FINAL SCIENTIFIC REPORT

SUMMARY

In the present study the effect of the loss of Nur77 transcription factor was investigated on the *in vitro* efferocytosis function, and on the *in vivo* polarization of bone marrow-derived macrophages (BMDMs) in the cardiotoxin-induced skeletal muscle injury model. Macrophage polarization is a process whereby macrophages develop a specific phenotype and functional response to different pathophysiological stimuli and tissue environments. In general, two main macrophage phenotypes have been identified: inflammatory (M1) and alternatively activated (M2) ones producing among many others IL-1β and IL-10, respectively. In the cardiotoxin-induced skeletal muscle injury model, monocytes arriving at the site of injury differentiate first to M1 macrophages that clear cell debris and trigger proliferation and differentiation of the muscle stem cells, while during the process of efferocytosis they change their phenotype to M2 to drive resolution of inflammation and tissue repair. Using this model, our laboratory shown previously that clearance of apoptotic cells (efferocytosis) and polarization of macrophages are strongly associated phenomena (1,2).

Nur77 transcription factor is expressed as an early response gene during efferocytosis. During this project we found that Nur77 null bone marrow-derived macrophages are characterized by elevated expression of the PPARγ transcription factor and enhanced efferocytosis capacity. In addition, Nur77 and PPARγ regulate transcription in different subsets of M2 skeletal muscle macrophages during muscle repair. Increasing evidence indicates that Nur77 can repress the expression of PPARγ, while PPARγ that of the Nur77. These observations might explain why Nur77 KO BMDMs express more PPARγ, and why these two transcription factors are expressed in distinct M2-like reparative skeletal muscle macrophage populations following cardiotoxin injury.

While PPARγ is known to promote phagocytosis of apoptotic cells and to facilitate M1/M2 conversion of macrophages, Nur77 is known to act as a negative regulator of the inflammatory responses. Our data confirm these observations, and demonstrate that the polarization of macrophages detected in the absence of Nur77 is conflicting - which is related to the loss of Nur77- mediated events - characterized by increased production of pro inflammarory IL-1β . In addition, we found enhanced Il-10 expression which is the consequence of the enhanced conversion of Nur77 KO M1 macrophages into the M2 direction.

The novelty of our findings is that we demonstrate for the first time that these two transcription factors function in different subsets of macrophages, They appear in simultaneously differentiating macrophages in the cardiotoxin-induced skeletal muscle injury model, and their mutual antagonism makes sure that in one subtype the PPARγ-, while in the other subtype the Nur77-mediated transcriptional events dominate. Our data provide additional information about the heterogeneity of the bone marrow-derived macrophages, and about their polarization.

DETAILED REPORT

Introduction

A key mechanism for maintaining tissue homeostasis is the effective execution of apoptotic cell death, followed by an efficient clearance by professional phagocytes. Every day billions of cells die in our body and efficiently cleared by macrophages during the process of phagocytosis without initiating inflammation or immune response. Apoptotic cells are not only removed by macrophages during the efferocytosis process and degraded, but macrophages also release various biologically active signaling molecules, which is triggered by the apoptotic cell uptake itself. In resting tissues these factors provide local trophic support, in the thymus they contribute to the thymic selection processes, whereas they drive the resolution of inflammation and tissue repair following tissue injury. Proper efferocytosis regulates the proper production of these engulfing macrophage-derived regulatory molecules.

Macrophages have the ability to both express distinct cell-surface receptors and release various bridging molecules for the recognition and adequate engulfment of apoptotic cells. Many of these receptors identify phosphatidylserine - a key ‘eat me’ signal on the surface of apoptotic cells - either directly or indirectly (via bridging molecules). Macrophages are capable for the continuous engulfment of dying cells as long as dying cells are around. So far, it has been demonstrated that these phagocytic receptors induce two parallel, evolutionarily conserved efferocytic signaling pathways, and both lead to the activation of the small GTPase Rac1, which initiate phagocytic cup formation via inducing actin polymerization.

Nuclear receptor Nur77 is one member of the nuclear receptor 4a (NR4a) subfamily, which belongs to the steroid/thyroid hormone receptor superfamily and is an orphan receptor for which no ligand is known. Besides forming a heterodimer with the retinoid X receptor to mediate retinoic acid-dependent transcription, it can also function as monomeric form, as well as a homodimer to regulate the Nur77 mediated signaling pathways. In addition, similar to other

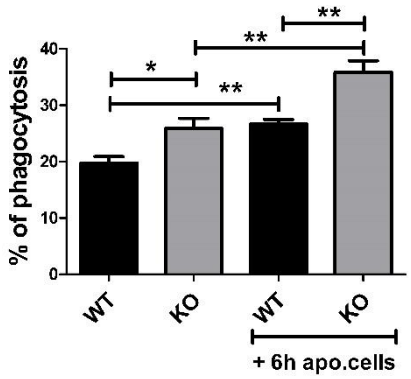
members of this transcription family, it is able to interact with other transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated 70 B cells (NF-κB), and regulate their transcriptional activity. Loss of Nur77 in macrophages has been reported to result in enhanced pro-inflammatory cytokine release following Toll like receptor 4 stimulation, and in an M1 type pro-inflammatory polarization in various atherosclerosis models. The effect has been linked to uncontrolled NFκB activation leading to enhanced transcription of various inflammation-related genes.

Recently the transcriptome of bone marrow-derived macrophages (BMDMs) from wild-type (WT) and Nur77-knockout (Nur77 KO) mice has been analyzed. In addition to the enhanced expression of a group of inflammation-related genes, IPA 80 Upstream Regulator Analysis revealed Rac1 as an activated upstream regulator possibly mediating changes in gene expression induced by the loss of Nur77. Since enhanced Rac1 activity of macrophages is known to be associated with enhanced efferocytosis capability, we decided to investigate phagocytosis of apoptotic cells mediated by the Nur77 KO macrophages. So far, it has been demonstrated that these phagocytic receptors induce two parallel, evolutionarily conserved efferocytic signaling pathways, and both lead to the activation of the small GTPase Rac1, which initiate phagocytic cup formation via inducing actin polymerization.

Results

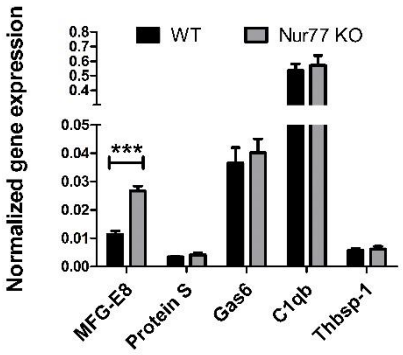
Nur77 null bone marrow derived macrophages have enhanced efferocytosis capacity

Since previous studies indicated that Nur77 null macrophages have an enhanced Rac1 gene signature, we checked whether Nur77 null BMDMs have an altered efferocytosis capacity. In line with the enhanced Rac1 gene signature, we also observed an increased efferocytosis capacity of BMDMs after a 45 min exposure to apoptotic cells. Loss of Nur77 resulted not only in an increase in the percentage of engulfing macrophages but also an increase in the number of engulfed apoptotic cells. This enhancement was more pronounced, if macrophages engulfed apoptotic cells first for 6 h, and their efferocytosis capacity was determined 18 h later.



Enhanced efferocytosis capacity of Nur77 null macrophages is related partly to enhanced MFG-E8 production

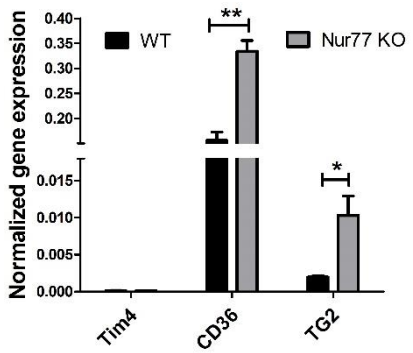
To check whether the observed difference in the efferocytosis capacity is related to a bridging molecule-driven efferocytosis pathway in Nur77 null macrophages, their phagocytosis was also determined after washing the cultured medium away. The efferocytosis capacity of both types of macrophages decreased, if the culture medium was washed away, and phagocytosis was determined in the absence of fetal bovine serum (FBS). What is more, in the absence of FBS, which might contain bridging molecules, the difference between the two types of macrophages completely disappeared. These data indicate that the enhanced efferocytic capacity of Nur77 null macrophages is related either to enhanced production of a bridging molecule, or to increased expression of a bridging molecule-dependent efferocytosis receptor.



To determine, whether enhanced production of a bridging molecule is responsible for the observed effect, we compared the mRNA expression of each bridging molecule in the wild-type and Nur77 null macrophages, and found that only that of MFG-E8 was increased, while no change in the mRNA expression of protein S, Gas6, C1qb or thrombospondin-1 (THSP)-1 was found.

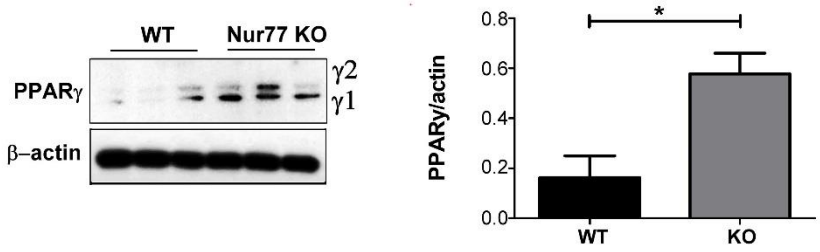
mRNA expression of the integrin coreceptor CD36 and TG2 is also enhanced in Nur77 null macrophages

Next, we checked whether gene expression of the target efferocytosis receptors of the various bridging molecules is altered. However, we have not found a change in the expression of Axl, MerTK, or CD91, targets of Protein S, Gas6, or C1qb. Previous studies have shown that MFG-E8 binds to various integrin receptors via its RGD domain. Integrin β1, β3 and β5 were all reported to participate in the uptake of apoptotic cells. In addition to bridging molecules, these integrins also utilize coreceptors for the function of efferocytosis, such as CD36 or transglutaminase 2 (TG2) (5). While the mRNA expression of integrin β1 and β3, and that of Tim4 was not altered by the loss of Nur77, the basal gene expression of both CD36 and TG2 was significantly higher in the Nur77 null macrophages, and we found a moderate increase in the expression of integrin β5 as well. Altogether, these data indicate that an enhanced integrin β3 and β5 signaling might be responsible for the enhanced efferocytosis capacity of Nur77-null macrophages.

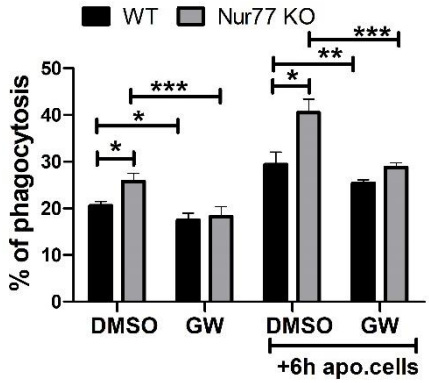


Nur77 null macrophages express elevated levels of PPARγ

Previous studies have shown that engulfing macrophages are capable of increasing their efferocytosis capacity via activation of their lipid-sensing nuclear receptors (liver X receptor (LXR)α/β, PPARγ and δ). Since the difference in the efferocytosis capacity between wild-type and Nur77 null macrophages was more pronounced, if macrophages have already engulfed apoptotic cells (demonstrated by first figure), when the content of the engulfed apoptotic material triggers activation of these nuclear receptors, we checked their mRNA expression. While the mRNA expression of PPARδ and that of LXRα did not change, the mRNA expression of PPARγ significantly increased in Nur77 null macrophages.



Enhanced PPARγ levels were detected in the Nur77 null macrophages at the protein level as well. In addition, the mRNA level of its known target gene fatty acid binding protein (FABP) 4 was also significantly elevated in the Nur77 null cells.

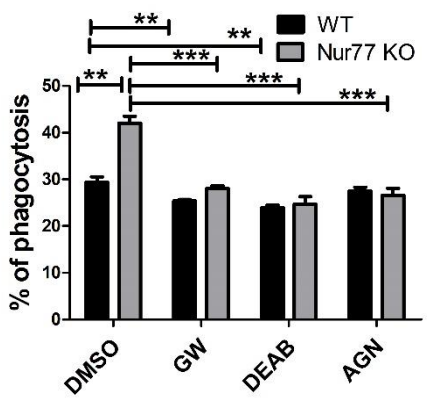


Accordingly, inhibition of PPARγ transcriptional activity by GW9662 for 24 h decreased the efferocytic capacity of Nur77 null macrophages. What is more, in the presence of the inhibitor the difference in the efferocytosis capacity of the two types of macrophages disappeared. Altogether these data indicate that the difference in the efferocytic capacity of the Nur77 null macrophages is related to an enhanced PPARγ transcriptional activity.

Nur77 null macrophages express significantly elevated level of RALDH2 and RARα

Previous studies from our laboratory have demonstrated that engulfing macrophages produce retinoids that upregulate the expression of various phagocytic receptors (6, 7). This enhancement in the phagocytosis gene expressions was partially mediated via an LXR-dependent induction of retinaldehyde dehydrogenases (RALDHs), enzymes that are

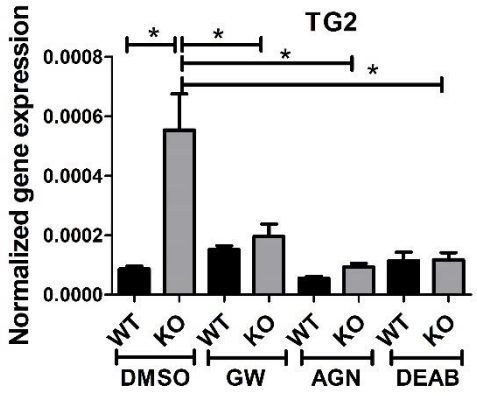
specifically involved in the synthesis of retinoic acids, and that of retinoic acid receptor (RAR)α, a nuclear receptor for which retinoic acids serve as ligands . We checked the mRNA and protein expression of RALDH2, the dominant RALDH in macrophages, and that of RARα. Expression of both RALDH2 and RARα was increased in Nur77 null macrophages as compared to their wild-type counterparts at both mRNA and protein levels. Accordingly, Nur77 null macrophages responded to all-trans retinoic acid (ATRA) treatment with a more significant increase in the efferocytosis capacity.



To determine whether PPARγ-regulated retinoid synthesis mediates the effect of the loss of Nur77 on the efferocytosis of macrophages, both wild-type and Nur77 null macrophages were pre-treated for 24 h with the PPARγ antagonist GW9662, the RALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB) or the panRAR antagonist AGN109, and their efferocytosis capacity was measured at the end of the treatments. These treatments decreased the efferocytosis capacity of both wild-type and Nur77 null macrophages, and the efferocytosis capacity of Nur77 null cells decreased to that of the wild-type ones.

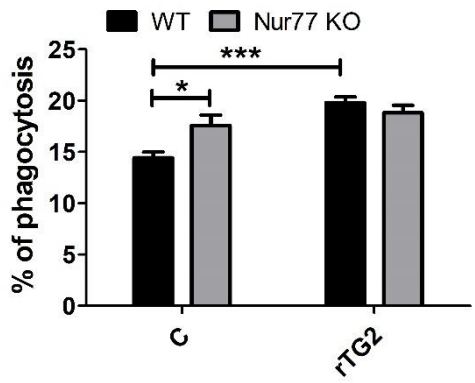
Increased efferocytosis capacity of Nur77 null macrophages is strongly related to the enhanced TG2 expression

Next we checked, how the above treatments affect the mRNA expression of those three phagocytosis genes, which we found to be significantly altered by the loss of Nur77. The most dramatic change was seen in the gene expression of TG2, in accordance with that its expression was enhanced most by the loss of Nur77. While these treatments hardly affected the basal levels of TG2 in the wild-type macrophages, each treatment reduced the TG2 mRNA expression of Nur77 null cells to the wild-type level. CD36 mRNA expressions, on the other hand, were only partially dependent on the PPARγ/retinoid signaling pathway, despite of the fact that CD36 expression was shown to be affected by both PPARγ and retinoids, while that of MFG-E8 was independent of it indicating that Nur77 must suppress the expression MFG-E8 in wild-type cells via a different mechanism.



Altogether, our data suggest that the enhanced PPARγ-dependent phagocytic capacity is largely dependent on the enhanced TG2 expression.

TG2 was shown to facilitate integrin signaling and to promote efferocytosis added as a recombinant protein. To determine whether addition of recombinant TG2 to wild-type macrophages could enhance their phagocytosis efficiency to the level of Nur77 null cells, efferocytosis capacity of both cell types was determined in the presence and absence of mouse recombinant TG2. Addition of recombinant TG2 enhanced the phagocytic capacity of wild-type macrophages, but not that of the Nur77 null cells. In addition, their phagocytic capacity was similar in the presence of recombinant TG2. These data provide further evidence for the TG2-dependence of the observed enhanced efferocytosis efficiency in Nur77-null macrophages.

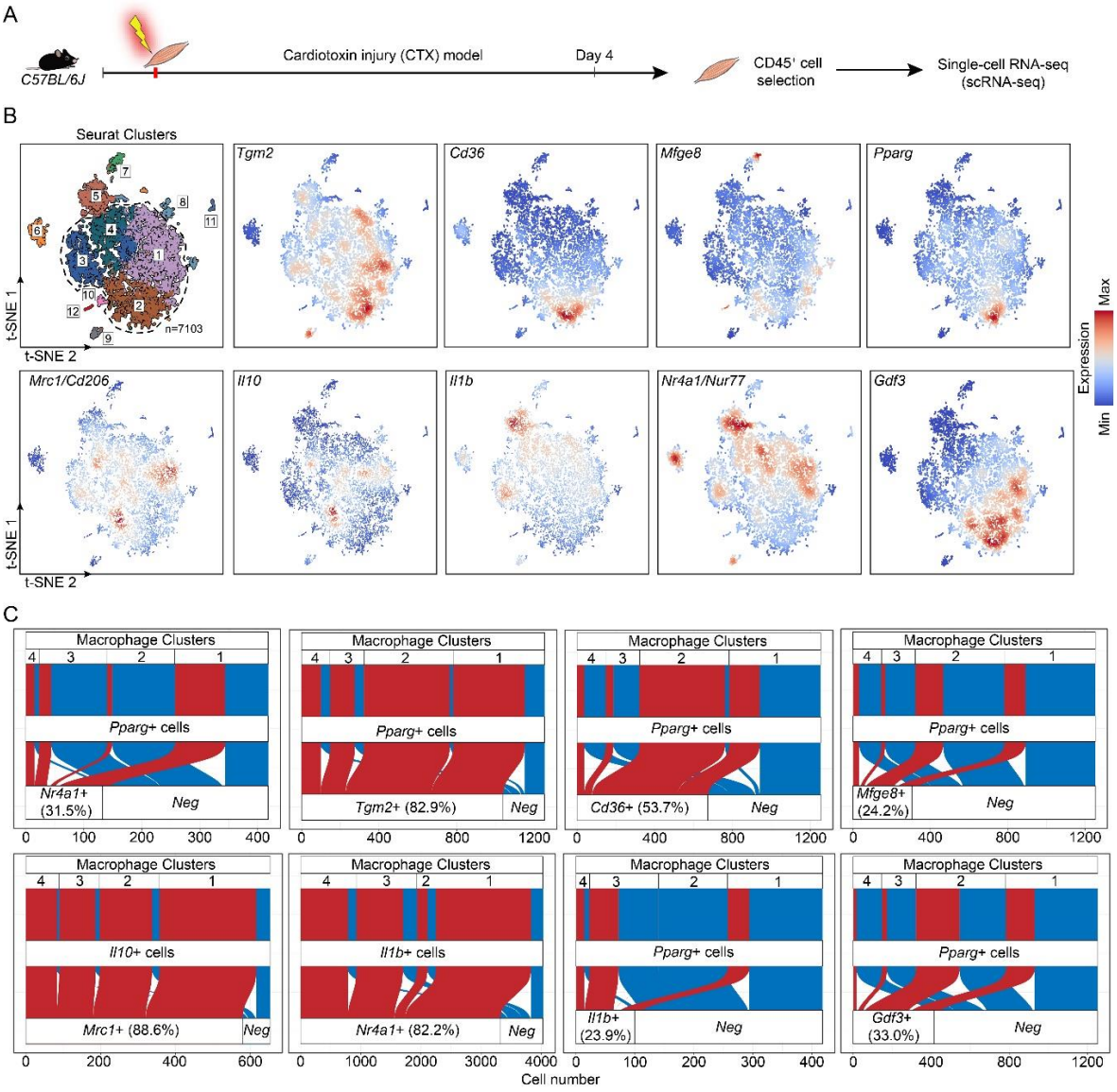


Nur77 and PPARγ are expressed by distinct groups of M2 macrophages which drive skeletal muscle tissue repair following cardiotoxin injury

Increasing evidence indicate that macrophages do not form one single group of cells, and even within their two main groups there is a big heterogeneity. The first group, the tissue resident macrophages are mainly derived from the yolk sac during embryogenesis. They act as sentinels in the tissues and play an essential role in tissue homeostasis. The second group, the BMDMs are recruited to the tissues in response to tissue injury induced by infection, autoimmune disorders, or by various injuries, and are crucial drivers and regulators of inflammatory and tissue regenerative responses. One model, in which the differentiation and function of BMDMs can be studied, is the cardiotoxin-induced injury model of the tibialis anterior skeletal muscle (3). Within this model, one day after the cardiotoxin injury monocytes arrive at the injury side and differentiate into M1 macrophages, which clear dead cells and, additionally, trigger muscle stem cell proliferation via the pro-inflammatory cytokines formed by them. As a result of the interaction with apoptotic cells during efferocytosis, these M1 macrophages are converted to M2 macrophages, and by day 4, they drive angiogenesis, myotube formation from myoblasts, and the resolution of inflammation.

Recent work by Patsalos et al. revealed the generation of three functionally distinct (growth factor producing, resolution-related and antigen presenting) populations of these M2 macrophages by analyzing their mRNA expressions using single-cell RNA sequencing (1).

Using these data, we demonstrate that on a single cell level PPARγ and Nur77 are expressed by different groups of M2 macrophages, and even when they are co-expressed, the PPARγ levels are low. PPARγ-dominant expression characterizes the growth factor producing macrophage population, while Nur77 is mainly expressed by the resolution-related and antigen presenting cells. What is more, TG2 and CD36 expressing cells partially overlap with that of the PPARγ-expressing ones, especially in the growth factor producing population, where the overlap is more than 90%, underlying our finding that PPARγ regulates their expression. However, there is little overlap between the PPARγ and MFG-E8 high expressing macrophages, in accordance with our *in vitro* finding that the increase in the MFG-E8 mRNA expression in Nur77 null cells is PPARγ-independent.

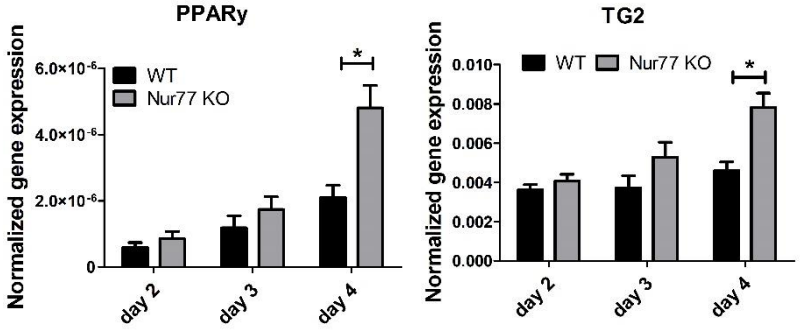


Immune single-cell transcriptomic map of regenerating murine skeletal muscle. **(A)** Workflow used for the isolation and analysis of previously published (1) single-cell RNAseq datasets from CD45^{+} cells isolated at day 4 following cardiotoxin-induced injury. (**B)** Upper left panel: unsupervised clustering

and t-SNE representation of CD45+ myeloid cells isolated from Day 4 post CTX injury (colored by 12 clusters defined using cluster resolution 0.4; dotted line indicates the four macrophage clusters 1-4; 1: Resolution-related, 2: Growth Factor-Expressing, 3: Pro-inflammatory, 4: Antigen-presenting macrophages). The rest of the panels indicate *Tgm2, Cd36, Mfge8, Pparg, Mrc1, Il10, Il1b, Nr4a1,* and *Gdf3* mRNA expression in the single-cell dataset. **(C)** Sankey plots that simultaneously define the number of cells with single and double positive expression for indicated genes and for each macrophage cluster. Red indicates cells with double positive/detectable gene expression and with blue the cells with single positive gene expression ("Neg": non-detectable gene expression in the third layer).

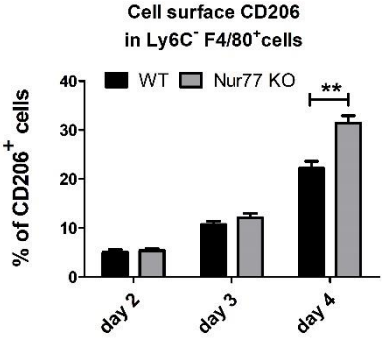
Loss of Nur77 leads to enhanced PPARγ and TG2 expressions, and to an accelerated CD206$^+$ macrophage formation in day 4 skeletal muscle macrophages following cardiotoxin-induced injury

Previous studies of our laboratory have demonstrated by studying macrophage polarization in the cardiotoxin-induced injury model of skeletal muscle that loss of TG2 not only affects the efficiency of the phagocytosis of dead cells, but also influences expression levels of PPARγ, and the M1/M2 conversion of macrophages by significantly delaying the appearance of CD206$^+$ M2 population (4). Using the same model, we tested how the loss of Nur77 affects the expression of PPARγ, and TG2, and the appearance of CD206$^+$ skeletal muscle macrophages following cardiotoxin-induced skeletal muscle injury. Simultaneously we also detected the disappearance of Ly6C, a marker, the disappearance of which allows us to follow the rate of M1/M2 macrophage conversion.



Expression of both PPARγ and TG2 was significantly higher in Nur77-null skeletal muscle-derived CD45$^+$ cells.

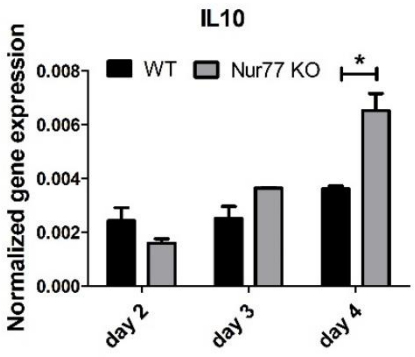
Accordingly, the appearance of CD206$^+$ cells was also significantly enhanced. However, in accordance with a previous report, we did not find a difference in the rate of M1/M2 conversion of skeletal muscle macrophages followed by the disappearance of the cell surface Ly6C molecule.



Interestingly, in TG2 null M2 macrophages the PPARγ expressions were found to be lower (4) indicating the existence of a positive autoregulatory loop that controls the expression of PPARγ, or the number of PPARγ-expressing cells. PPARγ upregulates TG2, while the produced TG2 protein promotes either the stable expression of PPARγ mRNA, or better survival of the PPARγ-expressing macrophages. Altogether, our data indicate that the Ly6C$^-$ PPARγ$^+$ TG2^{high} macrophages are the origin of at least one group of the Ly6C$^-$ CD206$^+$ macrophage population. Since TG2 acts as a coreceptor for integrins, our observations confirm that of others, who have demonstrated the involvement of integrins in the PPARγ-driven M2 conversion of macrophages.

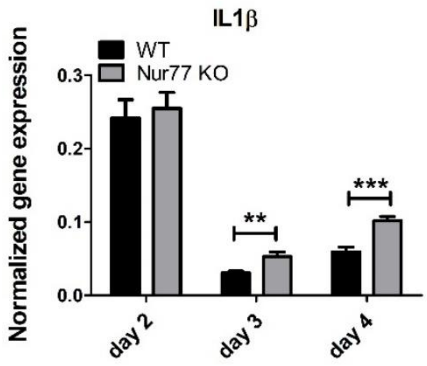
Loss of Nur77 results in contradictory production of pro- and anti-inflammatory cytokines by skeletal muscle macrophages following cardiotoxin-induced injury

M1 and M2 macrophages are characterized by the production of a different set of cytokines. While M1 macrophages produce pro-inflammatory cytokines, such as TNF-α, IL-1α, IL-1β, IL-6, IL-12, CXCL9, and CXCL10, M2 macrophages produce IL-10, TGF-β, CCL1, CCL17, CCL18, CCL22, and CCL24. During M1/M2 conversion the production of pro-inflammatory cytokines gradually decreases, while that of anti-inflammatory cytokines or growth factors increases. Since Nur77 was shown to suppress the expression of IL-1β, while PPARγ to induce IL-10 production, we decided to follow the mRNA expression of these cytokines in the skeletal muscle macrophages following cardiotoxin-induced injury.



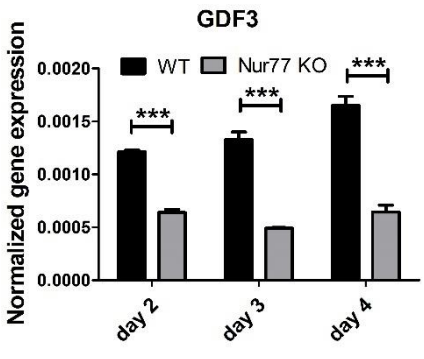
As shown in this figure, loss of Nur77 resulted in enhanced IL-10 production, a cytokine that plays a dominant role in promoting M2 conversion of the macrophages. Interestingly, we found that the producers of IL-10 are dominantly the CD206$^+$ macrophages (Demonstrated in figure from data by Patsalos et al.) suggesting that the observed enhanced IL-10 production is the consequence of the enhanced conversion of Nur77 null macrophages into the CD206$^+$ direction by day 4.

Altogether, these data demonstrate that PPARγ promotes IL-10 production by facilitating the formation of the IL-10-secreting $CD206^+$ macrophages. Surprisingly, while the IL-10 production was enhanced indicating an enhanced M2 polarization of Nur77 null macrophages in the cardiotoxin model of skeletal muscle injury, the IL-1β mRNA levels were also enhanced. This later indicates a prolonged inflammatory M1 state. However, as shown in figure from data by Patsalos et al., IL-1β is produced by separate populations of macrophages, which express dominantly Nur77, indicating that Nur77 plays a role in the suppression of this pro-inflammatory cytokine production during the resolution phase of the inflammation.



Loss of Nur77 results in decreased production of the growth factor GDF3

The way M2 macrophages are able to promote myoblast differentiation, myoblast fusion and myotube growth is that they produce various growth factors. Among them GDF3 was shown to be produced in a PPARγ-dependent manner. That is why we tested the expression of GDF3 in Nur77 null skeletal muscle macrophages following cardiotoxin injury.



To our surprise, despite the higher expression of PPARγ, the expression of GDF3 was lower in Nur77 null skeletal muscle M2 macrophages. Since the GDF3- and the PPARγ-expressing macrophage populations only partially overlap (Demonstrated in figure from data by Patsalos et al.), our data indicate that Nur77 might contribute to the GDF3 expression in those $Nur77^+$ macrophages, which do not express PPARγ. Accordingly, Nur77 null BMDMs also showed about a 55% decrease in the GDF3 mRNA expression, as reported by others as well.

References

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