

Immunolabeling of TGF- β during Muscle Regeneration

K. Nikovics, D. Riccobono, M. Oger, H. Morin, L. Barbier, T. Poyot, X. Holy, A. Bendahmane, M. Drouet, A. L. Favier

Abstract—Muscle regeneration after injury (as irradiation) is of great importance. However, the molecular and cellular mechanisms are still unclear. Cytokines are believed to play fundamental role in the different stages of muscle regeneration. They are secreted by many cell populations, but the predominant producers are macrophages and helper T cells. On the other hand, it has been shown that adipose tissue derived stromal/stem cell (ASC) injection could improve muscle regeneration. Stem cells probably induce the coordinated modulations of gene expression in different macrophage cells. Therefore, we investigated the patterns and timing of changes in gene expression of different cytokines occurring upon stem cells loading. Muscle regeneration was studied in an irradiated muscle of minipig animal model in presence or absence of ASC treatment (irradiated and treated with ASCs, IRR+ASC; irradiated not-treated with ASCs, IRR; and non-irradiated no-IRR). We characterized macrophage populations by immunolabeling in the different conditions. In our study, we found mostly M2 and a few M1 macrophages in the IRR+ASC samples. However, only few M2b macrophages were noticed in the IRR muscles. In addition, we found intensive fibrosis in the IRR samples. With *in situ* hybridization and immunolabeling, we analyzed the cytokine expression of the different macrophages and we showed that M2d macrophage are the most abundant in the IRR+ASC samples. By *in situ* hybridization, strong expression of the transforming growth factor β (TGF- β) was observed in the IRR+ASC but very weak in the IRR samples. But when we analyzed TGF- β level with immunolabeling the expression was very different: many M2 macrophages showed weak expression in IRR+ASC and few cells expressing stronger level in IRR muscles. Therefore, we investigated the MMP expressions in the different muscles. Our data showed that the M2 macrophages of the IRR+ASC muscle expressed MMP2 proteins. Our working hypothesis is that MMP2 expression of the M2 macrophages can decrease fibrosis in the IRR+ASC muscle by capturing TGF- β .

Keywords—Adipose tissue derived stromal/stem cell, cytokine, macrophage, muscle regeneration.

I. INTRODUCTION

THE injury of adult skeletal muscle initiates series of well-coordinated events that lead to the efficient repair of the damaged tissue. If the coordination failed, it can lead to unsuccessful regeneration and can induce the development of fibrosis. Consequently, to decrease the inflammation and to improve the muscle regeneration after injury is of great importance and interest not only scientifically but also clinically. One research axis is the use of stem cells. Indeed, it

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has been shown that local injections of ASCs could reduce inflammation and improve muscle regeneration [12]. However, the mechanisms involved are barely understood. Furthermore, bone marrow derived mesenchymal stem cells (BM-MSCs) have been shown to enhance wound healing by recruiting macrophages [5]. Macrophages are known a major source of many cytokines [11], which play critical roles in local regulation of the inflammatory process and skeletal muscle regeneration. Inflammation and tissue regeneration often linked processes, require precise timing and coordination of different cell types, especially macrophages. M1 macrophages are the first population of macrophages appearing after the muscle injury. These macrophages are responsible for early inflammatory responses [1]. Second population of macrophages participates in skeletal muscle repair is the M2 macrophages (M2a, M2b, M2c and M2d) [7], [9]. Subsequent differentiation/invasion by M2 macrophages attenuates M1 populations through the release of anti-inflammatory cytokines. These cytokines synthesized by M2 macrophages are essential for the muscle regeneration [18].

Muscle injury induces strong changes in muscle cells and in the extracellular matrix [15]. Recent studies have further indicated that the switch between proper regeneration and development of fibrosis is controlled by various factors. Among them are those belonging to the TGF- β s. TGF- β is able to inhibit myoblast proliferation but also able to promote fibrosis formation [16]. Thus, specific inhibition of TGF- β 1 signaling pathway can significantly improve muscle repair [6].

The mechanism behind the anti-fibrotic effect of matrix metalloproteinases (MMPs) is unclear but it has been shown that MMP expression can reduce fibrosis [10].

In the present study, we investigated the capacity of ASCs to improve muscle regeneration after injection of these cells into irradiated muscle. To do such a thing, we identified the different cell populations that can be found in the IRR, IRR+ASC and no-IRR samples, by immunofluorescence staining. Then we analyzed the cytokine gene expression in regenerating skeletal muscle by *in situ* hybridization.

II. RESULTS AND DISCUSSION

It was found that *in vitro* macrophages are able to stimulate the division and fusion of the myoblasts [3]. The same activation can be seen if macrophage-conditioned solution was used [4]. It was shown that macrophage depletion impairs skeletal muscle regeneration [8]. Our goal was to identify molecules secreted by macrophages which can help in the wound healing.

At first, to discover the phenotype of the macrophages of the

different (IRR+ASC, IRR and no-IRR) regenerating muscle, we performed immunofluorescence staining. In the IRR+ASC sample, the macrophages were identified on the basis of CD80 expression, M2 macrophages and satellite cells were identified on the basis of CD206 expression. The CD80-only positive cells were M1 macrophages, while CD80 and CD206 double positive cells were considered as M2 macrophages. Cells positives only with CD206 antibody but not with CD80 antibody were the satellite cells (Table I). Our result showed predominantly the presence of the M2 macrophages, but we could also detect a few M1 macrophages (Fig. 1). The expression of MMP2 was also studied.

TABLE I
 DETERMINATION OF CELL PHENOTYPES

	CD206	CD80
M1 macrophage	-	+
M2 macrophage	+	+
Satellite cell	+	-

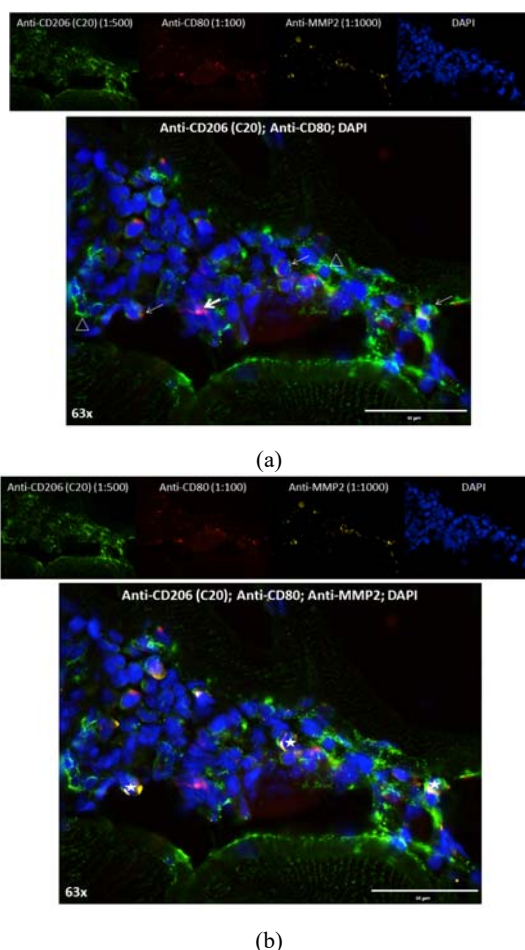


Fig. 1 M1, M2 macrophages, activated satellite cells and MMP2 proteins visualized in IRR+ASC regenerating skeletal muscle. (a) Anti-CD206 (Alexa488; green fluorescence) binds M2 macrophages and satellite cells. Anti-CD80 (Alexa568; red fluorescence) binds M1 and M2 macrophages. (b) Anti-MMP2 (Alexa647; yellow fluorescence). (Blue: nuclear staining with DAPI) Thin arrows: M2 macrophages; thick arrow: M1 macrophage; triangle: satellite cells, stars: MMP2 expression.

Compared to IRR+ASC group we observed significant difference when we investigated irradiated, (IRR) and non-irradiated (no-IRR) muscles. Indeed, we noticed only a few numbers of the satellite cells. Interestingly, macrophages have a little presence. The number and the proper activation of the satellite cells are essential during the wound healing, otherwise strong fibrosis can be detected [2]. The increased collagen content can induce autofluorescence in different tissue [17]. Using this feature, we found that the collagen of the fibrosis in the IRR sample had an intense fluorescence emission at 700 nm (excitation at 647 nm). No strong autofluorescences were detected in the no-IRR and IRR+ASC samples. In the present study, we noticed that the IRR muscle differs from other muscles in the development of the intensive fibrosis (data not shown).

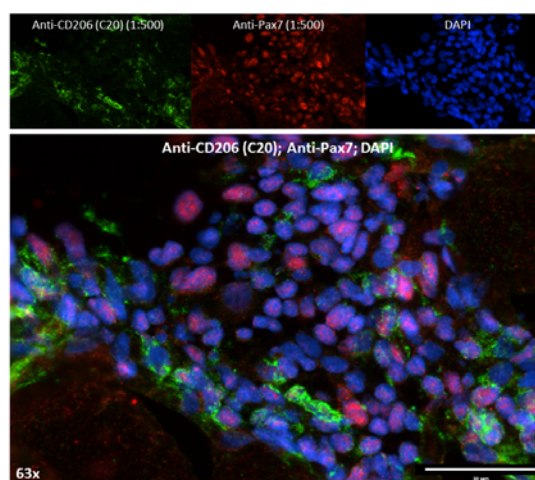


Fig. 2 Immunostaining pattern of activated satellite cells. CD206 (Alexa488; green fluorescence) and Pax7 (Alexa568; red fluorescence) Blue: nuclear staining with DAPI.

The paired box protein 7 (Pax7) expressions are an absolute prerequisite for the normal function of satellite cells during regenerative myogenesis. Upon activation, satellite cells exit from the quiescent state, defined by the expression of the Pax7, and start to proliferate [14]. The distribution of Pax7-positive cells/satellite cells have been investigated in the injured muscles. The antibody raised against Pax7 showed a number of PAX7 positive cells (Fig. 2). The cells were labeled also with CD206 antibody. The CD206 and Pax7 double positive cells were considered as activated satellite cells.

We tested the Ki67 antibody as a proliferation marker (Fig. 3). Staining with this antibody showed a small fraction of actively dividing cells. This result confirms that the activation of the satellite cells was efficient.

To clarify the cytokine expression network of the tissue we suggest detecting the different cytokines by *in situ* hybridization. Indeed, this technique has not been used so far for this kind of application. Very strong TGF- β 1b and TNF α expression was localized to the regenerating zone in the IRR+ASC sample (Figs. 4 A and B). IL-10 showed weaker but detectable expression. As positive control β -actin was used (Figs. 4 C and D). This work is still in progress.

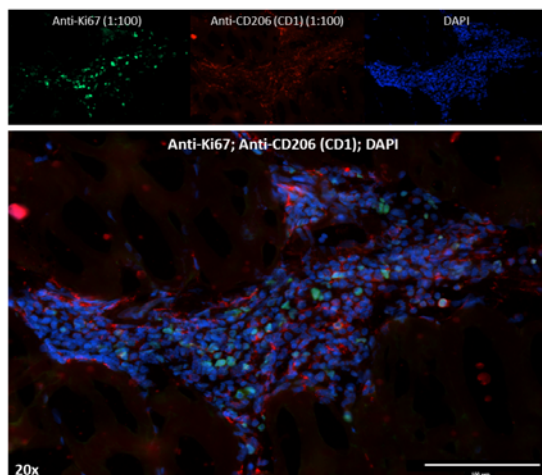


Fig. 3 A small fraction of the cells proliferates actively as determined with an antibody recognizing the proliferation marker Ki67 (Alexa647; artificial green fluorescence). M2 macrophage and activated satellite cells were recognized by CD206 antibody (Alexa568; red fluorescence) Blue: nuclear staining with DAPI.

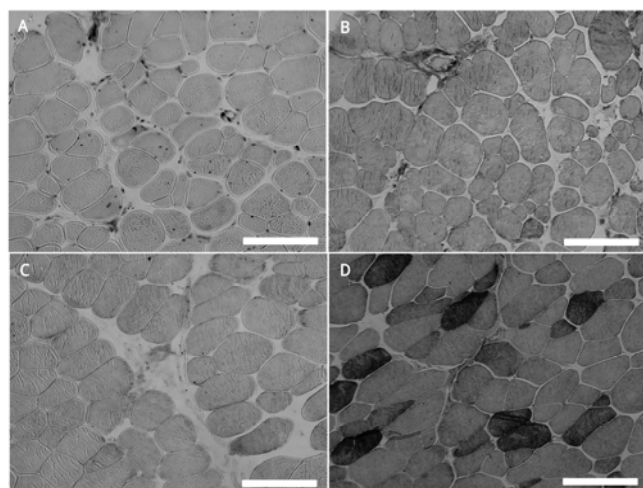


Fig. 4 Localization the mRNA of different cytokines in the IRR+ASC regenerating muscle by *in situ* hybridization. Expression of different mRNAs were visualized by black staining. A: TGF-β1b mRNA expression. B: TNFα mRNA expression. C: IL-10 expression. D: β-actin expression (positive control).

It is well known that each type of macrophage sub-population is involved in a specific biological process and express different cytokines (Fig. 5) [13]. With different immunolabelings, sub-population of the M2 macrophages (M2a, M2b, M2c and M2d), participating in muscle regeneration, were characterized. In the IRR muscle, only a few M2 macrophages were found and they did not express TGF-β (Fig. 6 (a)), therefore they were considered as macrophage M2b. In addition, the expression of IL-10 has been found in some cells that remain to be characterized (Fig. 6 (b)). These cells were expressing TGF-β also and the expression was strong. In the IRR+ASC muscle we find both M1 and many M2 macrophages. Two populations of M2 macrophages were identified: one expressed TGF-β (M2a, c and d), the other did

not (M2b) (Fig. 6 (c)). We noticed that the expression level in one cell was weak. We have also shown that almost all M2 macrophages expressing TGF-β also synthesized IL-10 (Fig. 6 (d)). Most of the M2 macrophages, found in the sample, expressed TNFα (Fig. 6 (e)). It is known, that only M2b and M2d macrophages synthesize TNFα. In the M2 macrophages we have observed IL-10 and TGF-β expression, too. Our results suggest that in the muscle, 76 days after irradiation, M2d macrophages are the most abundant population. M2d macrophages are very important for the blood vessels growth and maybe their abundance is the reason why very good tissue regeneration can be observed in the IRR+ASC muscle.

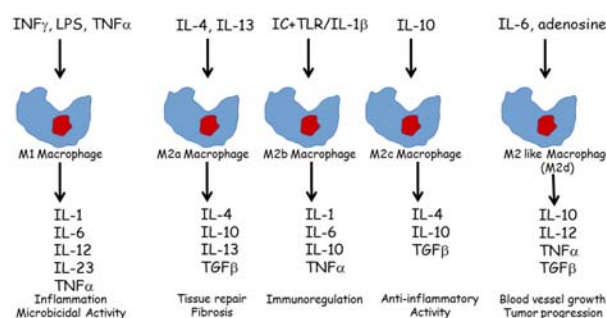


Fig. 5 Different cytokine expressions and functions of the macrophage populations

This observation raises the question: if, in the IRR+ASC sample we can detect much more M2 macrophage and TGF-β than in the IRR sample, why we cannot detect fibrosis in the IRR+ASC muscle? It was observed that MMP2 and MMP9 proteins are indispensable in promoting the muscle healing and that they can reduce fibrosis, most probably by inhibiting TGF-β1 [9]. We studied further the expression of the MMP2 protein (Fig. 1 (b)). Results showed that M2 macrophages in the IRR+ASC muscle expressed MMP2 proteins. However, cells in the IRR muscle did not express the MMP2 proteins (data not shown). Our results lead us to assume that MMP2 expression of the M2 macrophages are very important because it can avoid fibrosis in the IRR+ASC muscle by neutralizing TGF-β cytokine.

Stem cells induce the coordinated modulation of cytokine gene expression in different macrophage cells. Work is going on to further analyze the cytokine gene expression in regenerating muscle by *in situ* hybridization. With these results, we are going to investigate the pattern and timing of changes in gene expression occurring upon stem cells loading.

Our result leads us to assume that ASC injections after irradiation improve the activation of muscle regeneration pathway, since ASCs are very important for the macrophage activation. Furthermore, they trigger the clearance of the debris from the tissue and stimulate the activation of stem cells which are essential for the wound repair.

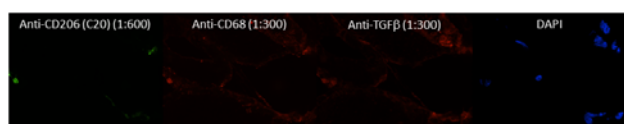
III. MATERIALS AND METHODS

This study was approved by the French Army Animal Ethics Committee (N°2011/22.1). All minipigs were treated in

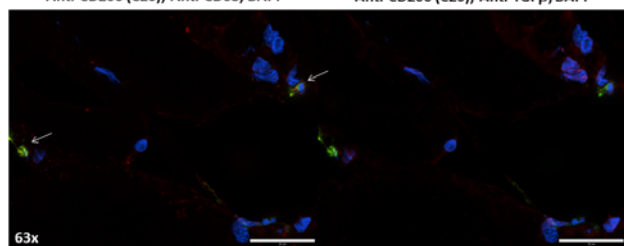
compliance with the European legislation related to animal care and protection in order to minimize pain and adverse effect for the animals.

A. Irradiation

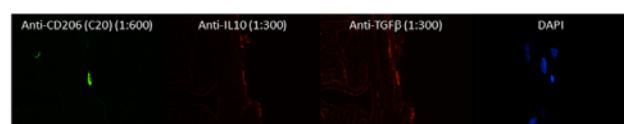
Experimental irradiation and ASC engraftment was previously described [12]. Minipigs were locally irradiated with 50 Gy γ rays and muscles were analyzed 76 days post irradiation.



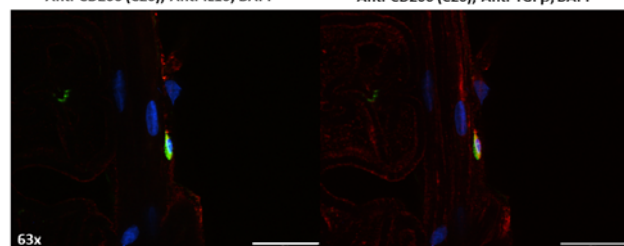
Anti-CD206 (C20); Anti-CD68; DAPI Anti-CD206 (C20); Anti-TGFβ; DAPI



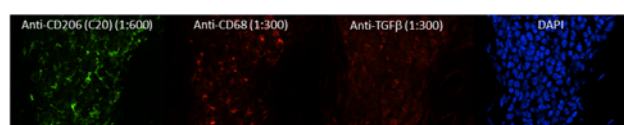
(a)



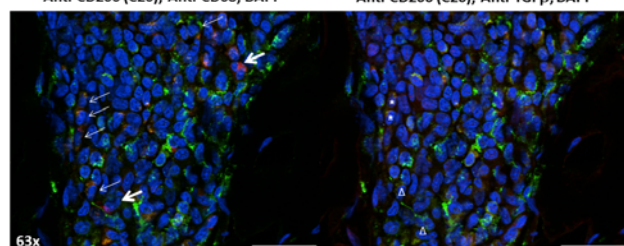
Anti-CD206 (C20); Anti-IL10; DAPI Anti-CD206 (C20); Anti-TGFβ; DAPI



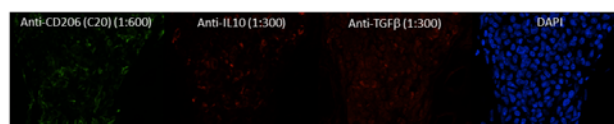
(b)



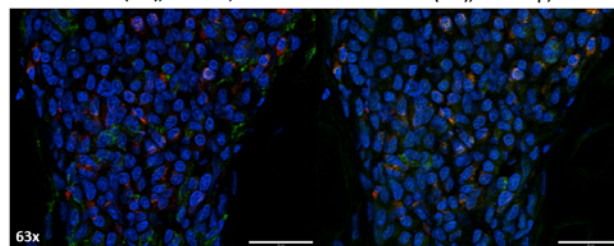
Anti-CD206 (C20); Anti-CD68; DAPI Anti-CD206 (C20); Anti-TGFβ; DAPI



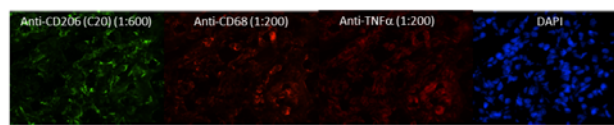
(c)



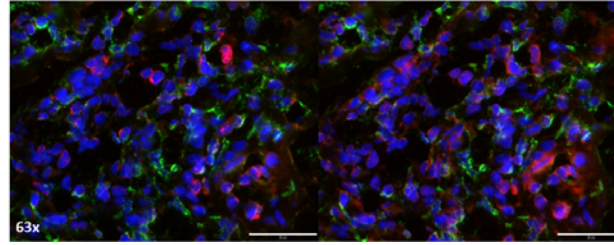
Anti-CD206 (C20); Anti-IL10; DAPI Anti-CD206 (C20); Anti-TGFβ; DAPI



(d)



Anti-CD206 (C20); Anti-CD68; DAPI Anti-CD206 (C20); Anti-TNFα; DAPI



(e)

Fig. 6 M1 and different sub-populations of the M2 macrophages visualized in IRR (a and b) and IRR+ASC regenerating skeletal muscle (c, d and e). (a) Anti-CD206 (Alexa488; green fluorescence) binds M2 macrophages. Anti-CD68 (Alexa568; red fluorescence) binds M1 and M2 macrophages. Anti-TGFβ (Alexa647; red fluorescence). (b) Anti IL-10 (Alexa568; red fluorescence). (c) Anti-CD206 (Alexa488; green fluorescence) binds M2 macrophages and satellite cells. Anti-CD68 (Alexa568; red fluorescence) binds M1 and M2 macrophages. Anti-TGFβ (Alexa647; red fluorescence). (d) Anti IL-10 (Alexa568; red fluorescence). (e) Anti TNFα (Alexa647; red fluorescence). Blue: nuclear staining with DAPI. Thin arrows: M2 macrophages; thick arrow: M1 macrophage; stars: M2a,c or d macrophage, triangle: M2b macrophage.

B. Immunofluorescence

Irradiated and non-irradiated muscle tissue were harvested just after euthanasia and submersed in liquid nitrogen and stored at -80°C . From the frozen samples, 10- μm sections were cut using cryostat microtome (Leitz-1720, Leitz, Stuttgart, Germany). To permeabilize cells, a 15-min treatment with 0.5% (v/v) Triton X100 buffered with PBS was carried out. After three washes with PBS, non-specific binding was blocked with 1% bovine serum albumin in PBS for 1h. Sections were then incubated overnight at 4°C with goat anti-CD206 antibodies (Santa Cruz Biotechnology, 1:500); mouse anti-CD68 antibodies (Thermo Fisher Sci.; 1:300); mouse anti-CD80 antibodies (Bioscience, 1:100); rabbit anti-TGF- β antibodies (Abcam; 1:500) or with rabbit anti-MMP2 antibodies Abcam; 1:1000). Cells were washed for 20 min in

PBS, and incubated with donkey anti-goat immunoglobulin Alexa Fluor 488 (Thermo Fisher Sci.; 1:1000); donkey anti-mouse immunoglobulin Alexa Fluor 568 (Thermo Fisher Sci.; 1:1000) and with goat anti-rabbit immunoglobulin Alexa Fluor 647 (Thermo Fisher Sci.; 1:1000) for 1 h. Finally, cells were washed for 20 min in PBS and mounted using Fluoroshield mounting medium (Abcam).

C. *In situ* hybridization

In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes. Briefly, muscles were fixed in 4% formaldehyde (freshly made from paraformaldehyde) and 0.1% Triton X-100 buffered with PBS (phosphate-buffered saline) under vacuum for 2×20 minutes, and left in fixative overnight. After fixation, tissues were washed, dehydrated and then embedded in paraffin. Ten µm sections were prepared and labeled with antisense probes, synthesized using digoxigenin (DIG)-UTP (Boehringer Mannheim) according to the manufacturer's instructions. Immunodetection of the DIG-labeled probes was performed using an anti-DIG antibody coupled to alkaline phosphatase as described by the manufacturer. Sections were viewing in a LEICA DM 6000B. No signal was detected in sections processed with a DIG-labeled sense cRNA probe (the negative control). The templates for transcription of different antisense probes were derived from a PCR-amplified fragments. The following primes were used: IL10_T3_UP

- TGAATTAACCCTCACTAAAGGGAatgccagctcagcact gctct IL10_T7_down
- TGTAATACGACTCACTATAGGGCtcagttcttctcatcttcat cTGFb1b_T3_UP
- TGAATTAACCCTCACTAAAGGGAcgtcggcgccgctg ctgctg TGFb1b_T7_down
- TGTAATACGACTCACTATAGGGCcactccaggactttgctttg tg TNFa_T3_UP
- TGAATTAACCCTCACTAAAGGGAatgagcactgagagcat gatcc TNFa_T7_down
- TGTAATACGACTCACTATAGGGCctcacagggaatgatcc caaa actin_T3_UP
- TGAATTAACCCTCACTAAAGGGAaaggccaaccgtgaga agatga actin_T7_down
- TGTAATACGACTCACTATAGGGCactcctgcttgcgatcca cat

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