

Statistics

Data are reported as mean ± S.E.M. All statistical analyses were performed using the GraphPad Prism v4.00 for Windows (GraphPad Software, San Diego, CA). D'Agostino and Pearson omnibus normality test was used to test normal distribution. Statistical analyses were performed with Student's t-test for unpaired samples and normally distributed data sets. The Kruskal–Wallis one-way analysis of variance was used for data sets that did not pass normality. Differences were considered to be statistically significant when $P < 0.05$.

Results

The survival rate after coronary artery ligation was 91% after 2 days, 87% after 5 days and 93% after 10 days. HE-sections of

Table 1 Targets involved in macrophage activation assessed by microarray analysis

Name (alias)	Symbol	Fold change MI *versus* LV	Fold change MAC *versus* MI	Derived from macrophages
Classical activation				
Tumour necrosis factor α	TNFa	16.7	129.7	+
Interleukin 1 β	IL1α	124.0	222.4	+
Interleukin 6	IL6	117.1	30.9	+
Alternative activation				
Chitinase 3-like 3 (YM1)	CHI3L3	14.2	198.7	+
Arginase 1	ARG1	67.9	2.5	+/−
Arginase 2	ARG2	10.5	32.4	+
Deactivated macrophages				
Interleukin 10	IL10	17.2	233.1	+
Interleukin 1 receptor antagonist	IL1rn	3.6	50.9	+
Suppressor of cytokine signalling 3	SOCS3	6.9	74.3	+

(+) Target genes predominantly expressed by macrophages; (+/–) targets up-regulated in myocardial infarction but not predominantly by macrophages; (−) targets expressed by other cell types during myocardial infarction but not by macrophages.

operated mice showed the typical time course of infarct remodelling, beginning with accumulation of inflammatory cells and followed by the loss of myocytes and scar tissue formation, which was characterized by an increased amount of interstitial tissue (Fig. 1C). More than 90% of the isolated cells showed a co-localization of CD11b and the macrophage-specific antigen F4/80 (Fig. 1B). This is in line with previously published results [3], demonstrating that monocytes have mainly differentiated into macrophages and are the predominant inflammatory cell type during this phase of infarct healing.

Microarray analysis

Microarray analyses revealed that, within the infarcted zone, 68 genes derive predominantly from macrophages, 391 targets derived from other cell types and 1876 targets, even though up-regulated in macrophages, were also transcribed by other cell types.

Among the 68 genes predominantly transcribed by macrophages, we identified strong up-regulation of targets that are specific for the classical and alternatively activated phenotype as well as genes that deactivate macrophages (Table 1). *Tumour necrosis factor α* (TNFα), *interleukin 6* (IL6) and *interleukin 1β* (IL1β), all typical for classically activated macrophages, showed strong up-regulation. At the same time, we found increased mRNA abundance of genes (*chitinase 3-like 3*, CHI3L3; *resistin like alpha,* RETNLA; *arginase 1*, ARG1; *arginase 2*, ARG2; *mannose receptor, C type 1*, MRC1) known to be specific for the alternatively activated macrophage 5 days after myocardial infarction. Deactivated macrophages were represented by strong up-regulation of *suppressor of cytokine signalling 3* (SOCS3), interleukin 10 (IL10) and *interleukin 1 receptor antagonist* (IL1ra), which are known to be involved in reduction of the inflammatory response.

Classical activation type-specific transcription of macrophages

To validate the finding of a co-existence of different macrophage sub-sets, we performed qRT-PCR and immunohistochemistry. Besides total RNA isolated from macrophages and the infarcted zone, we also investigated the healthy left ventricle, the infarct border zone (BZ, Fig. 1A) and RNA isolated from blood monocytes of healthy mice.

TNFα and IL6, which are characteristic for the classically activated type of macrophages, showed strong up-regulation after myocardial infarction. In the entire infarcted zone, TNFα was increased 16.7-fold compared with LV-controls (Fig. 2A). TNFα in macrophages was increased 183.3-fold compared with MI. Immunostaining of infarcted tissue with antibodies against TNFα and F4/80 showed a time-dependent expression (Fig. 3A and B). F4/80-positive macrophages expressed TNFα after 2 and 5 days (75.3 +/−9.2% and 68.6 +/−17%; $n = 5$). Ten days after myocardial infarction, the proportion of macrophages expressing TNFα decreased to 49.9 +/−16.5% ($n = 5$, $P < 0.05$ for trend). mRNA abundance of TNFα in macrophages was increased after 2 days (81.0+/−22.6-fold; $n = 5$) and peaked around day 5 (183.0+/−21.6-fold; $n = 5$). TNFα-mRNA decreased (67.4+/−1.8-fold; $n = 5$) 10 days after myocardial infarction (Fig. 3C).

IL6 was increased 157.1-fold in the entire infarcted area compared with LV-controls and, furthermore, increased 14.0-fold in macrophages compared with infarcted myocardium (Fig. 2B).

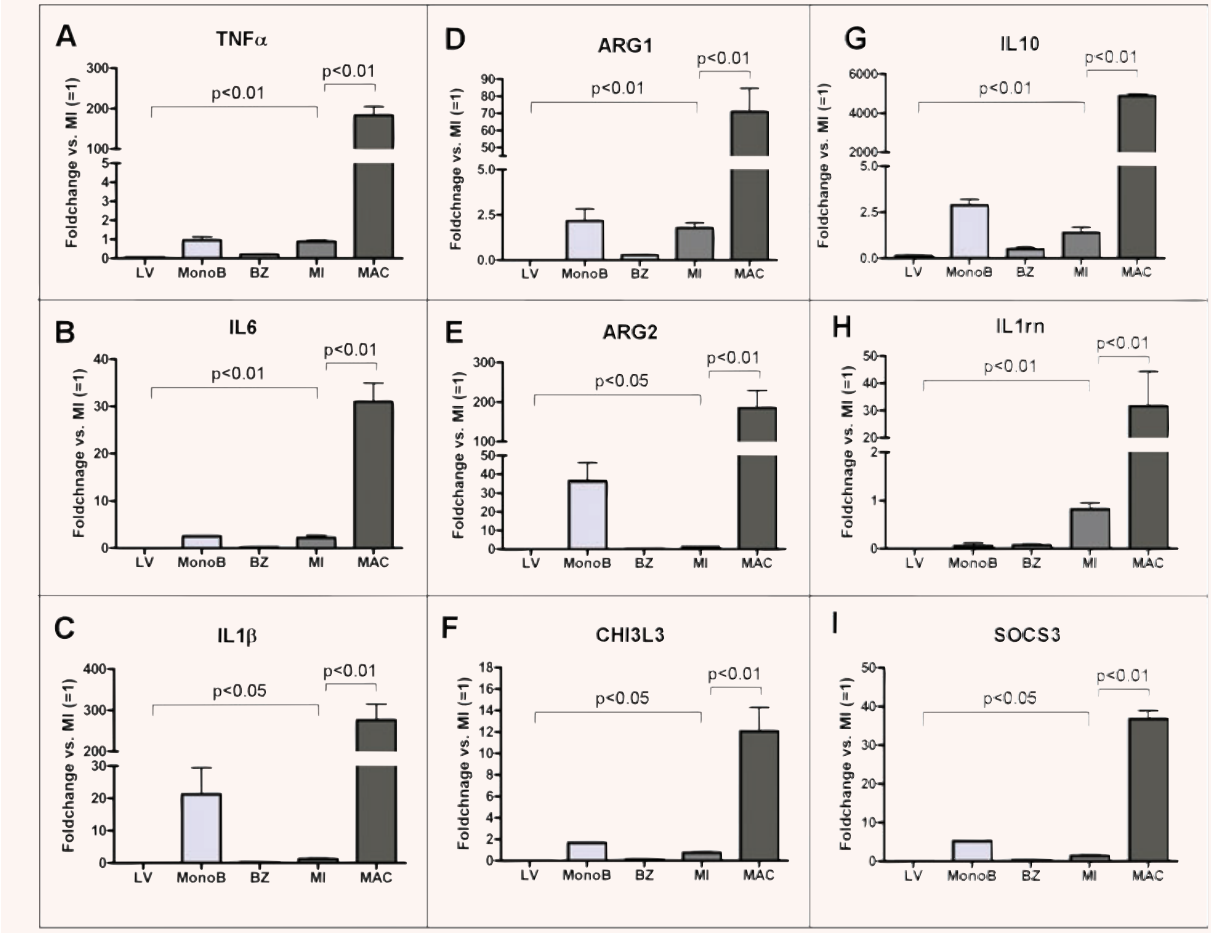

Fig. 2 qRT-PCR of genes specific for the classical, the alternative activated type of macrophages and deactivating targets 5 days after myocardial infarction. All fold changes (FC) are expressed relative to mRNA abundance in myocardial infarction (MI), which was set to 1. Tumour necrosis factor α (TNFα) (**A**), *interleukin 6* (IL6) (**B**) and interleukin 1β (IL1β) (**C**) are all strongly up-regulated in macrophages compared with myocardial infarction, BZ, MonoB and LV. This indicates a myocardial-specific regulation predominantly in macrophages. *Arginase 1* (ARG1) (**D**), *arginase 2* (ARG2) (**E**) and *chitinase3 like 3* (CHI3L3, also designated YM1) (**F**) are mainly transcribed by macrophages compared with MI, BZ and LV. In connection with ARG2 as well as CHI3L3, a moderate transcription was also detected in monocytes isolated from blood compared with MI, BZ and LV, which indicates an incomplete cardiac-specific expression. *Suppressor of cytokine signalling 3* (SOCS3) (**G**), *interleukin 1 receptor antagonist* (IL1rn) (**H**) and *interleukin 10* (IL10) (**I**), which are all known to suppress the inflammatory response, are predominantly transcribed by macrophages. Note that especially IL10 shows a very strong up-regulation in macrophages compared with controls. (FC, fold change; LV, left ventricle; MonoB, monocytes isolated from blood; BZ, border zone of infarcted area; MI, entire infarcted area; MAC, macrophages isolated from infarcted area; each $n = 6$).

IL1β showed a 221.9-fold increase of mRNA abundance in macrophages compared with infarcted zone. In the whole infarcted zone, IL1β was increased 93.6-fold compared with healthy left ventricle (Fig. 2C). These targets, representing the classical activation of macrophages, showed a cardiac-specific transcription when compared with transcription of monocytes isolated from blood.

Alternative activation type-specific transcription of macrophages

Targets characterizing the alternative activation type were also strongly increased in macrophages derived from infarcted area. ARG1 and ARG2 in healthy LV showed no transcription. ARG1 and ARG2 in macrophages were increased 41.6-fold and 119.2-fold

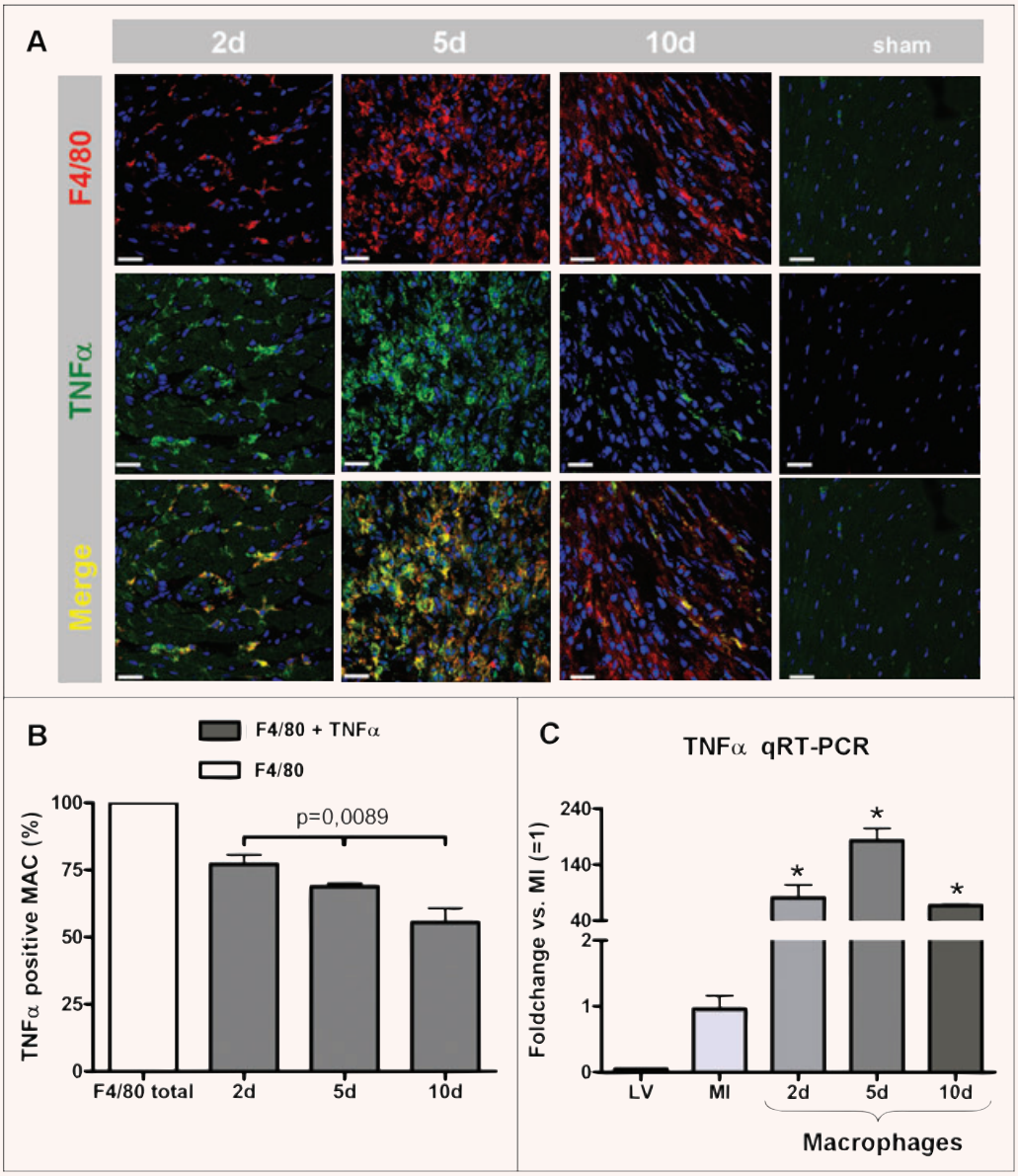

Fig. 3 Immunostaining of TNFα in the infarcted area at different time points after ligature of the left descending anterior coronary artery. (**A**) Confocal images of infarcted tissue 2, 5 and 10 days after MI, which were stained with specific antibody F4/80 (red) and TNFα (green), representing the classical activation type of macrophages. The last panel shows co-localization in merged images (merged images, yellow; nuclei: DAPI, blue). **(B)** TNFα-positive macrophages were quantified at different time points (2, 5 and 10 days after myocardial infarction). For each image, the proportion (in percentage) of F4/80-positive cells which, at the same time, express TNFα was evaluated. (**C**) Abundance of TNFα mRNA in macrophages isolated at different time points (2, 5 and 10 days, $n = 5$) after myocardial infarction. The relative amount of TNFα mRNA was investigated, using quantitative real-time PCR, and normalized to RNA isolated from entire infarcted tissue (MI), which was set to 1. (LV healthy left ventricle; $*P <$ 0.01 *versus* MI).

compared with infarcted area (Fig. 2D and E). Immunohistochemistry demonstrated that ARG1 is expressed only by a small proportion of macrophages after 2 days (8.4 +/– 0.7%; $n = 5$) (Fig. 4A and B). Five and 10 days after myocardial infarction, 47.0 +/– 6.6% and 49.4 +/– 7.8% of all F4/80-positive cells showed ARG1 expression ($n = 5$, $P < 0.05$ for trend). A relatively moderate increase after 2 days (14.5 +/– 1.3-fold *versus* MI set to 1; $n = 5$), but a strong increase of mRNA abundance after 5 and

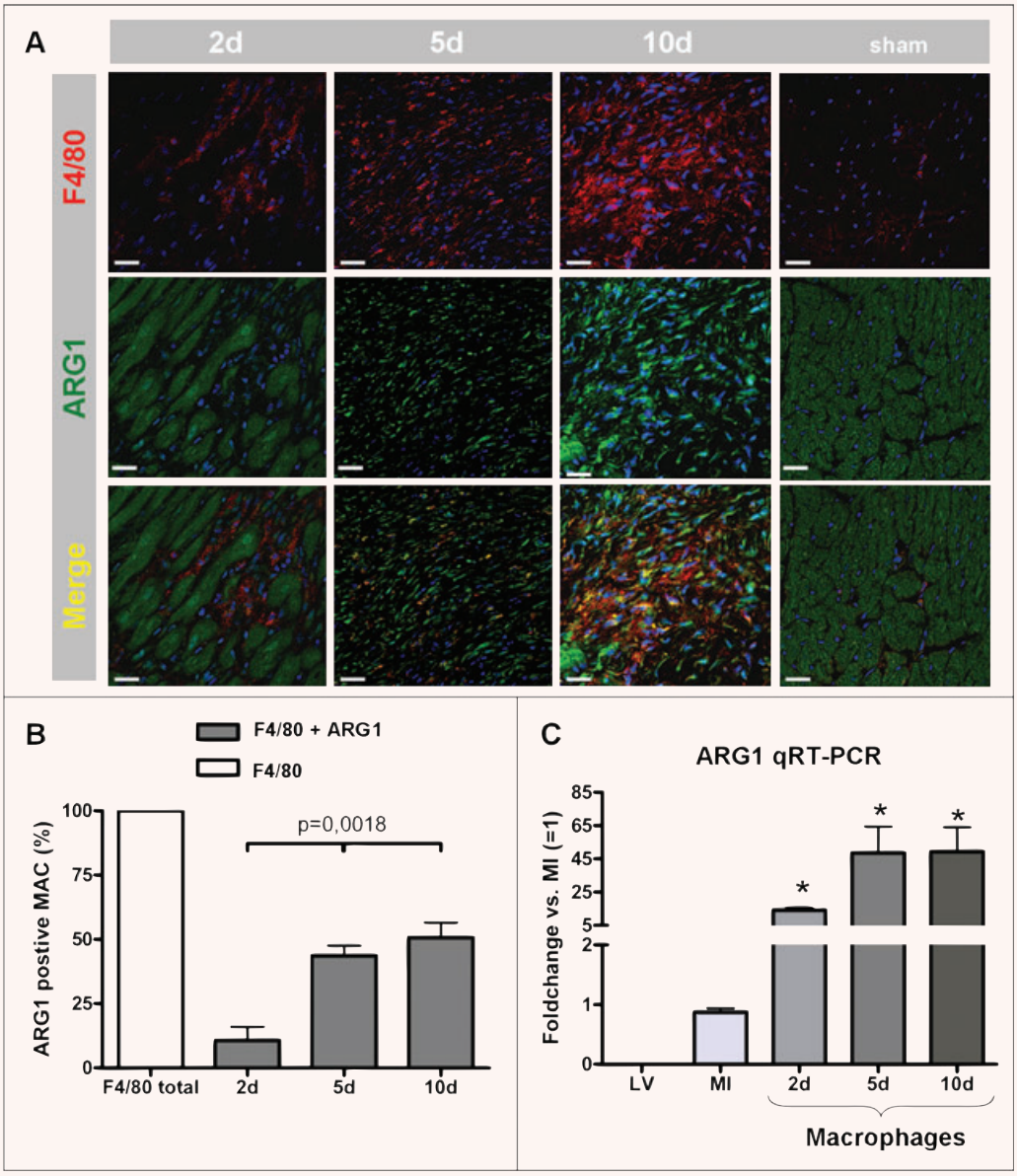

Fig. 4 Time course of alternative activated macrophages at different time points after myocardial infarction using immunostaining with ARG1. (**A**) Confocal laser microscopy images of infarcted tissue 2, 5 and 10 days after MI, which were stained with specific antibody F4/80 (red) and ARG1 (green), representing the alternative activation type of macrophages. The last panel shows co-localization in merged images (merged images, yellow; nuclei: DAPI, blue). (**B**) ARG1-positive macrophages were quantified at different time points (2, 5 and 10 days after myocardial infarction). For each image, the proportion (in percentage) of F4/80-positive cells which, at the same time, express ARG1 was evaluated. (**C**) Abundance of ARG1 mRNA in macrophages isolated at different time points (2, 5 and 10 days, $n = 5$) after myocardial infarction. The relative amount of ARG1 mRNA was investigated, using quantitative real-time PCR, and normalized to RNA isolated from entire infarcted tissue (MI), which was set to 1. (LV healthy left ventricle; $*P < 0.01$ *versus* MI).

10 days (48.6 +/– 15.3-fold and 49.3+/– 14.6-fold *versus* MI set to 1; $n = 5$) confirmed theses observations (Fig. 4C).

CHI3L3, which is also a gene for alternative activation, showed increased mRNA abundance in macrophages compared with the entire infarcted zone (16.5-fold) (Fig. 2F). There was no detectable transcription of CHI3L3 in healthy LV. In connection with ARG2 as well as CHI3L3, a reasonable amount of mRNA was detected in monocytes isolated from the blood of healthy mice. In both cases, there was

a significant transcriptional increase in macrophages isolated from the infarcted zone (5.1- and 7.2-fold) (Fig. 2E and F, MonoB).

Macrophage deactivation-specific transcription

Macrophages that express genes known to be anti-inflammatory, like IL10, IL1ra and SOCS3, are designated to be the deactivated type. Five days after infarction, macrophages are the main source of such anti-inflammatory genes in the infarcted zone. Especially IL10 mRNA abundance is extremely increased in macrophages compared with the whole infarcted zone (2150-fold) (Fig. 2G). Immunostaining of IL10 in macrophages showed a time-dependent increase of protein expression in the infarcted area. More than half (56.3 +/− 0.5%; $n = 5$) of all macrophages expressed IL10 after 2 days, whereas the proportion of IL10-positive cells increased to 71.8 +/− 22% ($n = 5$) after 5 days. Almost all macrophages (92.5 +/− 7.9; $n = 5$, $P < 0.05$ for trend) expressed IL10 10 days after MI (Fig. 5A and B). These observations are supported by strongly increased amounts of mRNA found in macrophages after myocardial infarction at the latter time points (5 and 10 days; 1727.0 +/− 79.2-fold and 1543.1 +/− 529-fold *versus* MI set to 1; $n = 5$). Two days after MI, only a 144.0 +/− 50-fold ($n = 5$) increase of mRNA was observed in macrophages.

Likewise, IL1ra, an IL1b-receptor antagonist (47.8-fold *versus* entire infarcted zone), and SOCS3 (26.7-fold *versus* entire infarcted zone) are predominantly transcribed by macrophages (Fig. 2H and I). A basal transcription was observed for IL10 and SOCS3 in healthy LV (Fig. 2G and I, MonoB), whereas IL1ra was not detected in LV-controls. IL10 and IL1ra were not detected in blood monocytes isolated from mice. However, SOCS3 was detected in blood monocytes, but at a much lower extent (4.2-fold) compared with macrophages isolated from infarcted zones.

Discussion

Attempts to modulate inflammation or to interfere with extracellular matrix (ECM) deposition after myocardial infarction had no clinical relevancy or even adverse negative effects on therapeutical outcome [20–22]. On the other hand, the dogma that scar formation after myocardial infarction is an irreversible process has been challenged by new findings [23] and the idea to influence the course of inflammation and scaring of the damaged heart therapeutically after myocardial infarction returns into the focus of clinical practise and basic science.

A critical phase during infarct healing is the phase when inflammatory processes are repressed and proliferating fibroblasts are triggered to synthesize ECM in order to replace the infracted area by a discrete but functional scar. Besides fibroblasts and myofibroblasts, the predominant cell type in the infarcted area during this phase of wound healing are activated macrophages, which are essential for a life-saving scar formation [24–26]. It is known that the role of these mononuclear cells is the removal of dead cells and debris and that they are also able to synthesize a wide range of cytokines and growth factors in order to stimulate fibroblasts and endothelial cells [3, 6, 27].

An important prerequisite to investigate the role of macrophages after myocardial infarction is to establish an exact transcriptional profile of these mononuclear cells. Therefore, a critical point within this study was the isolation of macrophages. Immunostaining as well as FACS analysis showed a more than 90% pure population of macrophages after isolation. Furthermore, the isolation of cells has the inherent risk of subsequent transcriptional artefacts. Therefore, immunohistochemistry of the infarcted zone proved the reliability of results obtained from microarray experiments and qRT-PCR without potential artefacts, which may have been derived from the isolation protocol. Despite its limited validity, we additionally compared the transcriptional fold change of investigated genes in macrophages isolated from infarcted tissue with the targets' transcriptional level of blood monocytes using qRT-PCR. Nevertheless, a contamination with a small proportion of other cell types other than macrophages or experimental artefacts due to the isolation procedure can not fully be excluded.

The importance of specific activation profiles of macrophages for tissue remodelling has recently been stated in context to renal injury and repair [28]. Our data demonstrate that also during wound healing after myocardial infarction, invading monocytes differentiate into macrophages, which show a time-dependent appearance of different activation types (Fig. 6A).

In the beginning, the inflammatory response triggers the recruitment of leucocytes into the infarcted area. Neutrophils and macrophages remove dead cells and matrix debris from the affected zone [3, 5]. We could show that, during this phase, the classically activated macrophage dominates the infarcted zone, which accounts for a strong pro-inflammatory release of cytokines like TNFα, IL6 and IL1β.

After the inflammatory phase, the proportion of alternatively activated macrophages increased significantly. The induction of alternative activation is known to trigger tissue repair [12, 29, 30]. Especially arginase-induced reduction of nitric oxide and the production of polyamines and proline, an important component of collagens involved in repairing extracellular matrix, point towards an important function of alternatively activated macrophages in connection with scar formation [31].

Finally, during the maturation phase of myocardial remodelling, anti-inflammatory genes like IL10 and IL1ra are predominantly expressed by macrophages, which delimit the inflammatory response, thereby preventing an over-expansion of the inflammatory processes into the border zone of the infarcted area.

Taken together, the role of macrophages is not restricted to the induction of inflammation and to the clearance of cellular debris in order to provide space for the scar-forming myofibroblasts. Our findings underline previously published results that macrophages

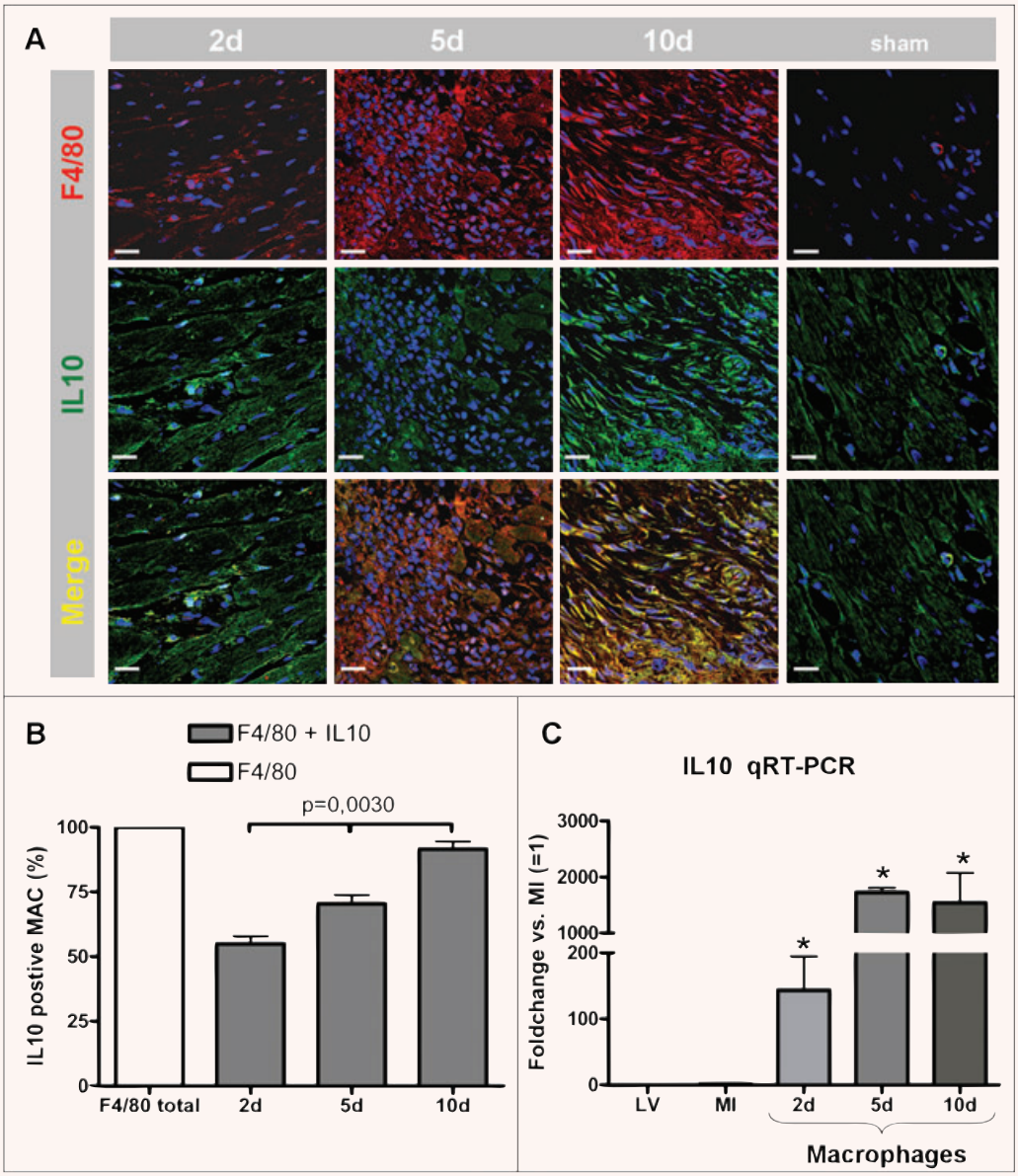

Fig. 5 After LAD ligature IL10 expression increases, demonstrating that deactivation of macrophages increases over a longer period of time. (**A**) Confocal laser microscopy images of infarcted tissue 2, 5 and 10 days after MI, which were stained with specific antibody F4/80 (red) and IL10 (green), representing the alternative activation type of macrophages. The last panel shows co-localization in merged images (merged images, yellow; nuclei: DAPI, blue). (**B**) IL10-positive macrophages were quantified at different time points (2, 5 and 10 days after myocardial infarction). For each image, the proportion (in percentage) of F4/80-positive cells which, at the same time, express IL10 was evaluated. (**C**) Abundance of IL10 mRNA in macrophages isolated at different time points (2, 5 and 10 days, $n = 5$) after myocardial infarction. The relative amount of IL10 mRNA was investigated, using quantitative real-time PCR, and normalized to RNA isolated from entire infarcted tissue (MI), which was set to 1. (LV healthy left ventricle; $^*P < 0.01$ *versus* MI).

play a central role in scar tissue formation [3, 5, 6, 25, 26] and orchestrate the different phases of tissue remodelling after myocardial infarction [32, 33].

The question, whether macrophages change their activation profile over a longer period of time, or if different sub-sets of monocytes, which differ in their activation profile, enter the infarcted area at different time points after MI, has to be subjected to further investigations. However, Nahrendorf *et al.* [34] demonstrated that the infarcted zone sequentially and actively recruits two different monocyte sub-populations *via* CCR2

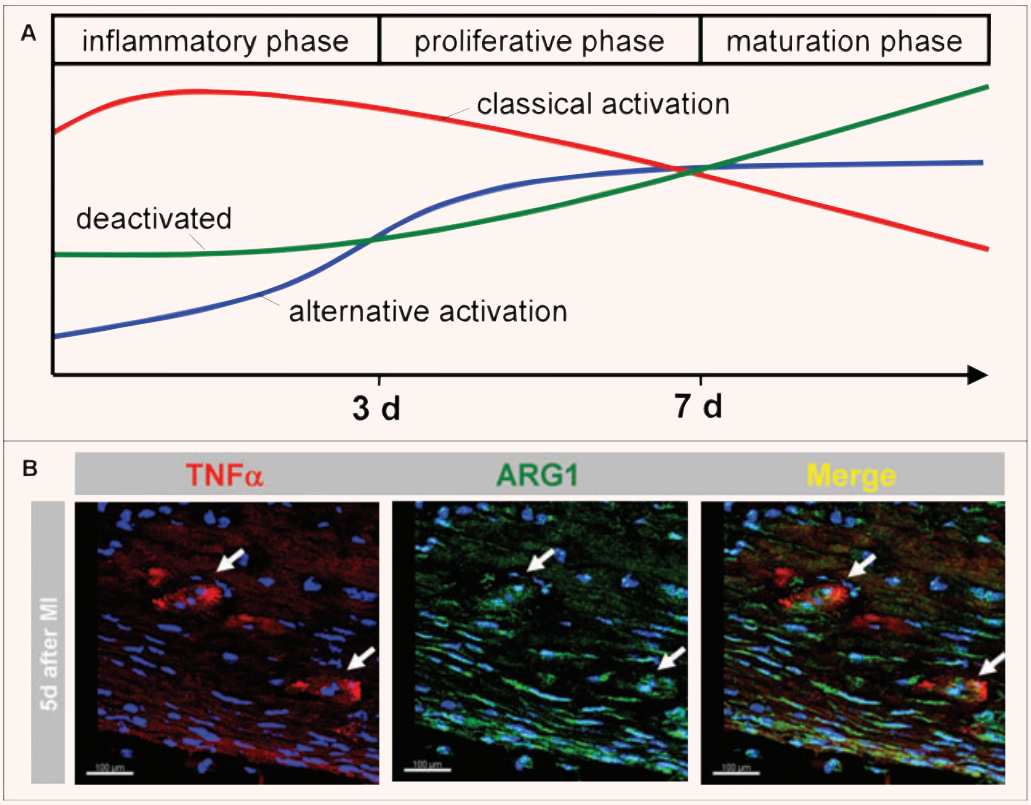

Fig. 6 Macrophages show a time-dependent occurrence of activation types after myocardial infarction. (**A**) Classically activated macrophages dominate the inflammatory phase after myocardial infarction. Alternatively activated and deactivated macrophages increase during the proliferative and maturation phase. (**B**) ARG1, representing the alternatively activated phenotype of macrophages, and TNFα, a target expressed in classically activated macrophages, were co-localized (arrows) using immunohistochemistry. (Scale bar 100 μm).

and CX 3 CR1 at different time points after MI. This might point at different sub-sets of invading monocytes, which account for different activation types of macrophages. On the other hand, our own preliminary data suggest that macrophages change their activation type, since we could co-localize activation type-specific targets (ARG1 and TNFα) in the same cells (Fig. 6B).

Our investigations, which focussed on the identification of the complete transcriptional profile of macrophages, revealed a finely adjusted expression balancing between anti- and pro-inflammatory genes, extracellular matrix remodelling and cytokines triggering fibroblast differentiation. They underline the fact that a detailed understanding of the role of all participants during scar maturation might lead to new therapeutic strategies in order to positively influence wound repair after myocardial infarction. Yet, it remains theoretical whether therapeutically modulating the activation type of macrophages during wound healing is a beneficial strategy to minimize secondary complications after myocardial infarction. Nonetheless, it is intriguing to speculate whether an increased amount of alternatively activated macrophages can initiate a more discrete but still resilient myocardial scar.

In conclusion, we tested the hypothesis and could show for the first time that macrophages are differentially activated during different phases of scar tissue formation after myocardial infarction.

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