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Role of Macrophages in Muscle Transfection with pDNA/ PLURONIC Formulation

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ROLE OF MACROPHAGES IN MUSCLE TRANSFECTION WITH pDNA/PLURONIC FORMULATION

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ROLE OF MACROPHAGES IN MUSCLE TRANSFECTION WITH pDNA/PLURONIC FORMULATION

Non-ionic amphiphilic block copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), Pluronics, arranged in a tri-block structure PEO-PPO-PEO, have raised a considerable interest in skeletal muscle Gene Therapy. Previous studies have demonstrated that co-administration of Pluronics with naked plasmid DNA (pDNA) by direct i.m. injection enhanced transgene expression not only in muscle but also in distal lymphoid organs (spleen and lymph nodes) and this response was strain-dependent; not observed in athymic (BALB/c nu/nu) mouse; suggesting a role of immune cells in gene transfer to skeletal muscles. Therefore, we first evaluated the role of inflammation and inflammatory cells, on muscle transfection. We showed that local inflammation in murine hind limb ischemia model (MHLIM) drastically increased DNA, RNA and transgene expression levels in muscles injected with pDNA/Pluronic. Ischemic muscles showed high number of GFP^+ muscle fibers with GFP expression co-localized with $desmin^+$ muscle fibers and $CD11b^+$ macrophages (MØs). This suggested that MØs assist muscle transfection with pDNA/Pluronic. Moreover, adoptively transferred MØs were shown to pass the transgene to inflamed muscle cells. Hence, we developed an *in vitro* model of inflammation by co-culturing transfected MØs with myotubes (MTs) and used constitutive (cmv-luciferase) or muscle specific (desmin-luciferase) reporter gene expression to show that Pluronic P85 enhances the horizontal pDNA transfer from MØ to MTs. Systemic inflammation (MHLIM and peritonitis) also increased the transgene expression with pDNA/Pluronic but not pDNA alone. Second, we carried out an immunological profiling of adjuvant (P85/LPS/Alum) induced cellular recruitment and showed that P85 helps modulate the response towards MØs predominated recruitment. Moreover, the contribution of MØ predominance in assisting gene transfer after i.m. injection of

pDNA/P85 was reinforced by *in vivo* MØ depletion that resulted in near complete attenuation in gene expression to the level of naked pDNA alone. Finally, MØ recruitment response was harnessed to further increase muscle transfection by preinjecting P85 at the subsequent sites of pDNA/P85 injection resulting in improved muscle transfection. This dissertation study provides a key evidence about the role of MØs in assisting gene transfer in pDNA/Pluronic delivery approach. Altogether, we introduce Pluronics as simple and commercially available excipients to enhance gene transfer using pDNA/Pluronic admix compositions; are recognized pharmaceutical excipients in US and British Pharmacopoeia and therefore have great potential in human gene therapy clinical trials in healthy and disease pathologies that often involves inflammation.

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LIST OF SYMBOLS AND ABBREVIATIONS USED

A	Adductor
APC	Antigen presenting cell
AUC	Area under curve
CGN	λ-Carrageenan
CM	Complete media
CMC	Critical micelle concentration
CMV	Cytomegalovirus promoter
DC	Dendritic Cell
DM	Differentiation media
DMEM	Dulbecco's minimal essential media
DNA	Deoxyribonucleic acids
dNTP's	Deoxyribonucleotide triposhpate
EDTA	Ethylene diamine tetra acetic acid
EGT	Electroporation Gene Transfer
EO	Ethylene oxide
Ex/Em	Excitation/Emission
ECM	Extra cellular matrix
FA	Femoral artery
FBS	Fetal bovine serum
Fig	Figure
FSC	Forward scatter
FV	Femoral vein

G	Gastrocnemius
GADPH	Glyceraldehyde 3-phosphata dehydrogenase
GFP	Green fluorescent protein
HBSS	Hank’s balanced salt solution
HE	Hematoxylin and eosin
HLB	Hydrophillic lipophillic balance
HS	Horse serum
HYAse	Hyaluronidase
i.j.v.	Intra jugular vein
i.m.	Intramuscular
i.p.	Intraperitoneal
IHC	Immuno histochemistry
IVIS	*In vivo* imaging system
LPS	Lipopolysaccharide
LP	Long pulse
MBs	Myoblasts
MCSF	Macrophage colony stimulating factor
MHLIM	Murine hind limb ischemia model
Min	Minute
MOs	Monocytes
MPs	Macrophages
MT	Myotubes
Neu	Neutrophil
NF-kB	Nuclear factor kB

n.s.	Non-significant
P85	Pluronic P85
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEC	Peritoneal exudate cells
PO	Propylene oxide
Q	Quadriceps
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SA	Saphenous artery
SEM	Standard error mean
SP	Short pulse
SV	Saphenous vein
SFM	Serum free media
SSC	Side Scatter
TA	Tibialis anterior
TLR	Toll like receptor
TNF	Tumor necrosis factor
vol	Volume
w	Weight
WBC	White blood cells
%	Percentage

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CHAPTER 1

1.1. Introduction

DNA was first identified in 1869 by a Swiss Physiological chemist, Friedrich Miescher from nuclear isolates of white blood cells, as a molecule which was resistant to protease digestion, therefore was not protein so he called it “nuclein” which was later named “nucleic acid” and finally “deoxyribonucleic acid” or DNA (1). The 3-dimensional structure of DNA, was not known until 1953, when Watson and Crick made a ground breaking conclusion that 1) DNA exists in double helix, 2) the double helix is a right handed structure, 3) the strands run in antiparallel direction, and 4) the two strands are connected together by hydrogen bonding (2). Since the structure showed how the nucleotides in the opposite strands are paired (A-T and G-C), it helped enormously understand the genetic code of life and its applications in gene therapy and other fields.

The concept of “Gene Therapy” was then proposed in 1960s and 1970s that DNA molecule encoding therapeutic protein has a great potential and can be used to replace defective genes in patients with genetic disorders. This lead to research focus on vectors to deliver genes and the first gene therapy clinical trial was approved in 1989. At present, a total of approx. 2000 clinical trials worldwide have been conducted until the end of 2015 for various diseases (http://www.wiley.com/legacy/wileychi/genmed/clinical/). The approaches for delivering genes in the clinical trials can be basically divided into two categories; viral gene delivery and non-viral gene delivery. Most of the clinical trials studies (~70%) have been conducted using viral vectors because of natural tropism whereby, viruses have inherent capacity to bypass various cellular barriers for transporting DNA into nucleus that results in very high transfection of various tissue/cell types. However, the viral vectors pose severe safety concerns due to their ability to generate strong immune

responses and the possibility of integration into host proto-oncogenes. Most notable being the death of a patient in USA in 1999 due to inflammation against modified adenovirus vector in phase-I clinical trial study conducted by University of Pennsylvania-Philadelphia (3-5). In another study conducted in France, out of 11 patients treated for X-linked SCID disease or bubble boy disease syndrome using retrovirus vector, 3 patients developed T-cell leukemia due to integration of virus downstream of a transcription factor, whose over expression resulted in T-cell lymphoblastic leukemia (6, 7). Similarly, one patient in England also developed T-cell leukemia when treated with similar retrovirus treatment. These events led to immediate halt of other gene therapy clinical trials worldwide using retroviruses for gene delivery purpose to X-SCID patients. Another patient in USA in 2007 died of receiving an AAV vector for treatment of rheumatoid arthritis but the reason for her death has been under debate.

On the other hand, most of the non-viral gene delivery platform (~ 23% of total worldwide clinical trials) utilizes plasmid DNA (pDNA) for therapeutic protein expression or DNA vaccination. The major benefit of using pDNA is its excellent safety profiles unlike viral gene delivery approaches and are discussed in detail in chapter 2. Direct injection of naked pDNA results in muscle transfection and reporter protein expression *in vivo* was serendipitously discovered in 1990s. Even now, naked pDNA injection is by far the most common method of non-viral gene delivery in clinical trials for 1) local or systemic therapeutic protein expression and 2) expression of antigen protein for DNA vaccination. However, this approach suffers a major drawback of low gene expression versus viral gene delivery which in contrast results in stable and high gene expression using some viruses. Not many advancements in the approach of naked pDNA delivery have been made so far that can help increase muscle transfection except electroporation mediated gene transfer (EGT). EGT employs high voltage electric pulses using electrodes to drive

pDNA across the muscle membranes but suffers a major drawback of inducing significant muscle damage and cannot be used in muscular dystrophies where the aim is the rescue already degenerating tissue.

pDNA is a negatively charged macromolecule and cannot cross the cell membrane barrier which is why researchers tried to complex it with cationic lipids “Lipoplex” or cationic polymers “Polyplex” to make neutral nanoparticles to enhance its cellular uptake. This approach significantly enhanced the transfection of pDNA *in vitro* in various cell types but no such increase was observed when these complexes were injected directly into muscle. Rather, naked pDNA alone transfected muscles to higher levels than any lipoplex or polyplex used (8). Therefore, there is a need to understand the complex uptake of naked pDNA in the skeletal muscle so that the delivery approach can be improved. This would help open up new avenues for therapeutic application of naked pDNA especially in diseases where currently viral vectors are the only option as gene delivery vectors.

One unique platform that enhance gene expression of pDNA in muscle are the Pluronic block copolymer. Pluronic block copolymers are non-ionic triblock structures that consist of hydrophilic Ethylene Oxide (EO) and hydrophobic Propylene Oxide (PO) blocks arranged in A-B-A structure, i.e. EO_n-PO_y-$EO_{n,}$ where the length of EO and PO blocks vary in different types of pluronics. Pluronic belong to a category of non-ionic copolymers known by names poloxamers, Synperonics, CRL, SP. Pluronics have been categorized into families according to the arrangement as A-B-A (pluronic) and B-A-B (pluronic R). Every pluronic has a different hydrophilic-lipophilic balance (HLB) ratio depending upon the relative balance of EO and PO units and different critical micelle concentration (CMC). Accordingly, hydrophobicity of a pluronic is inversely correlated with the HLB value. Nomenclature of Pluronic uses letters **F** solid, **P** paste and **L** liquid followed by three or

two numeric digit. The last digit of the Pluronic name corresponds to percentage of ethylene oxide i.e. 8 means 80% of the EO and the first one or two corresponds to approximate molecular weight of PO when multiplied with a factor 300. e.g. F127, L61 etc. When coadministered with pDNA, these copolymers increase gene expression in orders of magnitude in DNA injected muscle. Because these are non-ionic so unlike cationic lipids/polymers, they do not interact with pDNA, implying that condensation of pDNA in lipoplexes and polyplexes may be the reason for decrease in muscle transfection compared with naked pDNA alone. In addition to other advantages, pluronics also provide relatively safe formulations in comparison to cationic polymers and the toxic side effects of pluronics have been reported to be correlated to the lipophilicity of the copolymers and occur far beyond the concentration used for gene therapy. Notably, pluronics are mentioned in United States Pharmacopoeia as FDA approved excipients for drug-formulations.

Pluronics offer a very simple and cheap formulation for muscle transfection, and they have been studied extensively for drug and gene delivery (9). As an adjuvant, Pluronics have been widely used in protein formulations for vaccination purposes. Pluronics were shown to increase the systemic expansion of Dendritic Cell (DC) and local expansion of DC, macrophages and natural killer cell population. Therefore, copolymer based muscle transfection in comparison to naked pDNA approach offers two great models to study the mechanism of muscle transfection. In our previous studies, we have observed that immune system plays a crucial role in muscle transfection and gene expression due to following observations: 1) when coadministered with pDNA, Pluronic P85 increased gene expression not only in injected muscle tissue but also in the distal lymphoid organs such as draining lymph nodes and spleen. 2) The gene expression colocalized with $CD11b^{+}$ APCs in muscle, lymph nodes and spleen and the proportion of

GFP^+APCs increase upon coinjecting pDNA with P85. 3) Furthermore, we observed that increase in muscle gene expression in naked pDNA upon coadministering with P85 was strain-dependent and was not observed in athymic nude mice. All the observations suggested that block copolymer based gene delivery approach may employ immune system cells for increasing the gene expression in muscles. Therefore, in this dissertation study, we focused on the role of immune cells in block copolymer based muscle transfection for assisting gene/protein transfer to the skeletal muscle fibers using different *in vivo* and *in vitro* models. Specifically, chapter 1 introduces problem, hypothesis and specific aims of the thesis. Chapter 2 serves as an introductory basis on the role of inflammation (immune cells and inflammatory factors) on gene expression of non-viral gene delivery. Chapter 3 focuses on the role of inflammation (local and distal) and inflammatory cells on gene expression of naked pDNA alone and when coformulated with block copolymers (P85 and SP1017). Chapter 4 describes a rational approach to dissect the innate immune response to naked pDNA alone or when coformulated with block copolymers using peritoneal lavage and characterize and proves the role of specific cells (macrophages or MØ) in muscle transfection. Also, this chapter presents an engineering approach where MØs were recruited to the subsequent sites of pDNA injection to further increase the muscle transfection levels of pDNA/P85. Finally, Chapter 5 focuses on the overall discussion, conclusion and future studies.

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CHAPTER 2

REVIEW OF LITERATURE: ROLE OF INFLAMMATION IN GENE DELIVERY TO MUSCLE

2.1. Gene delivery

DNA delivery for therapeutics is a relatively new field of biotechnology in comparison to small molecule and proteins therapeutics. Specifically, genes can be delivered to target cells for therapeutic protein expression such as dystrophin in muscular dystrophy (1) or to express antigenic proteins for prophylactic or therapeutic vaccination response (2). DNA delivery is particularly a viable approach for substituting protein delivery in host tissue due to practical hurdles like short half-life, instability and high cost of manufacturing and immunogenicity of therapeutic proteins (3).

2.1.1. An overview of viral and non-viral gene delivery

As of January 2015, majority, ca.70% (n=1446) of the worldwide gene therapy clinical trials have been conducted using viral vectors and the second major ca. 23% (n=546) using non-viral vectors, mostly naked plasmid DNA (pDNA) (http://www.wiley.com/legacy/wileychi/genmed/clinical/). In lieu of obvious advantages offered by viral vectors, high immunogenicity (humoral and cellular immune response from high vector dose), complex delivery systems, inability of repeated administration of same serotype and high cost of production limits its application (4). On the contrary, non-viral gene delivery using naked pDNA, is preferred due to their ease and low cost of production, flexibility to customize, very stable at room temperature, low immunogenicity and most important being the ease of repeated administration. However, despite of all advantages, pDNA delivery is infamous for practical hurdles like inefficiency to transfect skeletal

muscles and transient increase in gene expression. Therefore, recent technological developments in non-viral gene delivery (physical, chemical and biological methods) has focused mainly on enhancing muscle transfection with naked pDNA to clinical meaningfulness. At the same time, all technological innovations added to the complexities and cost, to a moderate levels to otherwise simple pDNA delivery system. Nevertheless, in spite of all hurdles, naked pDNA is still preferred for gene delivery in human clinical trials.

2.1.2. Bottlenecks of non-viral gene delivery

Inflammation is a complex phenomenon and even at lower extents, it can have some negatively impacts on non–viral gene delivery. Specifically, inflammation can be actuated due to unmethylated CpG dinucleotides or CpG motifs of viral promoters in pDNA backbone, foreignness of chemical vectors (e.g. cationic lipid/polymer) and the invasiveness of physical delivery approach (e.g. electroporation). Notably, these events are at far lower levels than immune responses against viral vectors. Even though the local adjuvant effect, including release of pro-inflammatory factors and recruitment of inflammatory cells, are a prerequisite for an effective gene delivery for vaccination, these negatively affect transgene expression. For e.g. un-methylated CpG sequences in bacterially derived pDNA activates pro-inflammatory factor secretion (TNF-α, IL-12, IL-6, IFN-γ) resulting in premature silencing of gene expression (5). However, the inhibitory effect on gene expression was reduced by using methylated pDNA or by using purified expression cassettes without CpG pDNA backbone (5). The exact mechanism of epigenetic silencing of the pDNA has not been elucidated yet, and use of hybrid promoters with partial or exclusively mammalian sequences have helped prolong gene expression (6). The transient gene expression can also be very tissue specific and dependent on cell turnover, which is why the gene expression in tumor tissue is very short lived (7, 8).

Moreover, inflammation response due to direct intra-muscular injection is unescapable. Therefore, it is imperative to understand and dissect its effects on naked DNA gene expression for unravelling the inhibitory responses and use it in the favor of stable gene expression.

2.1.3. Focus of the review

Because of inflammation being a complex response and there are known negative impacts of inflammatory signaling on gene expression, technological developments in gene delivery studies rationalize increased gene transfer to the technology itself keeping the effects of inflammation on the side track. These observations along with our experience where inflammatory cells help gene expression, prompted us to bridge the knowledge gap between the positive influence of inflammation on transcriptional activation or gene transfer to the skeletal muscles. Therefore, we compiled reports on each successful gene delivery approach using naked pDNA with/without a vector and their effects alone or in combination on inflammation and transgene expression.

2.2. Inflammation and non-viral gene delivery.

We believe that inflammation, can be utilized as a friend than a foe to assist gene expression for multiple reasons. Firstly, most of the pDNA vectors carry a constitutive cmv-promoter, composed of multiple nuclear factor κB (NF-κB) binding sites, which is highly responsive to NF-κB phosphorylation and transcriptional activation during inflammatory responses (9). Secondly, basal NF-κB levels in cell cytoplasm are enough to drive pDNA import from cytoplasm to nucleus and this response can further be enhanced in inflammation (10). Thirdly, cellular infiltrates during inflammation are majorly composed of highly phagocytic myeloid cells such as neutrophils, monocytes,

macrophages which can uptake pDNA and express reporter genes thus representing as an additional reservoir to muscle fibers resulting in increased total gene expression (11, 12). Finally, monocytes and macrophages can also assist in the transfer of genes/proteins to the neighboring cells thus increasing the transfection efficiency (discussed later).

2.2.1. Mechanism of gene transfer in skeletal muscle

Muscle transfection by direct i.m. injection of naked pDNA was a serendipitous discovery in 1990 by John Wolff and coworkers at University of Wisconsin-Madison (13). The results were striking and proposed mechanisms suggested a pDNA transfer through membrane disruptions caused by needle penetration but no histological evidence were provided to support the conclusions. A decade later, highly efficient liver transfection by large volume (2.5 ml) hydrodynamic tail vein injection of naked pDNA was also reported (14). It was suggested that a pDNA uptake in muscle and in liver is mediated through a specific receptor, which can be inhibited upon co-delivering polyanionic molecules (15). Specifically, pDNA uptake was inhibited upon co-administration of heparin, polyglutamic acid, polyinosinic acid, dextran sulphate but not by all polyanionic molecules e.g. polycytidilic acid and chondroitin sulphate. This suggested a role of scavenger receptors in pDNA uptake in a charge and/or structure dependent manner (16). A successful pDNA uptake and gene expression was found to be a slow mechanism that required accumulation of pDNA molecules in muscles in first 4 h (15). With no conclusive mechanism of pDNA uptake the research focus of naked pDNA-based gene delivery was diverted towards the technological advancements (physical chemical or biological vectors) to enhance the muscle gene expression levels which alter the mechanism of pDNA uptake as discussed later.

2.2.2. Parameters affecting gene expression in skeletal muscle

Immediately after direct i.m. injection of naked pDNA was proposed, parameters affecting the gene expressions were studied for many years with a hope to further optimize and increase the levels of gene expression. These include the effect of needle type, injection speed, direction of injection (perpendicular or parallel to muscle fibers), injection volume, osmotic gradient of injection fluid, type of solute, type of muscle tissue, physiologic condition of the muscle, age and sex of the animals, pre-injection (hypertonic sucrose solution, bupivacaine, barium chloride), promoter type, size and form of DNA (linear, open or supercoiled) (17-23).

2.3. Effect of inflammation on gene expression of naked pDNA

Regardless of the painstaking efforts, no significant improvements were made probably because the serendipitous observation of muscle transfection upon direct injection of naked pDNA gave a quick lead to gene therapy field over complex skeletal muscle biology which was not understood at that time. Although, skeletal muscles were known for a mysterious regenerative potential unlike any other tissue the beneficial role of inflammatory cells in helping muscle regeneration was not known until lately and discussed below. Similar muscle regeneration and inflammatory response can also follow after physical injury caused by direct i.m. injection of pDNA and the unmethylated CpG sequences in the pDNA backbone (24). As discussed below, we believe that inflammatory response and muscle transfection can be mutually inclusive and can enhance gene expression levels.

2.3.1. Direct injection of naked pDNA in muscle

Many past studies related to naked pDNA injection in muscle have reported overlapping observations of muscle inflammation dependent increase in transgene expression not only in skeletal muscle but also in the distal lymphoid organs. The earliest

study was reported by Takeshita et al when inflammation resulted in an order magnitude increase in gene expression in ipsilateral ischemic muscles compared to intact contralateral muscles upon direct i.m. injection of pDNA (25). However, the possible effects of inflammatory cells in contributing to muscle gene expression were not discussed. The first indirect role of inflammatory cells in contributing to the gene expression in healthy muscle or draining lymphatic tissues was reported by Torres et al. The authors showed that surgical removal of *TA* muscle shortly after pDNA administration (10 min) did not abrogate the Ag specific immune response suggesting that the genes in the muscles were probably taken up and expressed by the draining APCs in the distal organs such as lymph nodes or spleen to induce protective immunity (26). Later, many follow up studies reported transgene expression in dendritic cells and/or MØ in the draining lymph nodes and spleen after administration of pDNA by scarification (27), i.m. injection (12) and intradermal injections (28). The results were further confirmed by tissue distribution studies of fluorescently labeled pDNA in *TA* muscle by Dupuis et al who showed that majority of the Rh-pDNA 24 h after injection co-localized with $CD11b^{+}$ APCs in muscle and draining lymph nodes suggesting that earlier observations on colocalization of reporter protein in APCs was not exclusively because of protein uptake from the transfected muscles but also by reporter protein expression due to pDNA uptake (11). Interestingly, Gaymalov et al showed that co-administration of synthetic adjuvants such as amphiphilic block copolymers with pDNA substantially increased gene expression in CD11b+ APCs in injected muscles, draining lymph nodes and spleen compared to naked pDNA alone which suggested an additive effect of gene expression cause by artificial APC recruitment response by copolymers (29). All-together, a body of evidence exists for the involvement of immune cells in transgene expression in muscle and distal organs which can be maneuvered by use of additional adjuvants.

2.3.2. Rapid intra vascular injection in muscle

Upon direct i.m. injection, the diffusion of pDNA in skeletal muscle is limited because pDNA has to cross the extracellular matrix to reach each muscle fiber. Since muscles have a high density of capillaries that reach out to each muscle fiber, the diffusion barrier can be avoided by intra vascular delivery which is also clinically attractive approach for delivering the pDNA to different muscles. In 1998, Budker et al for the first time showed a successful arterial gene delivery approach in small animals (rats) using large volume (10ml) injection and outflow of liquid blocked that resulted in 40 times increase in muscle transfection compared to i.m. injection (30). Later, another successful delivery in large animals (rhesus monkeys) was reported by Zhang et al. who delivered 150 ml pDNA solution using catheters via femoral artery with a simultaneous tourniquet to stop the outflow of liquid resulting in transfection of up to 40% of the muscle fibers (31).

Vascular delivery approach allowed transgene expression throughout the muscle in comparison to local expression around needle track upon direct i.m. injection and it reproduced in both small and large animals. However, other studies showed an increase in serum creatine kinase, minor swelling and accumulation of neutrophils ($CD43^+$) and macrophages ($ED1^+$ and $ED2^+$) in muscles following tourniquet approach confirming an inflammatory response due to high volume liquid injection in vasculature (32, 33). We believe, that these immune cells can also contribute to the total gene expression but these studies didn't analyze any co-localization of transgene expression with macrophage or neutrophil markers.

2.3.3. CpG sequences

Upon phosphorylation of inhibitor kappaB protein, active NF-kB transcription factor enters nucleus and binds unmethylated CpG motifs of TATA promoter sequence for transcriptional activation of genes (34-36). Therefore, most commercially available pDNA

vectors are designed with viral promoters (human cytomegalovirus [CMV] and rous sarcoma virus [RSV]) that carry unmethylated CpG sequences for driving a constitutive expression of reporter transgenes in various cell and tissue types (37). During inflammation, activation of various signaling pathways results in NF-kB upregulation which can enhance reporter gene expression (9, 34-36). Sriadhibatla et al. showed that Pluronic enhanced gene expression in stably and transiently transfected fibroblasts was due to activation of stress signaling (upregulated hsp-68) resulting in enhanced NF-kB and AP-1 levels (38). The CpG driven gene expression is a very specific response and methylation and unmethylation of CpG in rous sarcoma virus promoter was shown to decrease and increase transgene expression respectively (39).

2.4. Effect of inflammation on chemical gene delivery

Naked pDNA alone has a short plasma half-life (< 5min), therefore *i.v.* injection for lung/liver delivery requires encapsulation with cationic non-viral vectors. Therefore, calcium phosphate, cationic lipids (most popular), cationic polymers and cationic amino acids have been utilized to neutralize the charge, condense the pDNA, protect from nuclease degradation and increase the cellular uptake via endocytosis. Nevertheless, surface chemistries and combinations of non-viral vectors can activate immune responses resulting in inflammation as discussed below.

For instance, pDNA without CpG motif and cationic liposome alone were shown not to activate inflammatory pathways in MØs as expected. However, complexes of both triggered release of inflammatory cytokines by MØs *in vitro* suggesting a CpG independent inflammation (40). Another study suggested that inflammatory cytokines induce epigenetic silencing of pDNA resulting in transient gene expression. Therefore, for a successful gene delivery, DNA formulations need to be specifically designed to keep the inflammation at the lowest. Kako et al. showed that cationic lipid carrier and unmethylated

CpG motifs of pDNA cause systemic inflammation upon *i.v.* injection of lipoplex resulting in high serum TNF-α levels and initial increase in gene expression in various organs compared to pDNA alone injections (41). The effects of inflammation-dependent increase in gene expression were confirmed when depletion of MØ by clodronate and inhibition of inflammation by dexamethasone decreased the initial gene expression in liver, spleen and lungs (41). However, dexamethasone treatment resulted in prolonged gene expression. This was likely due to effects of lower systemic inflammation and TNF-α levels that resulted in lower epigenetic silencing of pDNA. This suggests that MØ are closely associated with CpG induced TNF-α secretion. Similar responses were observed on liver gene expression (42). Specifically, gene expression after direct RII infusion (retrograde intrabiliary infusion) of PEI-pDNA, chitosan/pDNA and naked pDNA alone increased in MØ depleted mice than healthy mice from day 3 until day 15 (42) again suggesting that MØ induced inflammatory responses cause epigenetic silencing. Therefore, MØ induced inflammation plays a central role behind the transient gene expression and also provides an evidence for enhancing the initial gene expression.

2.5. Effect of inflammation on physical gene delivery

Electroporation mediated gene transfer (EGT) is the most promising technique of muscle transfection using naked pDNA. The procedure involves electrophoretic movement of naked pDNA across the transiently generated aqueous cell membrane pores in muscle. The technique was introduced as early as 1998, but the physiological and histological effects of electric pulses on muscle tissue had not been studied or/and reported in detail until lately (43, 44). The procedure induces local inflammation and massive recruitment of APCs depending on the pulse strength, pulse duration and distance of electrodes. Importantly, pulse voltages directly correlate with the levels of muscle transfection and relatively stronger electric pulses (voltages from as low as

100v/0.5cm to as high as 2000V/0.5cm) can increase the gene expression by 2-3 orders of magnitude along with unavoidable inflammation response in muscle (45, 46). Apparently, inflammation benefits the efficacy of otherwise non immunogenic pDNA vaccines mostly due to increased antigen processing and presentation provided by additional inflammatory cells (44, 47). On the downside, EGT results in ~80% decrease in muscle torque and contractility immediately after the procedure due to inflammation. Therefore, physiological assessments suggested lowering the voltages to at least 75V/cm along with pre-injection of hyaluronidase for minimal muscle damage with relatively higher gene expression (44, 48).

So far, not many studies except few have studied in detail the contribution of transgene expression by inflammatory APCs or mononuclear cells in addition to transfected skeletal muscle fibers. Gronevik et al showed that the number of transfected APCs per muscle after naked pDNA injection increased from 72±12 and 35±12 in soleus and quadriceps respectively to 447±95 and 499±11 due to EGT procedure (49), or 6-fold and 14-fold increase in GFP+ mononuclear cells in rats and mice respectively (49). Therefore, it implies that APCs can provide an additional reservoir for gene expression in the pDNA injected muscle and relatively stronger electric pulses should recruit higher number of these inflammatory cells resulting in higher additive effect in transgene expression. Similar observations were reported by others when longer (400µs instead of 200 µs) and stronger (200 V/cm instead of 50 or 100 V/cm) electric pulses recruited APCs to a higher and lower levels respectively (50). Moreover, the gene expression of IgG2b in the initial weeks (1-3 weeks) was higher after long pulse (LP) EGT compared to short pulse (SP) EGT probably because of higher proportion of initial gene expression in cellular infiltrate along with muscle tissue. Later, at 4-12 weeks, once the inflammation was resolved, gene expression profiles of SP-EGT and LP-EGT flipped suggesting that high

inflammation after LP damaged the tissue of transfected muscle fibers what lead to a decrease in net gene expression at later time points (50). In another study, muscle gene expression of cmv promoter-endothelial nitric oxide synthase showed a linear increase upon i.m. injection of pDNA with EGT on day 0, 3 and 7 post ischemia surgery compared to intact muscles (51). Such an increase can be explained by the additive effect of gene expression by increasing recruitment of local inflammatory cells in muscle due to ischemia surgery.

2.6. Effect of inflammation on combination delivery.

Since alternative approaches for pDNA delivery employ different mechanisms of pDNA transfer across cell membranes, one would expect a synergistic or additive response of combining two separate approaches on transgene expression levels.

2.6.1. EGT and CpG motifs

EGT can enhance gene expression of naked pDNA independent of CpG sequences. However, insertion of NF-kB binding sequences upstream of promoter should be able to assist the pDNA transfer and gene expression due to the reasons discussed above. Mahindhoratep et al proved this concept by which, addition of NK-kB consensus sequences resulted in increased muscle transfection both with and without EGT (9). The results suggested that basal NF-kB levels are sufficient for enhanced pDNA import resulting in trans-gene expression of naked pDNA alone. However, additive effect of NF-kB binding sequences and EGT on gene expression was also observed (9).

2.6.2. EGT and hyaluronidase pre-injection

Conceptually, enhanced intramuscular distribution of pDNA may result in enhanced electro transfer of DNA. This can be achieved by pretreatment of muscle with hyaluronidase (HYAse) that catalyzes the hydrolysis of hyaluronic acid, a major

component of extracellular matrix (ECM), leading to temporary decrease in viscosity of connective tissue and, therefore enhancing diffusion of fluids upon subsequent pDNA injection. To support this phenomena, Mennuni et al reported 5 and 17-24 times increase in muscle transfection in mice and rabbits respectively using different reporter protein expression constructs upon pretreatment with HYAse 1-4 h before EGT (52). It was discussed in many subsequent reports that HYAse pretreatment helps reducing the voltages of EGT but keep similar transfecting efficiencies with comparatively lower tissue damaging effect than high voltage EGT alone (48, 52, 53). However, the inflammatory effects of HYAse treatment had not been studied or discussed in detail until recently (54). A recent report confirmed that HYAse contribute to the local inflammatory effects of EGT in the initial 2 weeks. Specifically, HYAse amplified the inflammatory cell recruitment ($F4/80^+$, $CD11c^+$ and $MHC\text{-}II^+$ cells) between before and after EGT resulting in enhanced early secretion of IL-1β, IL-6 and TNF-α cytokines (54). However, as discussed above, it has been shown that inflammatory cells directly contribute to transgene expression in healthy and ischemia mice (49, 51).

2.6.3. EGT and block copolymers

Non-ionic block copolymers are excellent polymers for gene and protein therapeutics (55). For gene delivery, coadministration of copolymers with naked pDNA (pDNA/copolymer) increases transgene expression in both muscle and distal lymphoid organs (29). Hunter et al showed that more hydrophobic, high molecular weight block copolymers were more active adjuvants for raising antibody responses than hydrophilic polymers (56, 57). Similarly, more hydrophobic block copolymers like L61 were also more active in enhancing muscle gene expression of naked pDNA than relatively hydrophilic polymers such as P85 or F127 (29). Hydrophobicity, has also been associated with the danger signals that results in APC recruitment and immune system activation which may

explain why the above explained response in protein and gene delivery were observed by relatively more hydrophobic Pluronics. Moreover, adjuvant response of Pluronic L61 was harnessed to recruit APCs to the subsequent site of EGT resulting in an order of magnitude increase in muscle gene expression compared to pDNA/L61 or pDNA and EGT performed at the same time. In another study, formulation of naked pDNA with SP1017; a mixture of relatively hydrophobic L61 (0.25% w/v) and relatively hydrophilic F127 (2% w/v), was shown to significantly increase EGT of naked pDNA alone to muscles from day 6 to day 30 (58). These observations suggested an overlap of inflammatory responses with the muscle transfection.

On the other hand, hydrophilic copolymers have membrane stabilization and sealing properties as described elsewhere (59, 60), and hence can also be used to reduce muscle damaging effects or necrosis after electrical injuries due to EGT. This effect was confirmed when muscle damage quantified as a measure of serum creatine phosphokinase after EGT procedure was reduced using poloxamer 188 (HLB 29) (50). Similarly, high dose irradiation (upto 40-80Gy) lead to extensive cell membrane damage and acute cell necrosis, which was significantly improved by poloxamer 188 treatment (60).

Non-ionic tri-block copolymers or Pluronics (EO_x-PO_y-EO_x) have characteristic central hydrophobic poly-propylene oxide $(PO)_y$ block flanked by relatively hydrophilic poly-ethylene oxide $(EO)_x$ blocks. The relative length of each block dictates the hydrophilic lipophilic balance (HLB) which further affects the physical and functional properties of the triblock. For e.g. Pluronics with lowest HLB have highest hydrophobicity, highest adjuvant effects and highest muscle transfection and vice versa (29, 61, 62).

2.6.4. EGT and anionic polymers/lipids

Relatively recently it was shown that coadministration of anionic, but not cationic or non-ionic lipids enhances the electroporation mediated drug transport across the cell membranes (63). This concept was leveraged by Nicole et al. to show that poly-glutamate, an anionic polymer, can enhance the transfer of pDNA during EGT (64). It was suggested that anionic molecules provide templates to neutralize the electroporation-induced processes that otherwise deactivate pDNA. Similarly, coadminstration of non-coding DNA aka “stuff DNA” which is stuffed into or mixed with coding pDNA was also shown to enhance the EGT gene transfer up to 21 times (65). The authors discussed that neutralization of positively charged components after electrical pulses offers competitive protection to coding pDNA resulting in enhanced gene expression. However, the role of inflammatory effects of anionic molecules was not discussed herein.

2.6.5. Block copolymers and inflammation

Block copolymer adjuvants enhance gene expression of naked pDNA in muscle in a dose dependent and HLB dependent manner resulting in a typical bell-shaped curve (29). It was recently shown that combination of inflammation and block copolymers show synergistic effect on the levels of muscle transfection. Local and distal inflammation drastically enhanced the gene expression of pDNA formulated with P85 or SP1017 in mice (Chapter 3). There was a substantial gene expression in $CD11b^{+}$ cells in ischemic muscle compared to intact healthy muscles and the role of macrophages in enhancing the muscle transfection was studied (Chapter 3) and discussed below.

2.6.6. Ultrasound and block copolymers

Ultra sound is a very safe and widely used in clinical practice, which offers an attractive option for gene delivery. Studies have shown that ultra sound alone or in combination with microbubbles contrast agents help increase the cell membrane

permeability to pDNA (66). In principle, ultra sound helps pDNA transfer across the cell membranes via transient pores made by the constriction and swelling of gases from the micro bubbles (cavitation) around the skeletal muscles, therefore one may expect a higher gene expression in inflamed muscles due to transfection of local inflammatory cells. Danialou et al confirmed this by showing 3 times increase in transfected muscle fibers and 22 times increase in total Lac Z protein in ultra sound mediated/microbubble gene transfer only during local inflammation in muscular dystrophy (mdx/mdx mice) and not healthy muscles (67). Moreover, combining Pluronic block copolymers (P85 but not F127 and L61) with ultrasound (without additional microbubble vectors) also increased gene expression but marginally (68). These effects may not be explained by the adjuvant effects of P85 because L61 is more hydrophobic and more potent adjuvant. Also, there was no use of microbubbles as in earlier case. Therefore additive effects of both P85 and ultrasound may result in the observed effects.

2.6.7. Pre-injection

Muscles undergoing tissue damage and regeneration show enhanced muscle transfection of naked pDNA than intact muscles (21). For example, muscles pre-injected with barium chloride transfect to ~ 10 times higher levels than undamaged muscles (21). Similarly, snake venom (cardiotoxin) injection 5-11 days before pDNA injection also triggered similar increase in muscle transfection than uninjured muscles (23). These studies were conducted in late 1990s when the skeletal muscle biology was not completely understood. Since MØs are constantly present in muscle and help regeneration, therefore they can also uptake and express reporter genes, which would increase the overall muscle gene expression. We have shown recently a similar effect when block copolymers assisted muscle transfection by guiding pDNA transfer from MØs to adjacent muscle cells (Chapter 3). Therefore, we further engineered an improved muscle transfection approach

by pre-injection scheme. Specifically, MØs were recruited by block copolymer pre-injection to the site of subsequent pDNA/P85 injection to show a further increase muscle transfection (Chapter 4). Similarly as discussed above, pre-injection of block copolymer adjuvant 1 h before EGT of pDNA also increased muscle gene expression suggesting the role of inflammatory cells in facilitating muscle gene expression (69).

Overall, all discussed factors suggests a more complex scenario of pDNA transfer across muscle membranes than just an electrophoretic movement of pDNA across the destabilized cell membranes as in EGT. Not if all, but non-electrical factors such as inflammation or inflammatory cells, DNA (CpG etc), DNA formulation, definitely affect gene expression in each case discussed above and have been discussed elsewhere in detail (70).

2.7. Cell mediated in situ gene delivery

Cell-to-cell communication among mammalian cells is analogous to commonly known “Quorum sensing” in prokaryotes. In recent years the prevailing view that eukaryotic cells are restrained from communicating and exchanging genetic information has been challenged (71). Mammalian cells have shown to have a one or a two way cell-to-cell communication that operates through exosomes, microvesicles and tunneling or membrane nanotube structures (71-74). This structures carry and transfer a range of nucleic acids and proteins to other cell types (72, 74-76). Haney et al recently demonstrated that MØs transfected with GFP-DNA or catalase expressing DNA can travel across BBB, deliver DNA via secreted exosomes to adjacent cells resulting in GFP expression in brain parenchyma or reduced brain inflammation respectively in murine model of Parkinson disease (76). Thus, one can speculate that upon direct i.m. injection of pDNA, the tissue resident MØs can uptake and transfer the DNA via *in situ* horizontal gene transfer to other immune cells or adjacent host cell and express the transgenes.

The above mentioned approach can offer a practical advantage over existing cell based therapies which utilize the natural ability of circulatory cells for their ability to home to the sites of inflammation for drug/gene delivery (76-78). These approaches involve cumbersome *ex vivo* manipulation of cells before adoptive transfer which requires technically complex preparation, characterization of cells and the risk of contamination. Therefore, an in situ drug/gene delivery approach via host cells will offer a simpler and safer therapeutic option.

2.7.1. MØs/Monocytes in skeletal muscle injury and gene delivery.

MØs and their precursor cells, monocytes (MOs) play a central role in first line of defense and at the site of injury or inflammation. MØs are professional phagocytic cells that encounter pathogen and help antigen, processing and presentation to other immune cells for the resolution of infection. Specifically, bone marrow derived MOs constantly patrol in the circulation for the search of inflammation (79). After chemotaxis and extravasation towards the sites of infection, MOs mature into MØs while they help resolve infection and switch from pro-inflammatory type (M1-MØs) to anti-inflammatory type (M2-MØs) to assist tissue damage, repair and then undergo apoptosis, drain into lymphatics as dendritic cells/macrophages or stay in the host tissue as resident MØs (79). During resolution of infection, MØs secrete cytokines to recruit T cells in the vicinity where they further assess the level of local inflammation by direct contact with MØs or in a “bystander effect” with/without any cell-to-cell contact and then respond by secreting factors that further initiates a concerted events of innate immune cell recruitment response including MØs as explained above. Because of their ability to enter any type of tissue, MØs have been used as “Trojan Horses” to deliver drugs across blood brain barrier, hypoxic regions or tumors, site of inflammation in joints or muscle etc. (76, 78, 80).

Skeletal muscle tissue has been long known for its unique regeneration potential unlike other tissues. Though, much interest had focused on the role of stem cells in muscle regeneration, the innate immune response determined the extent to which regeneration occurs (81). Later, Bone marrow transplant studies identified that hematopoietic stem cell intermediates with $Mac1^{+}Gr1^{+}$ myeloid cells/MØ phenotype (82-85) or Mac1+ cells $Sca\text{-}1^{+}$/$c\text{-}Kit^{+}$ (86) phenotype assist muscle regeneration through MØ-to-muscle cell fusion. This response was confirmed when, injured skeletal muscles failed to regenerate in MØ depleted mice (87). Therefore, MØs have gained particular interest for responding to tissue insult, persistence until resolution of muscle damage and protective effects on muscle membranes by cell-to-cell contact dependent manner (87, 88). Similar effects of MØs were also shown in other tissues like liver, intestines and brain as explained elsewhere (89-92).

Fusogenic response of MØs has also been seen elsewhere when multiple MØs fuse together to form Langhans giant cells with <20 nuclei arranged in circular peripheral arrangement or foreign body giant cells with >20 nuclei arranged in irregular arrangement. These giants cells are observed around the implanted biomaterials to form a avascular capsules which can lead to failure of implants (93). It was suggested that when MØs adhere to a surface, which is too large to be degraded effectively via phagocytosis, they become fusogenic (94, 95).

2.7.2. MØs help gene transfer upon co-administration of block copolymers and pDNA.

Upon direct injection of naked pDNA in muscle, APCs infiltrate the tissue, uptake the pDNA and express the transgenes in muscle, draining lymph nodes and spleen (12, 26). Pluronic block copolymers, widely studied for their adjuvant activity for both protein (57, 61) and DNA delivery (29, 96), have been shown to significantly enhance the

process and increase the gene expression not only in DNA injected muscles but also in draining lymph nodes and spleen to upto an order or few orders of magnitude respectively (29). Notably, all the effects of copolymer induced increase in gene expression were strain-dependent with no effects in athymic nude mice suggesting that T cells were responsible for APC recruitment and increase in transgene expression (96). Similar T-cell dependent inflammation response “Bystander effect” to poly(ethylene oxide) prosthetic implants was observed in immunocompetent and not in immunedeficient (athymic) strains of mice as explained by Sandhu et al (97).

We have studied in detail the effects of APC recruitment on transgene expression upon i.m. injection of naked pDNA alone and when co-formulated with block copolymers (Chapter 3, 4). In particular, block copolymers drastically enhance and modify the cellular recruitment response towards MØ predominated response as opposed to other adjuvants (alum and LPS). The copolymers then selectively stimulate the gene transfer from pDNA transfected MØs to adjacent muscle cells resulting in muscle specific increase in gene expression in *in vitro* co-culture (Chapter 3). The specificity of MØ dependent muscle transfection was confirmed by reversal of copolymer enhanced gene expression to the levels of naked pDNA alone by *in vivo* depletion of MØs by clodronate-liposome (Chapter 4). As expected, the transgene expression by pDNA/P85 further increased in presence of local inflammation in muscle suggesting that MØs and block copolymers are mutually inclusive for copolymer based muscle transfection approach. Significant but marginal increase in gene expression with naked pDNA alone was also observed in presence of inflammation. This knowledge was further utilized in engineering the MØ recruitment to the subsequent site of DNA injection to further increase the muscle gene expression of pDNA/copolymer formulation (Chapter 4). Therefore, we propose an *in situ* gene delivery approach using Pluronics that leverages help from immune cells

specifically MØs to deliver genes to adjacent cells, in this case skeletal muscles as shown in Fig. 1. Notably, resident MØs are not present in muscle parenchyma but in the epimysium/perimysium where they initiate and orchestrate the inflammatory response after a muscle injury (98, 99).

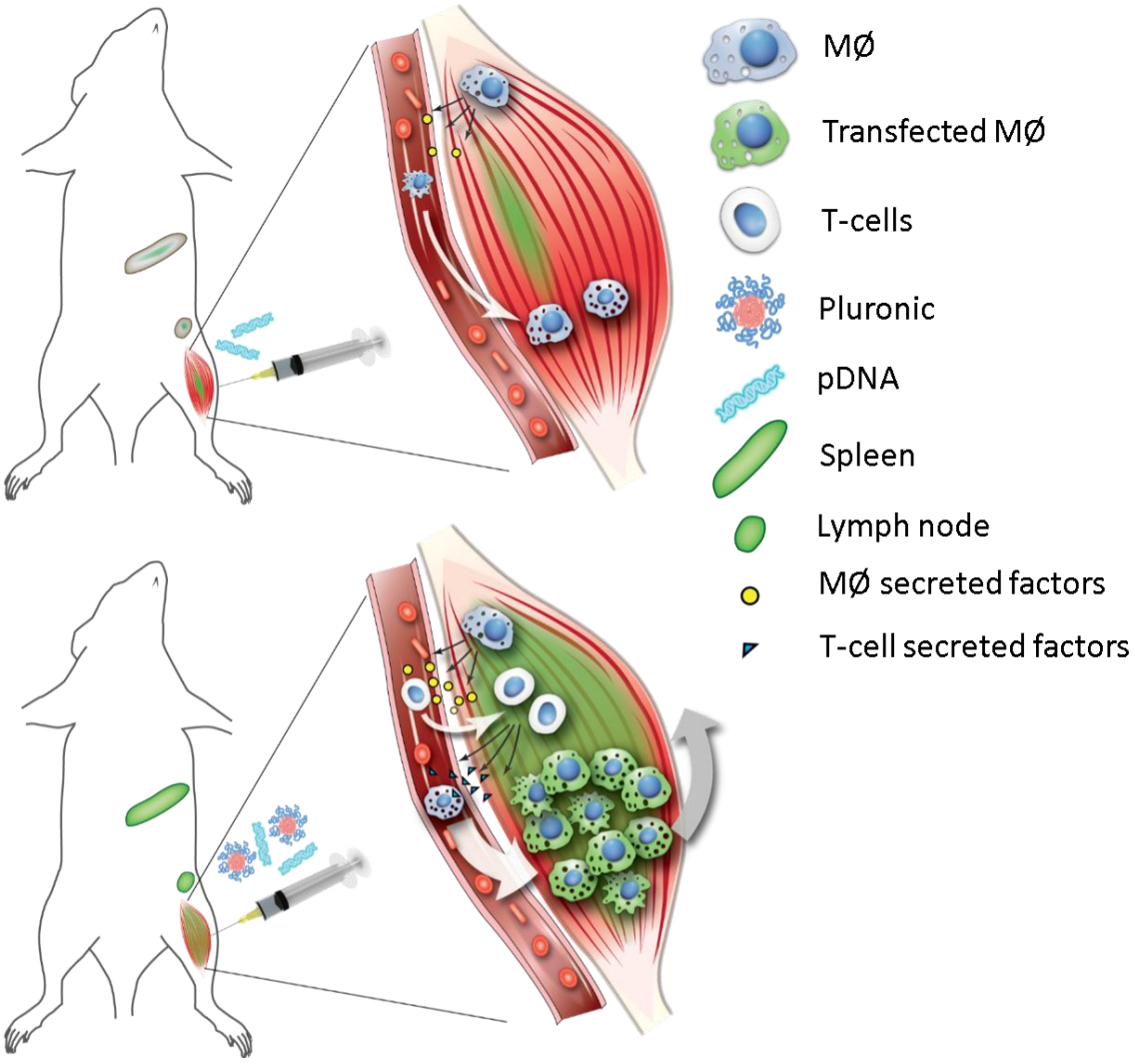

Figure 2.1. Block copolymer induced MØs recruitment response in muscle upon direct i.m. injection. A physical intervention (direct i.m. injection of naked pDNA alone or coformulated with Pluronic P85, CpG in pDNA backbone) can stimulate resident MØs present in the periphery of muscles epimysium/perimysium (98, 99) to start a T cell dependent or independent MØ recruitment response in muscle. After extravasating in the muscle upon pDNA/p85 injection, T cells in the vicinity of stressed resident MØs may show a "bystander effect' by sensing local inflammation and secrete additional factors that recruits additional inflammatory cells including MOs. In contrast to naked pDNA alone, block copolymers helps enhance the cellular recruitment response predominated by MOs. As discussed in chapter 3, P85 can enhance pDNA transfer and gene expression in MØs and also enhance the horizontal transfer of pDNA from MØs to muscle cells. In a time dependent manner, transfected MØs may undergo apoptosis, mature into resident MØs or drain to the distal lymphatic organs. Since pDNA/P85 recruits massive number of MOs, more pDNA transfected MOs will drain to the lymphatics resulting in enhanced transgene expression in the draining lymph nodes and spleen.

Similar to Pluronics other non-ionic polymers have also shown up to 10-fold enhancement of transgene expression over naked pDNA alone (100). We believe that block copolymers help MØs to fuse with muscle cells during muscle regeneration as explained above by excluding water molecules from the MØ-to-muscle cell contacts resulting in enhanced gene transfer (Fig. 2). A similar synthetic approach of stimulating cell-to-cell fusion can be explained by PEG based cell-to-cell fusion in hybridoma technology for creating somatic cell hybrids. Since the triblock structures of Pluronics have PEG on the flanking ends, depending on the length of PEG block, different copolymers can increase the natural MØ-to-muscle cell fusion response to different extents.

MØ-to-muscle cell fusion mechanism can also assist gene transfer response after naked pDNA alone injections in muscles undergoing inflammation and regeneration after hind limb ischemia, preinjection with cardiotoxin, barium chloride or bupivacaine.

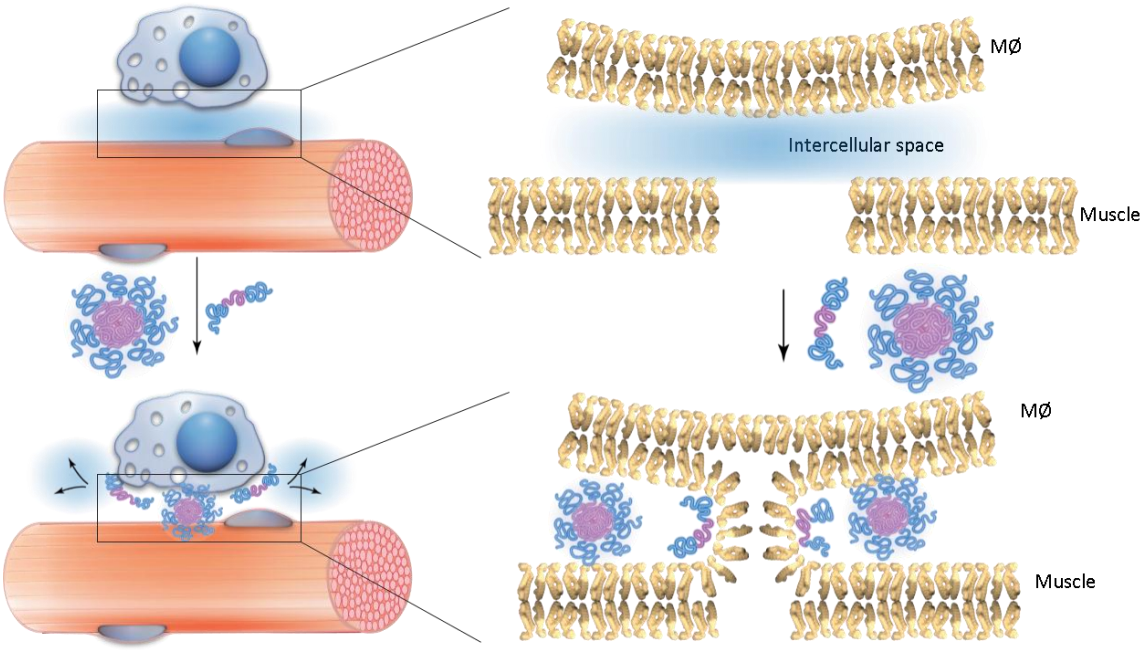

Figure 2.2. Proposed model of horizontal gene transfer from MØ to muscle cell fusion upon i.m. injection with pDNA/P85. Since, MØs are recruited to the sites of naked pDNA injections as a result of physiological insult caused by needle injury, these MØs phagocytose extracellular naked pDNA and then fuse (very low frequency <0.1%) with the existing muscles as a part of regeneration process thus, delivering pDNA into otherwise hard to transfect skeletal muscles. However, Pluronic can enhance the horizontal gene transfer from the transfected MØs to muscle fibers by assisting cell to cell fusion. The latter step is similar to polyethylene glycol (PEG) mediated cell-to-cell fusion, in which it involves exclusion of water molecules between lipid bilayers and changing the orientation of molecular dipoles that results in membrane fusion as in somatic cell hybrid technology (101-104). Since Pluronics contain PEG as well as polypropylene glycol chains capable of incorporating into lipid membranes (105), we believe that they can enhance the MØs to muscle cell fusion events eventually delivering pDNA to muscle fibers (horizontal gene transfer) and increasing transfection and gene expression both in healthy and inflamed muscles.

2.8. Overall conclusions.

New approaches in non-viral gene delivery are developing at a fast scale, with relatively less understanding in gene transfer mechanism. With a better knowledge of skeletal muscle biology at present, there is a need for research focus towards mechanistic of gene transfer *in vivo* upon direct i.m. injection so that key bottlenecks and the limitations of non-viral gene delivery can be overpowered. To this end, development of *in vitro* models relevant to *in vivo* gene transfer upon direct injection is also very critical. For e.g. 3D muscle models have been shown to have more active endocytosis compared to in 2 D cultures which may provide more insights into pDNA uptake in otherwise hard to transfect 2D muscle models *in vitro*. Additionally, block copolymers are very interesting tools to enhance gene transfer process both *in vivo* and *in vitro* and can be used in clinical applications. Notably, most interesting aspect of gene transfer in skeletal muscle is the associated inflammatory responses and the effects on gene expression, which needs careful immunological investigation. Inflammation is a complex response and there are drawbacks and benefits to it. However, many inflammatory responses can be modulated, wherein Pluronic adjuvants has already found its application and many more adjuvants or immune modulators yet need to be explored.

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CHAPTER 3

Horizontal Gene Transfer from Macrophages to Ischemic Muscles upon Delivery of Naked DNA with Pluronic Block Copolymers

Abstract

Intramuscular administration of plasmid DNA (pDNA) with non-ionic Pluronic block copolymers increases gene expression in injected muscles and lymphoid organs. We studied the role of immune cells in muscle transfection upon inflammation. Local inflammation in murine hind limb ischemia model (MHLIM) drastically increased DNA, RNA and expressed protein levels in ischemic muscles injected with pDNA/Pluronic. The systemic inflammation (MHLIM or peritonitis) also increased expression of pDNA/Pluronic in the muscles. When pDNA/Pluronic was injected in ischemic muscles the reporter gene, Green Fluorescent Protein (GFP) co-localized with desmin^{+} muscle fibers and CD11b^{+} macrophages (MØs), suggesting transfection of MØs along with the muscle cells. P85 enhanced (~4 orders) transfection of MØs with pDNA *in vitro*. Moreover, adoptively transferred MØs were shown to pass the transgene to inflamed muscle cells in MHLIM. Using a co-culture of myotubes (MTs) and transfected MØs expressing a reporter gene under constitutive (cmv-luciferase) or muscle specific (desmin-luciferase) promoter we demonstrated that P85 enhances horizontal gene transfer from MØ to MTs. Therefore, MØs can play an important role in muscle transfection with pDNA/Pluronic during inflammation, with both inflammation and Pluronic contributing to the increased gene expression. pDNA/Pluronic has potential for therapeutic gene delivery in muscle pathologies that involve inflammation.

3.1. Introduction

Direct intramuscular (*i.m.*) injection of naked plasmid DNA (pDNA) for skeletal muscle transfection was pioneered by J.A. Wolff in 1990 (1). As of today *i.m.* injection of pDNA makes up around 18% of worldwide gene therapy human clinical trials (2). These trials are related to DNA vaccines, Duchene muscular dystrophy, hind limb ischemia and cardiac ischemia (3-5). The *i.m.* injection of pDNA has shown excellent safety profiles, but low gene expression levels, which limits its use in various therapeutic applications. To overcome this limitation, we and others have proposed a very simple and inexpensive approach of co-administering naked pDNA with non-ionic Pluronic block copolymers ("Pluronics" or "poloxamers") that were shown to drastically increase the levels and duration of muscle gene expression (6, 7). Pluronics consist of non-ionic ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a basic triblock A-B-A structure: EO_x-PO_y-EO_x. They do not form complexes with the pDNA (8). So far the mechanisms by which Pluronic increases the transfection with naked pDNA in skeletal muscles remained not well understood.

It has been known that skeletal muscles have a remarkable ability to regenerate after tissue injury, which coincides with the inflammatory events and presence of immune cells, in particular, macrophages (MØs). These cells play a key role in the process of skeletal muscle regeneration (9-13). Due to constant persistence throughout the inflammatory response, MØs help muscle membrane repair in a cell-to-cell contact dependent manner (14). Similarly, we know that direct *i.m.* injection of pDNA also triggers an inflammation response due to first, the physical injury caused by the needle, and second, the exposure to unmethylated CpG islands in bacterially derived pDNA. A previous study reported an increase in muscle transfection of pDNA during hind limb ischemia (15). It was already noted before that Pluronic effects pDNA expression in the muscle were lacking in immune-deficient athymic mice, which implied the role of immune

cells in muscle transfection (8). Moreover, co-administration of Pluronic with pDNA in the muscle greatly increased expression of the transgene along with the cDNA and mRNA levels in distal lymphoid organs, such as draining lymph nodes and spleen, with the transgene being co-localized there with antigen presenting cells (APCs) (16).

Most disease pathologies involve recruitment of inflammatory cells to the tissues and secretion of pro-inflammatory factors either locally or systemically. Since the therapeutic genes in the pDNA constructs are driven by constitutive cytomegalovirus (cmv) promoter with multiple NF-kb binding sites (17), inflammation may influence the gene expression. However, many studies involving *i.m.* injections of pDNA have been conducted in healthy muscles even when the ultimate goal was to use this method for therapeutic protein expression in disease-affected tissues. Therefore, studies of the gene expression using relatively new platforms such as Pluronic block copolymers in various disease models will further help understanding the limits and opportunities for application of these platforms in gene therapy.

The aim of this study was to evaluate the effect of local and systemic inflammation on muscle gene expression after *i.m.* delivery of naked pDNA with or without Pluronics. To induce local inflammation, we used a murine hind limb ischemia model (MHLIM) and for systemic inflammation both MHLIM and peritonitis. In previous work, we ranked the potency of various Pluronics on the expression of a plasmid containing a luciferase reporter gene under the control of cmv promoter, and found that Pluronic P85 (P85) and SP1017 were the most effective and safe (16). Here we examine the effects of these copolymers on the MØ ability to take up and express pDNA and horizontally transfer the transgene to the muscle cells both during *in vitro* coculture and adoptive transfer to healthy and disease affected animals.

3.2. Materials and Methods

3.2.1. Plasmids.

The gWIZ™ high expression vectors encoding the reporter genes, luciferase (gWIZ™Luc) and green fluorescent protein (GFP; gWIZ™GFP), both under control of an optimized human cmv promoter followed by intron A from the cmv immediate-early gene were used throughout the study (Gene Therapy Systems, San Diego, CA). The pDRIVE5Lucia-mDesmin and pDRIVE5GFP-mDesmin (InvivoGen, San Diego, CA) encodes luciferase and GFP reporter proteins respectively, transcribed under the control of murine desmin promoter for muscle-specific expression. All plasmids were expanded in E. coli DH5α and isolated using Qiagen's EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA) according to the supplier's protocol, reconstituted in phosphate buffered saline (PBS) and stored at -20^{o}C until use.

3.2.2. Block copolymers.

Pluronic L61 (batch # WPNT-511B), P85 (batch # WPNT-511B), F127 (batch # WPNT-511B), were a gift of the BASF Co. (Mount Olive, NJ). A mixed composition of L61 (0.25% w/v) and F127 (2% w/v), SP1017 (2.25% w/v) was obtained from Supratek Pharma Inc. (Montreal, Canada) or prepared using the corresponding copolymers.

3.2.3. pDNA/Pluronic formulations.

The pDNA formulations were prepared as described (16) and used immediately for *i.m.* injections.

3.2.4. Cells.

RAW264.7 immortalized mouse MØs cell line and C2C12 immortalized myoblasts (MBs) cell line were purchased from ATCC and cultured at 37^{0}C and 5% CO_2. To obtain terminally differentiated skeletal myotubes (MTs), C2C12 MBs were plated in

96-well plates (50,000 cells/well) in complete media (CM) containing Dulbecco's minimal essential media (DMEM) and 10% fetal bovine serum (FBS). After 24 h or ~100% confluence, CM was replaced with 200 µl/well differentiation media (DM) containing DMEM and 2% horse serum (HS) to facilitate fusion of MBs (18). DM was changed every 12 h thereafter until day 5-10 when healthy long differentiated MTs were formed.

3.2.5. Animals.

All animal experiments were carried out with approval of the University of Nebraska Medical Center (UNMC) and University of North Carolina at Chapel Hill (UNC-CH) Institutional Animal Care and Use Committee and in accordance with the NIH Guide for Laboratory Animal Use. Female Balb/c mice (6- to 8-weeks-old, Charles River Laboratories, Wilmington, MA) were used throughout this study. The animals were kept in groups of five and fed *ad libitum*.

3.2.6. Inflammation models and scheme of experiments.

MHLIM was generated by surgical procedure as described (19, 20). Briefly, under general anesthesia, the femoral artery (FA) of right hind limb was completely excised after ligation at its proximal origin as a branch of the external iliac artery and before the point distally where it bifurcates into the saphenous and popliteal arteries. Saphenous artery and saphenous vein were also excised. An interval of 10 days was allowed for postoperative recovery before DNA administration in ischemic *tibialis anterior* (*TA*) muscle by direct *i.m.* injections. In some groups MHLIM surgery was performed on ipsilateral hind limb and test articles were simultaneously injected in contralateral *TA* muscles of the same mouse. Peritonitis was induced by intraperitoneal (*i.p.*) injections of filter-sterilized λ-carrageenan (CGN; 1 mg/200 µl PBS) on the 1st and 2nd day and followed by test articles injections in *TA* muscles on the 3rd day. In some groups pDNA and Pluronic were in administered in separate legs in both healthy and peritonitis model.

3.2.7. pDNA injections.

Animals were anesthetized by *i.p.* injection of mixed solution of ketamine (100 mg/kg) and xylazine (25 mg/kg) (Sigma, St. Louis, MO). Single injection of pDNA in 50 µl of Hank's balanced salt solution (HBSS) alone or 50 µl of the block copolymer solution in HBSS was administered directly into right *TA* muscle of the mice using 28G 1 cc sterile syringe (BD Bioscience, Franklin Lakes, NJ).

3.2.8. Luciferase activity *in vivo*.

Unless indicated otherwise mice were euthanized at the time points indicated in the figure legends and tissues were processed. The luciferase activity in 10 µl tissue homogenates was quantified using a TD20/20 or Glomax 20/20 luminometer (Promega, Fitchburg, WI) for an integration period of 20 s and 10 s respectively and normalized per mg of tissue as described before (16). Alternatively, luciferase activity was measured in live animals using *in vivo* imaging system IVIS-200 (Xenogen Corporation, Alameda, CA) 5 min after *i.p* injection of D-Luciferin and the imaging data were quantified as described before (16).

3.2.9. Polymerase Chain Reaction (PCR).

PCR and Reverse transcription PCR (RT-PCR) were performed as described in (16).

3.2.10. Percent White Blood Cell (WBC) count in blood samples.

Fresh serial blood samples from the tail vein (30-40 µl), collected into EDTA coated capillaries by tail milking method, were analyzed in VetScanHM5 hematology analyzer (Abaxis Ltd, Union City, CA) and % WBC counts were plotted on a time scale (days) after ischemia surgery

3.2.11. Tissue Histology.

Muscle tissues [quadriceps (Q), adductor (A), gastrocnemius (G) and *TA*)] at specified time points were removed en bloc and processed as described earlier (16).

3.2.12. Adoptive transfer of GFP transfected MØs in ischemic mice.

RAW 264.7 MØs were plated *in vitro* in T75 flask in macrophage colony stimulating factor (MCSF) supplemented CM for 24 h and transfected with gWIZ™ GFP pDNA using genePORTER300 transfection reagent as per supplier's protocol (extrapolated from 60 mm^2 dish to T75 flask based on surface area). After another 24 h, $5X10^6$ MØs were injected to ischemic mice by intrajugular vein injection (*i.j.v.*) 48 h post ischemic surgery. Ipsilateral ischemic and contralateral healthy muscle tissues were isolated 3 days after adoptive transfer of MØs (5 days post-surgery), embedded and frozen. (Time point of tissue isolation was determined in a separate study where biodistribution of fluorescently labelled and MCSF polarized or M2-MØ, and unpolarized MØ was determined by IVIS imaging Fig. S3.15). 10 µm tissue sections were processed for immunohistochemistry (IHC) as described in (16).

3.2.13. Cytotoxicity evaluation.

Cytotoxicity was determined using the CellTiter® 96 Aqueous Cell Proliferation Assay (MTS assay) (Promega, Fitchburg, WI) as per manufacturer recommendations. Briefly, cells (20,000 MØs, 16,000 MBs or day 7 MTs per well) were seeded in a 96-well plate 24 h before the experiment. Cells treated with Pluronic solutions in 100 µl of serum free media (SFM) for 2, 4, 6 and 8 h were rinsed thrice with SFM and cultured in CM. After another 24 h, cells were incubated with a mixture of 100 µl SFM + 20 µl MTS assay reagent for 1 h at 37°C at 5% CO_2 and finally, calorimetric readout at 490 nm was done using Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA). The results were expressed as the mean percentage cell viability relative to untreated cells ± SEM (n=6).

3.2.14. *In vitro* luciferase gene expression.

To study the effects of P85 on gene expression, 500,000 MØs were plated in 24-well plates in antibiotic free media. 24 h later, cells were treated with mixture of gWIZ™Luc in different concentrations of P85 in SFM for 2h, and cultured in CM. After another 24 h, luciferase activity was measured in cell lysates as described in (16). Luciferase activity in coculture experiments (below) was measured in 10 µl cell lysates (cmv-coculture model) or 10 µl supernatant media (desmin-coculture model) using luciferase assay reagent or Quantiluc® respectively. The data was integrated over 10 s. Total protein content was measured using PierceTM BCA Protein Assay Kit (Thermo-Fisher Scientific Inc, Waltham, MA) and data was expressed as RLU/mg protein.

3.2.15. Gene expression in coculture.

RAW 264.7 MØs were transfected with either gWIZ™Luc or pDRIVE5Lucia-mDesmin plasmids using genePORTER® 3000 transfection reagent (Genlantis, San Diego, CA) as per supplier's recommended protocol. To remove membrane bound complexes after transfection prior to coculture, transfected MØs were briefly rinsed with 1mg/ml heparin sulphate solution in PBS, followed by two times rinse with PBS and this procedure was repeated twice. MØs were then resuspended in CM and 50,000 MØs were added on top of 50,000 MBs or MTs in each well. Exactly after 2 h when MØs adhere to MBs or MTs, the coculture was rinsed with SFM (thrice) to remove traces of serum and exposed to SFM with/without P85 (0.01%, 0.1%, 0.3% and 1.0% w/v) for another 2 h, rinsed again with SFM (thrice) and cultured in 280 µl CM. After 24 h, gene expression was analyzed as described above. To study time-course of gene expression in coculture, the media was replaced every 12 h and samples were collected at the defined time points. For confocal imaging, gWIZ™GFP or pDRIVE5GFP-mDesmin plasmid were used. At

different time points, post coculture samples were fixed and labeled as explained in immunocytochemistry (ICC) in Supplementary Methods.

3.2.16. Western blotting.

Cell lysates prepared using mammalian protein extraction reagent (M-PER®; Pierce Biotechnology, Rockford, IL) were mixed with 2x Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), boiled for 5 min and separated on a precast 12% SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred on PVDF membranes, blocked with 5% w/v skim milk and probed overnight (o/n) with antibodies against β-actin (Abcam, Cambridge, MA) and desmin (Abcam, Cambridge, MA) at 4^{o}C with gentle shaking. Membranes were washed with tris-buffered-saline (TBS) containing 0.1% w/v tween and probed with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, TX) and donkey anti-goat IgG-HRP (Abcam, Cambridge, MA) for 1 h at room temperature (RT) with gentle shaking before visualizing the protein bands using Pierce^{TM} ECL Western Blotting substrate (Thermo Fisher Scientific, Waltham, MA).

3.2.17. Statistical analysis.

Student's t-test was used to calculate statistical significance with $p < 0.05$ (unless indicated) in all cell culture and animal experiments except **Fig. 3.1a, b** where p values were obtained using Student's t-test following logarithmic transformation of the data.

3.3. Results

3.3.1. Pluronic enhances gene expression during local inflammation in MHLIM

It was reported that co-injection of Pluronics with pDNA into the *TA* muscle of healthy animals increases and prolongs the gene expression compared to the naked pDNA (16). Here, to determine the effect of copolymers on the gene expression in the inflamed muscle of the disease-affected animal, we established and characterized MHLIM using previously described procedures (19, 20). The ischemia and tissue inflammation in this model were validated by histological examination of various ischemic muscle sections (as presented in supplementary information **Fig. S3.1**). This model shows a typical response consistent with inflammatory cellular infiltrate. The pDNA, with or without Pluronic (P85 or SP1017), was injected into ischemic *TA* muscle of MHLIM on day 10 after surgery ("ischemic muscle" injections). In parallel experiments, same pDNA formulations were injected in the healthy mice ("healthy muscle" injections). The expression of luciferase was determined in the muscle homogenates on day 4 after injection, as previously described (8). The results suggested that P85 and SP1017 increased the luciferase expression in a dose-dependent manner, revealing typical bell-shaped dependencies of the expression vs. copolymer concentration, (**Figs. 3.1a, b**) in both ischemic and healthy muscles. However, there was a shift in the optimal concentration of Pluronic in the ischemic muscles, where the maximal expression was observed at higher concentration of the copolymers compared to the expression in the healthy muscles (0.6% vs. 0.3% w/v for P85 and 0.1% vs. 0.01% w/v for SP1017). At the maximum, in ischemic muscles, the expression levels of pDNA/copolymer mixtures were increased compared to the naked pDNA by ca. 72 fold for pDNA/P85 and 26-fold for pDNA/SP1017. Moreover, the maximal gene expression in ischemic muscles exceeded that in healthy muscles by ca. 8- and 3-fold for pDNA/P85 and pDNA/SP1017 respectively. Interestingly, the

expression of naked pDNA also somewhat increased in ischemic muscles compared to that in healthy muscles.

We further determined the pDNA dose response on the gene expression by injecting different doses (5 μg, 10 μg and 50 μg) of naked pDNA or pDNA/Pluronic (SP1017, 0.1% w/v) into ischemic muscle. The results suggested that the optimal dose of pDNA/SP1017 was 10 μg in 50 μl HBSS (**Fig. 3.1c**), and this dose was same as the one previously reported for healthy animals (8). At the lower dose of 5 μg, the levels of gene expression declined. At the higher dose of 50 μg, the gene expression leveled off and was not significantly different from that observed at 10 μg pDNA. Therefore, 10 μg pDNA was selected for further injections.

Next, the naked pDNA or pDNA/Pluronic was injected into ischemic and healthy muscles and the levels of luciferase pDNA and mRNA were determined 4 days after injection as previously described (16). The results suggested that Pluronic (SP1017, 0.1% w/v) increased pDNA and mRNA levels in healthy as well as in ischemic muscles compared to the animals injected with pDNA alone (**Fig. 3.1d**). Interestingly, the levels of DNA and mRNA in MHLIM injected with pDNA alone were considerably higher compared to these levels in the healthy animals (**Fig. 3.1d**).

Our previous studies showed that Pluronic prolongs transgene expression in the muscle of healthy Balb/c mice up to several weeks (21). Here we determined the time course of the luciferase expression in the ischemic *TA* muscle of MHLIM by quantifying luciferase bioluminescence in live animals (**Fig. 3.1e** and Supplementary information **Fig. S3.2**). The areas under the curve (AUC) for the 60 days period were determined for all groups using the trapezoidal rule. When comparing the ischemic and healthy muscles injected with the naked pDNA, the former displayed much higher gene expression at every time point. Moreover, the gene expression was further significantly enhanced and

prolonged when pDNA was formulated with SP1017, resulting in increased exposure of the animal to the transgene by nearly an order of magnitude for at least 50 days. Thus, the AUC values in the MHLIM injected with pDNA/SP1017 and naked pDNA were 2186 and 312 day*photons/s*10^6, respectively, suggesting ca. 7-fold increase in the transgene expression as a result of the pDNA formulation with SP1017. For comparison, in healthy animals the corresponding AUC values for pDNA/SP1017 and naked pDNA were 597 and 150 day*photons/s*10^6 respectively.

The GFP reporter gene expression in ischemic *TA* muscles, harvested on day 4 after gWIZ™ GFP pDNA injection, was mainly seen in the muscle fibers located in and around the site of the injection, and co-localized with desmin muscle marker (**Fig. S3.3a**). Also, a substantial GFP expression was observed in small punctate cells, uniformly spread throughout ischemic tissue section, and co-localized with CD11b MØ marker (**Fig. S3.3b**). Interestingly, the number of GFP expressing CD11b+ MØs in ischemic muscles appeared to be much higher than previously reported by us in non-inflamed muscles of the same mouse strain (16). Moreover, we also observed a higher number of GFP expressing muscle fibers in ischemic muscles injected with pDNA/P85 compared to pDNA alone (**Fig. S3.4** and **Fig. S3.5**).

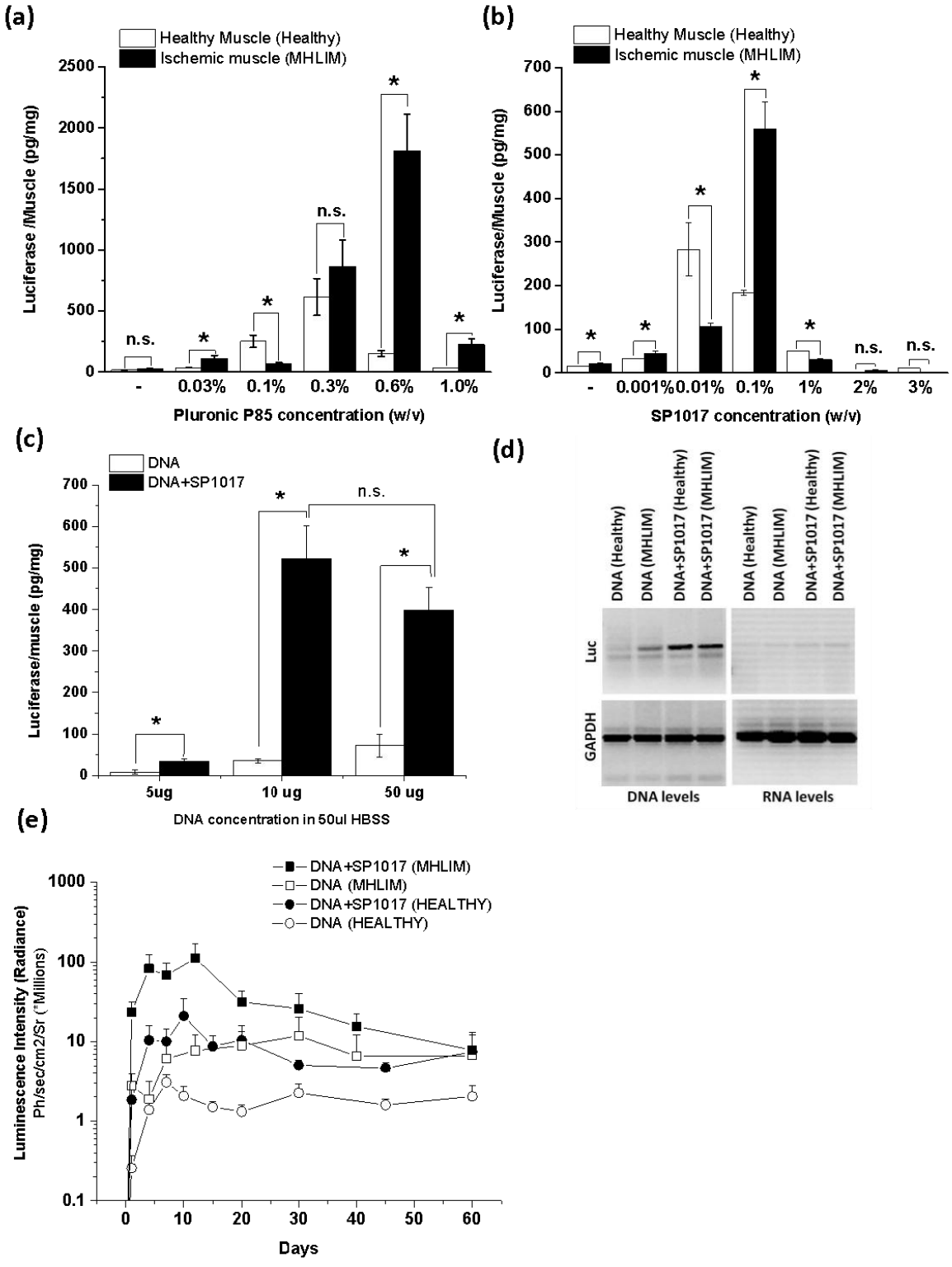
(a)
Healthy Muscle (Healthy)
Ischemic muscle (MHLIM)
Luciferase /Muscle (pg/mg)
2500
2000
1500
1000
500
0
n.s.
*
*
n.s.
*
*
-
0.03%
0.1%
0.3%
0.6%
1.0%
Pluronic P85 concentration (w/v)
(b)
Healthy Muscle (Healthy)
Ischemic muscle (MHLIM)
Luciferase/Muscle (pg/mg)
700
600
500
400
300
200
100
0
*
*
*
*
*
n.s.
n.s.
-
0.001%
0.01%
0.1%
1%
2%
3%
SP1017 concentration (w/v)
(c)
DNA
DNA+SP1017
Luciferase/muscle (pg/mg)
700
600
500
400
300
200
100
0
*
*
n.s.
*
5ug
10 ug
50 ug
DNA concentration in 50ul HBSS
(d)
DNA (Healthy)
DNA (MHLIM)
DNA+SP1017 (Healthy)
DNA+SP1017 (MHLIM)
DNA (Healthy)
DNA (MHLIM)
DNA+SP1017 (Healthy)
DNA+SP1017 (MHLIM)
Luc
GAPDH
DNA levels
RNA levels
(e)
DNA+SP1017 (MHLIM)
DNA (MHLIM)
DNA+SP1017 (HEALTHY)
DNA (HEALTHY)
Luminescence Intensity (Radiance)
Ph/sec/cm2/Sr (*Millions)
1000
100
10
1
0.1
0
10
20
30
40
50
60
Days

Figure 3.1. Effect of co-formulation of pDNA with Pluronic on the transgene expression, DNA and RNA levels in healthy and ischemic skeletal muscles. Luciferase expression **(a-c, e)** or DNA and RNA levels **(d)** in ischemic *TA* muscles of MHLIM **(a-e)** or normal *TA* muscles of healthy animals **(a, b, d, e)** after single injections of the pDNA, pDNA/P85, or pDNA/SP1017. **(a-e)** All injected solutions contained 10 µg gWIZ-Luc pDNA in 50 µl HBSS with or without copolymers except **(c)** where the pDNA concentration was varied. **(c-e)** SP1017 concentration in injected solution was 0.1 % w/v. **(a-d)** Measurements were done on day 4 by tissue sampling. **(e)** Time course of luciferase expression was determined in live animals by quantifying the bioluminescence imaging data (supplementary **Fig. S2**). **(a-c, e)** Data are mean $\pm$ SEM **(a-c)** $n = 5$, * $p < 0.05$, n.s. – not significant or **(e)** $n = 3$-4.

3.3.2. Pluronic enhances gene expression in muscle during systemic inflammation

MHLIM was accompanied by systemic inflammation as evidenced by increase in neutrophils and MOs count in blood (**Fig. 3.2a**). Therefore, we examined the effect of inflammation during MHLIM on the transgene expression in the distal muscle. In this experiment the MHLIM was developed as described before, but the site of pDNA injection was spatially separated from the inflammation site (**Fig. 3.2b**). Naked pDNA alone or pDNA/Pluronic were injected in contralateral *TA* muscle simultaneously with the ischemia surgery performed on ipsilateral hind limb, and gene expression levels in muscles and draining lymph nodes were quantified in tissue homogenates at day 3. Consistent with our previous studies (8, 16) in healthy mice P85 significantly increased gene expression compared to pDNA alone. The ischemia surgery in MHLIM resulted in a further dramatic increase in the expression, both in contralateral muscle (**Fig. 3.2c**) and lymph nodes (**Fig. 3.2d**). Importantly, the effect of inflammation on gene expression was only observed with pDNA/P85 and not with pDNA alone. The effects appeared to be dependent on the type of the copolymer used since they were not significant for the pDNA/SP1017 (Supplementary information **Fig. S3.6**).

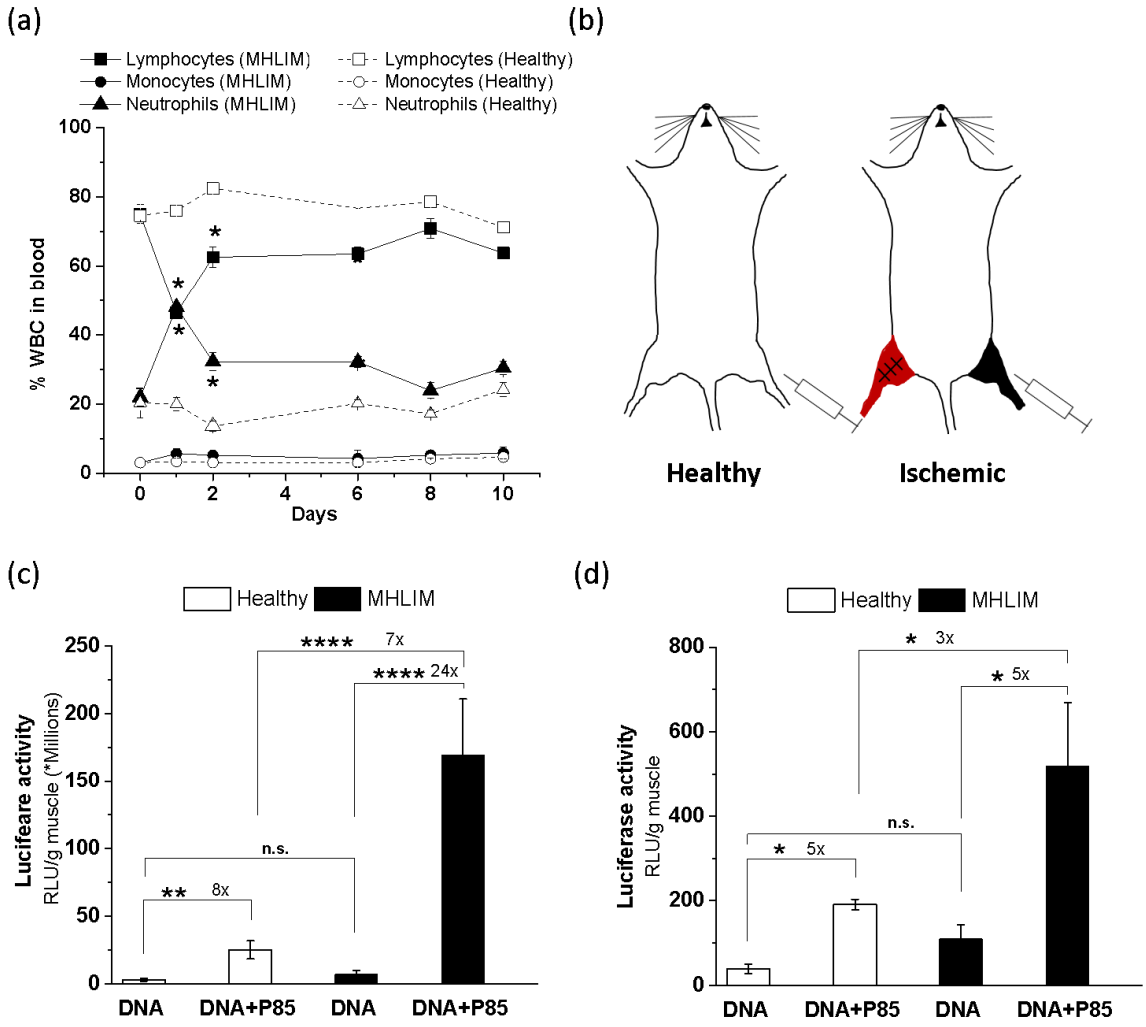

Figure 3.2. Effect of skeletal muscle ischemia on transgene expression in contralateral muscles. (a) Changes in percent WBC counts in serial blood samples at different time points after ischemia surgery in MHLIM (solid lines, flied symbols) in comparison to healthy mice (dotted lines, open symbols). Day 0 corresponds to surgery. **(b)** Test articles were injected in *TA* muscles in healthy mice or in contralateral *TA* muscles simultaneously with the ipsilateral ischemia surgery in MHLIM. **(c, d)** Luciferase gene expression in the **(c)** *TA* muscles and **(d)** draining lymph nodes of MHLIM (black) and healthy mice (white) 3 days after single administration of 10 µg gWIZ™ Luc with or without 0.3 % w/v P85. Data are mean ± SEM (n = 3 **(a)** or n = 10 **(c, d)**), * $p < 0.05$, **$p<0.01$, ****$p<0.001$, n.s. – non significant. **(a)** Statistical comparisons were made between MHLIM and healthy mice.

The effect of inflammation on Pluronic-mediated gene expression at a distal site was striking and led to another experiment using peritonitis model to confirm the generality of the observation. Similar to MHLIM, systemic inflammation in peritonitis mouse model was confirmed by quantifying percent WBCs by hematological analysis of serial blood samples from tail vein (**Fig. 3.3a**). In this experiment, in addition to pDNA alone and pDNA/P85 groups, we included an additional group injected with P85 and pDNA in spatially different hind limb muscles (**Fig. 3.3b**). Since effect of P85 was more pronounced, we did not use SP1017 in this experiment. Luciferase reporter gene expression was quantified by *in vivo* imaging after *i.p.* injection of D-luciferin substrate. Similar to our observations in ischemia model, animals with peritonitis showed a trend to increase gene expression in muscle compared to healthy animals (**Fig. 3.3c**). Mice injected with naked pDNA in ipsilateral leg and P85 in contralateral leg (DNA, P85) showed gene expression levels similar to naked pDNA alone suggesting that increased muscle transfection is dependent on codelivery of P85 with pDNA. Still, the contralaterally injected P85 significantly increased gene expression of pDNA in ipsilateral muscle in the peritonitis animals (**Figs. 3.3c, d**).

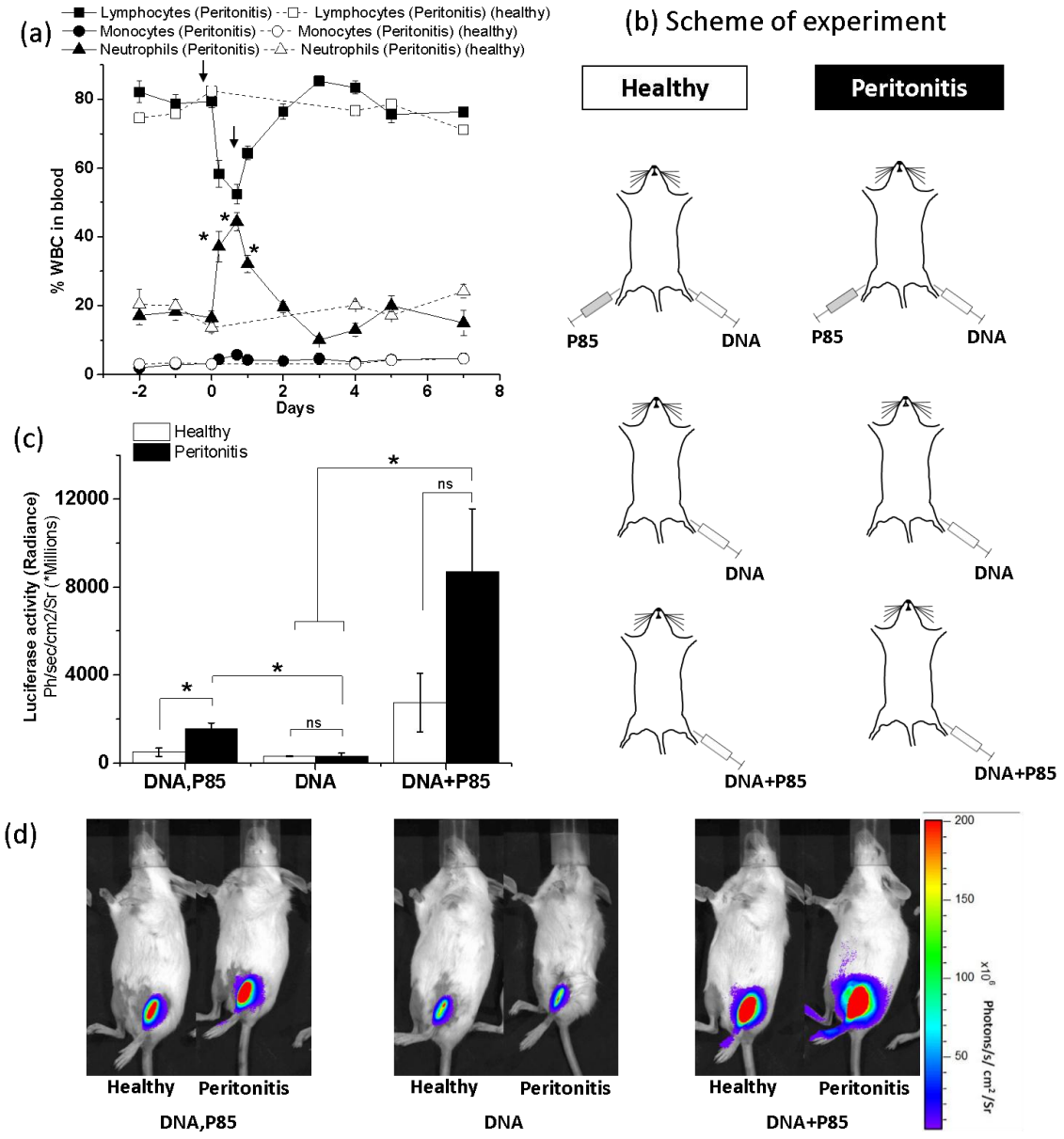

Figure 3.3. Effect of peritonitis on transgene expression in skeletal muscles. (a) Changes in percent WBC counts in serial blood samples at different time points after induction of peritonitis (solid lines, filled symbols) in comparison to healthy mice (dotted lines, open symbols, same group as in **Fig. 2**). Day 0 corresponds to the first injection of CGN to induce peritonitis. **(b)** Healthy mice or mice with peritonitis were injected in *TA* muscles with 10 µg gWIZ™ Luc pDNA alone, pDNA coformulated with 0.3 % w/v P85 or simultaneously injected in ipsilateral and contralateral *TA* muscles with pDNA and 0.3 % w/v P85. **(c)** Luciferase gene expression in pDNA injected muscle of healthy (white bars) and peritonitis (black bars) mice was quantified using by quantifying the bioluminescence imaging data 4 days after pDNA administration. **(d)** Representative IVIS images used for quantification of gene expression data 4 days after pDNA administration. **(a, c)** Data are mean ± SEM (n=3), * $p < 0.05$, n.s. - non significant. **(a)** Statistical comparisons were made for peritonitis *v.s.* healthy mice groups**.**

3.3.3. Pluronic enhances transfection of MØs with pDNA *in vitro*

Since *in vivo* study suggested colocalization of the reporter gene with MØs (**Fig. S3.3**), we examined whether Pluronics can enhance transfection of MØs with pDNA *in vitro*. To this end we exposed RAW 264.7 cells for 2 h to pDNA at various concentrations of P85 and quantified luciferase in cell lysates 24 h after the exposure. Addition of P85 to the media, at the concentration as low as 0.01%, resulted in a significant enhancement of the gene expression (**Fig. 3.4a**). This effect increased as the copolymer dose increased and reached over 4 orders of magnitude at 1% P85 concentration when the gene expression levels nearly matched that observed using a commercial transfection kit, genePORTER® 3000. Moreover, at the higher dose of pDNA (10 µg) the gene expression with 1.0% w/v P85 exceeded that using genePORTER® 3000 transfection (**Fig. 3.4b**). Analysis of pDNA uptake in the cells showed higher pDNA uptake in MØs than muscle cells and significant although marginal increase of pDNA uptake in MØs in the presence of P85 (Supplementary information **Fig. S3.7**). No such increase in uptake was observed in cultured muscle cells with the addition of P85 (MBs or MTs). Other Pluronic block copolymers also showed a similar effects on gene expression in RAW 264.7 cells (**Fig. S3.14**)

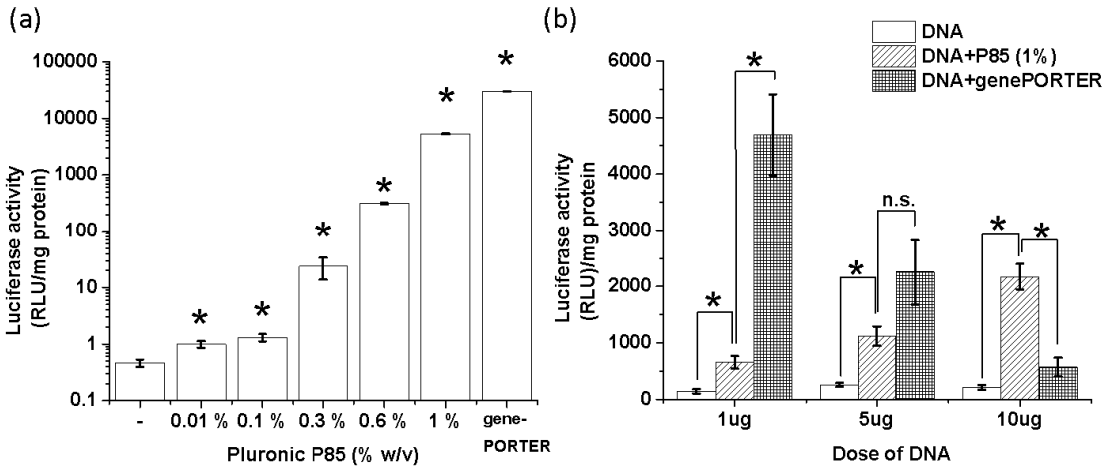

Figure 3.4. Effect of Pluronic on the gene expression in RAW 264.7 MØs. (a, b) Cells were exposed to gWIZ™ Luc pDNA with or without P85 in SFM **(a)** for 2 h using 1 µg pDNA and various concentrations of P85 or **(b)** for 4 h using different amounts of pDNA (1 µg, 5 µg and 10 µg) and 1.0% w/v P85. **(a, b)** Cells transfected using genePORTER® 3000 transfection reagent are presented as positive control. Data are mean ± SEM (n=4), * $p<0.05$, n.s. – non significant, statistical comparisons were done for treated vs. untreated groups unless specified different.

3.3.4. Adoptive gene transfer to muscle by *ex vivo* transfected MØs

To examine whether MØs can transfer genes to ischemic animals, the RAW 264.7 MØs were transfected *ex vivo* with gWIZ^{TM} GFP using genePORTER 3000 and adoptively transferred via jugular vein to MHLIM animals 24 h post transfection and 48 h post ischemia surgery. The lower hind limb muscles (ischemic and non-ischemic) were isolated 3 days after MØs administration, sectioned and analyzed by confocal imaging for co-localization of GFP with CD11b^{+} MØs and desmin^{+} muscle fibers. Firstly, we found that GFP expressing MØs were recruited to ischemic muscles and not to non-ischemic muscles of MHLIM (**Fig. 3.5**). Secondly, the GFP expression in ischemic tissues co-localized with desmin^{+} muscle fibers as seen in **Fig. 3.5**. Larger ischemic muscle fibers clearly expressed GFP protein unlike non-ischemic control muscles. This implied that MØs can transfer GFP gene across the muscle cell membrane, and along with the muscle cells can collectively represent a reservoir or conduit for the gene expression. We observed lower levels of muscle-specific marker (desmin) in ischemic muscle compared to non-ischemic muscle, which is typical for ischemia (24, 25). Importantly, in ischemic muscles CD11b^{+} MØs were localized in close proximity to muscle fibers as was seen from co-localization of desmin and CD11b. This may imply that cell-to-cell contacts play role in exchange of genes or proteins from MØs across the cell membranes to otherwise hard to transfect skeletal muscle fibers.

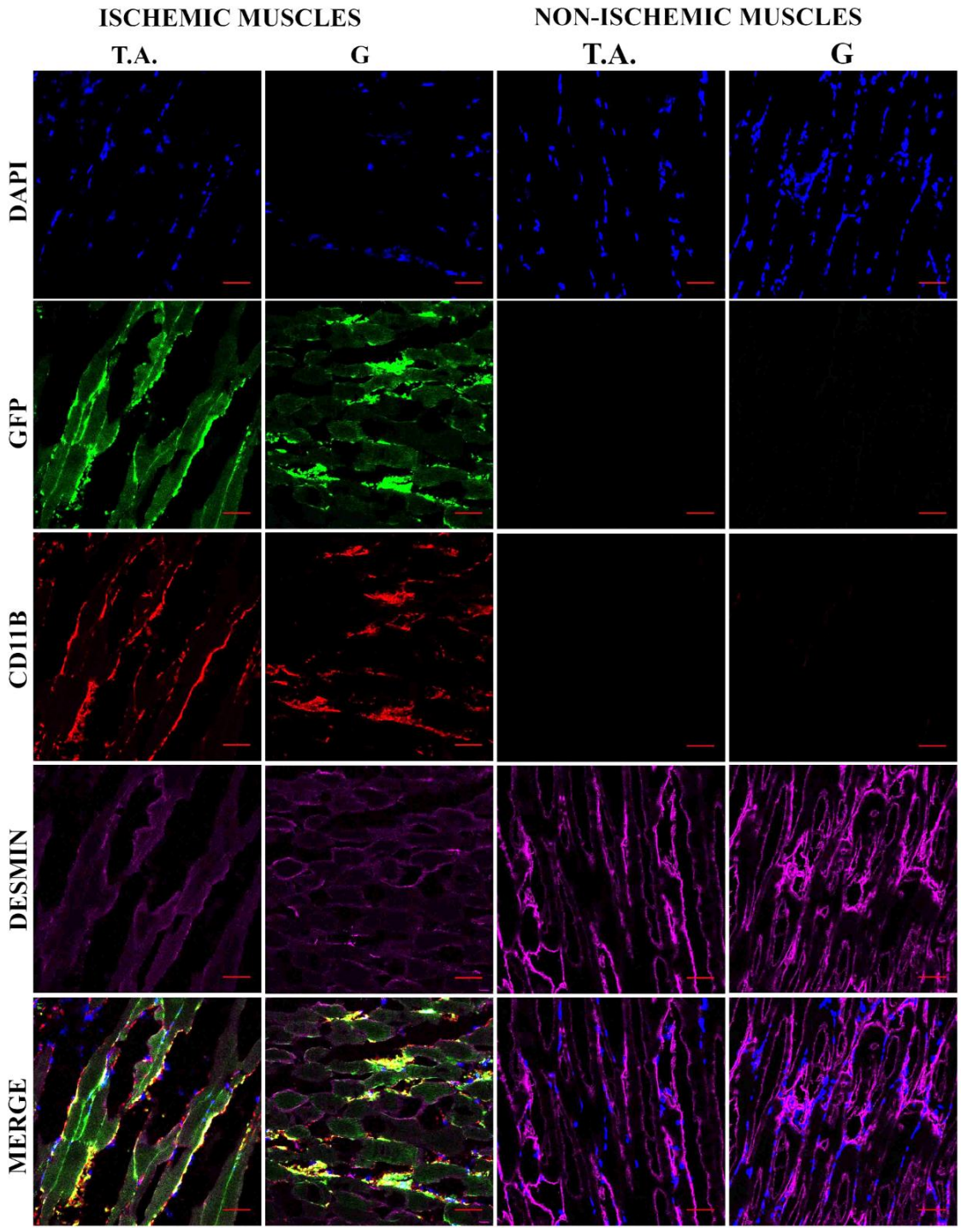

Figure 3.5. *In vivo* transfection of muscle cells upon adoptive transfer of GFP transfected MØs. RAW 264.7 MØs transfected *ex vivo* with gWIZ™ GFP pDNA and administered *i.j.v* in MHLIM Balb/c mice (24 h after transfection, 48 h post ischemia surgery). Tissues (*TA* and G) were isolated 3 days thereafter and 10 µm thick sections of frozen tissues were processed for IHC. The color staining corresponds to nuclear DAPI (blue), GFP (green), CD11b (red) and desmin (magenta). The bottom panels present digitally superimposed images of preceding panels to visualize the co-

localization (yellow or white). The images (20 x) are representative of 3 sections per muscle and 3 mice per group. Scale bar = 50 µm.

3.3.5. *In vitro* horizontal gene transfer from transfected MØs to muscle cells

To further examine pDNA transfer from MØs to muscle cells at the cellular level, we developed an *in vitro* coculture model. Briefly, gWIZ^{TM} GFP transfected MØs were co-cultured with MBs, and GFP expression in MØs and MBs was visualized by confocal imaging at different time points. As shown in **Fig. 3.6**, we clearly observed GFP expression in desmin^{+} MBs after 48 h and 72 h coculture with transfected MØs. Notably, MBs stained positive for MØ marker (CD11b) and not *vice versa* implying a unidirectional transfer of membrane components from MØs to MBs upon coculture, which may explain the mechanism of delivering pDNA/protein across the membranes resulting in muscle cell transfection. Our observation was similar to other reports that describe bi/uni-directional exchange of membrane components as a general cell-to-cell communication amongst various cell types (26-28).

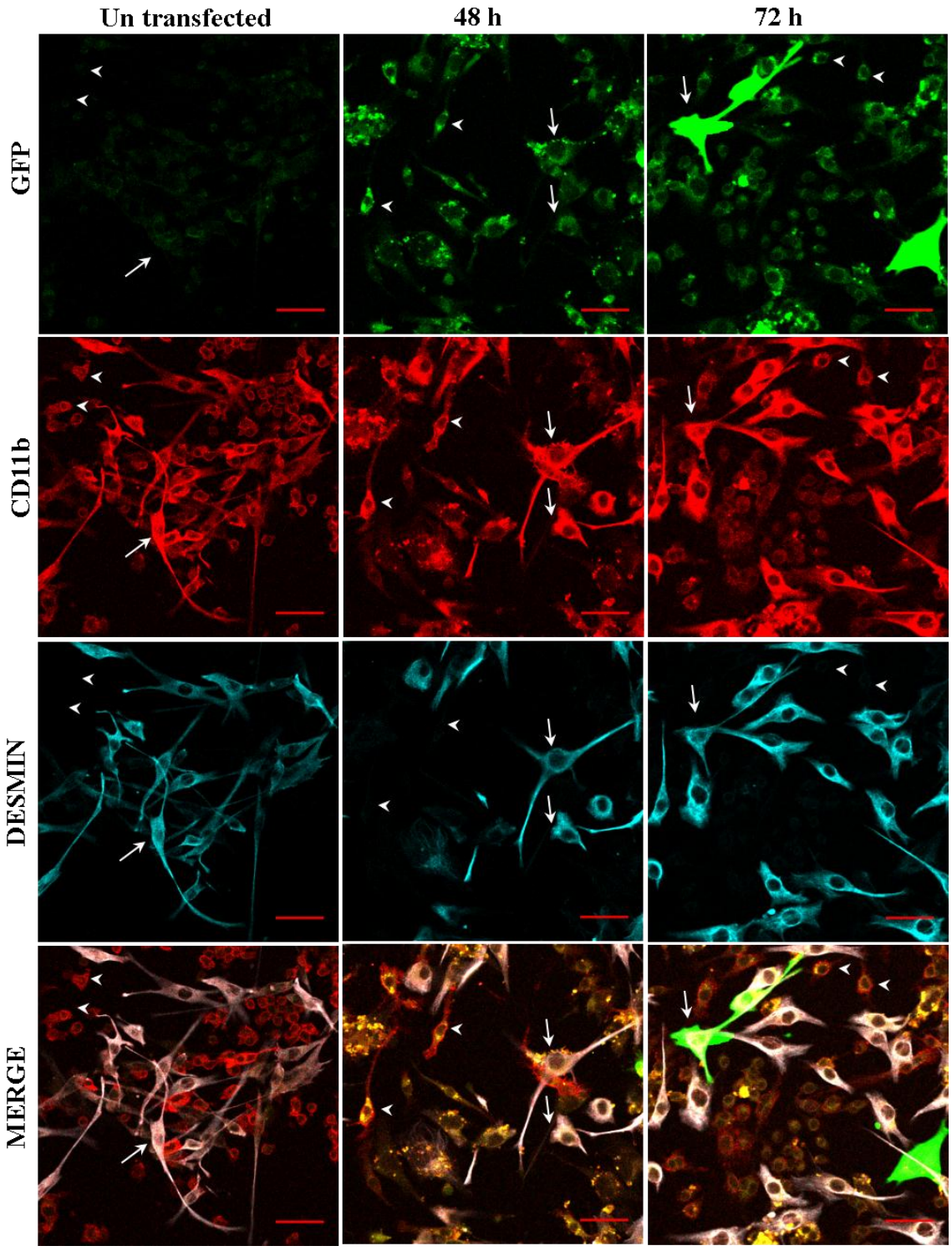

Figure 3.6. *In vitro* transfection of muscle cells upon coculture with GFP transfected MØs. MØs (arrow heads) were transfected with gWIZ™ GFP pDNA and then cocultured with un-transfected MBs (arrows) for up to 72 h. At specific time points cells were harvested and processed for ICC. MBs stained positive for both GFP and CD11b at each time point. The color staining corresponds to GFP (green), CD11b (red), and desmin (cyan). The bottom panels present digitally

superimposed images (20 x) of preceding panels to visualize the co-localization (yellow or white). Scale bar = 50 µm.

3.3.6. Pluronic enhances horizontal transfer of pDNA from MØs to muscle cells

After demonstrating that Pluronic can enhance the transfection of MØs with pDNA, we further examined whether it could also increase the pDNA transfer from the transfected MØs to muscle cells. To evaluate such possibility, we studied *in vitro* effect of P85 on the transgene expression in the coculture of transfected MØs with muscle cells. First we transfected the constantly multiplying RAW 264.7 MØs with pDNA [MØ+DNA] and then cocultured them on top of the monolayers of 1) either terminally differentiated C2C12 MTs (depicted herein [MØ+DNA]+MT), or 2) precursor MBs ([MØ+DNA]+MB). Notably, MTs display high level of expression of desmin, which is much lower in MBs (**Fig. 3.7a**). Thus we used two different plasmids, one with the constitutive (cmv) promoter, gWIZ™ Luc pDNA (same as the plasmid used above), and the other with the muscle specific-desmin promoter, pDRIVE5Lucia-mDesmin (to quantify the muscle specific gene expression). In both cases, the transfected MØs or the cocultures were exposed to different concentrations of P85 (0.01, 0.1, 0.3, 1.0% w/v) for 2 h and the total gene expression was determined after 24 h and normalized to the total protein. Using the desmin-driven pDNA we observed relatively low levels of normalized gene expression in all cell systems - [MØ+DNA], [MØ+DNA]+MT, and [MØ+DNA]+MB (**Fig. 3.7b**). In the transfected MØs the gene expression was increased at 0.01% P85, while at higher copolymer concentrations it was either less or not significantly different compared to untreated controls. In [MØ+DNA]+MB coculture the gene expression was not statistically different from the control in all but the highest P85 concentration group, in which it was significantly decreased. In contrast, [MØ+DNA]+MT coculture after exposure to the copolymer displayed a trend for a monotonous increase in the normalized gene expression, this increase becoming significant at 1% P85. At this copolymer concentration

the total gene expression in [MØ+DNA]+MT coculture was much higher than that in [MØ+DNA] and [MØ+DNA]+MB (Supplementary information **Fig. S3.8**).

Therefore, we further examined the gene expression in [MØ+DNA] and [MØ+DNA]+MT coculture at different time points after exposure to 1% P85 (**Fig. 3.7c**). In this case starting from day 2 there was a dramatic increase in gene expression in [MØ+DNA]+MT coculture but not in the transfected MØ alone. Compared to the coculture the level of gene expression in MØ+DNA was negligible with or without exposure to P85. The latter is probably explained by the promoter selectivity of the pDRIVE5Lucia-mDesmin plasmid used in this study as well by some toxicity of the 1% P85 to macrophages (Supplementary information **Fig. S3.9**). Notably, when similar co-culture experiments were carried out using constitutive (cmv) promoter driven gWIZ™ Luc pDNA, both the total and normalized gene expression in MØ+DNA groups after 24 h was high (Supplementary information **Fig. S3.10** and **S3.11**). However, at later time points the normalized gene expression in MØ+DNA sharply decreased while in the coculture it increased, especially in the groups treated by P85 (Supplementary information **Fig. S3.10**). Using plasmids expressing GFP in the [MØ+DNA]+MT coculture groups, we were able to clearly see gene expression in the MTs (**Fig. 3.7d** and Supplementary information **Fig. S3.11c**). This result is particularly amazing given the fact that MTs are most difficult to transfect cells using normal cell transfection means (Supplementary information **Fig. S3.12**).

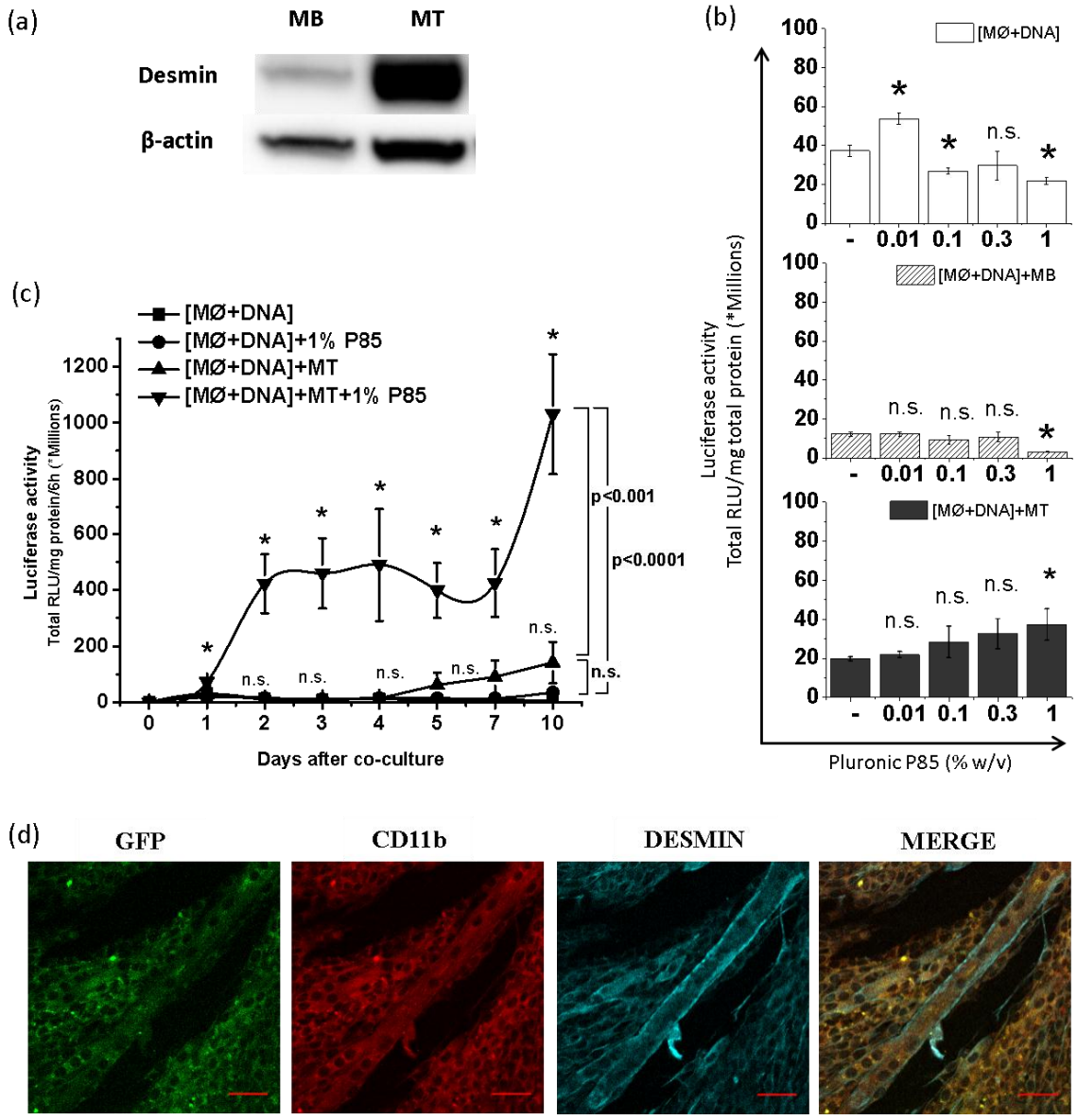

Figure 3.7. Effect of P85 on horizontal gene transfer from transfected MØs to muscle cells upon co-culture. (a) Increased desmin expression upon differentiation of MBs to day 7 MTs. **(b, c)** pDRIVE5Lucia-mDesmin transfected MØs were plated alone, [MØ+DNA], or cocultured with MBs, [MØ+DNA]+MB, or MTs, [MØ+DNA]+MT, and exposed to P85 (0.01, 0.1, 0.3 and 1.0 % **(b)** or 1 % w/v **(c)**) for 2 h. Luciferase secreted in cell culture media was quantified after next 24 h **(b)** or at different time points. In the latter case **(c)** the media was replaced every 12 h and luciferase secreted in the media over 6 h period was determined daily until day 10. Data represents mean ± SE, * $p<0.05$, n.s. – non significant **(b)** (n=6), **(c)** (n=12), statistical comparisons were done for coculture with and without P85 treatment vs. MØ+DNA groups alone and the curves were compared using AUC and one-way ANOVA analysis. **(d)** GFP expression (green) in desmin$^+$ MTs (cyan) was validated 3 days after their coculture with CD11b$^+$ MØs (red) transfected with gWIZ™ GFP pDNA. The last panels in each row present digitally superimposed images (20 x) of preceding panels to visualize the co-localization (yellow). Scale bar = 50 µm.

3.4. Discussion

Administration of pDNA to skeletal muscle is a strategy to deliver various genes for prophylactic and therapeutic purposes in healthy and diseased muscles (29-31). Currently, the application of direct *i.m.* pDNA injections is hindered by low levels of gene expression. Improvements in this area include electroporation-facilitated gene transfer known for its simplicity and use in human clinical trials (32). However, electroporation is associated with the risk of extensive and irreversible muscle damage induced by electrical pulses (33). This has limited its application in therapeutics, especially, when the goal is to rescue the muscle fibers during muscular pathologies, such as hind limb ischemia, polymyositis, muscular dystrophies, etc. Most of these pathologies involve inflammation and recruitment of inflammatory cells, which further contribute to tissue degeneration and muscle damage (34, 35). We have reported that coadministration of pDNA with non-ionic amphiphilic Pluronic block copolymers leads to drastic increases in the level and duration of transgene expression in the healthy muscles (6, 8). In contrast to electroporation, muscle tissue did not appear to be affected by pDNA/Pluronic mixture as observed by histological analysis (16) and animals injected with these formulations did not show any signs of discomfort. Furthermore, Pluronic was coadministered with pDNA in the muscle during electroporation, where it alleviated the pulse-induced tissue damage and further increased the transgene expression (36).

Here, we report supplementary effects of Pluronic and inflammation caused by muscle ischemia on the transgene expression in the muscle. The greatest expression was observed in MHLIM when pDNA/Pluronic was injected directly in the ischemic muscle, although a significant increase compared to expression in healthy animals was also seen when the site of injection was distal from the ischemia site. The latter suggests that systemic inflammation contributes to the pDNA/Pluronic gene delivery in the muscle. This

conclusion was also reinforced by an observed increase in muscle transgene expression with pDNA/Pluronic during systemic inflammation in the peritonitis model. Interestingly, the effects of Pluronic were much higher when Pluronic was co-delivered with pDNA in the same muscle than upon their separate administration in different muscles. Still in the latter case gene expression in peritonitis model was increased possibly suggesting systemic distribution and effect of Pluronic. In the absence of Pluronic inflammation had little if any effect on pDNA expression during peritonitis and relatively small effect in MHLIM. Notably, other studies also reported that inflammation could enhance muscle gene expression upon direct *i.m.* injection of naked pDNA (15, 37).

Previously published data suggested that Pluronics do not enhance muscle transfection in immune-deficient athymic nude mice (8, 38), which implied a mutually inclusive role of both immune cells and copolymers in the muscle transfection. We have also reported that 1) transgenes can be transferred from *in vitro* transfected MØs to the surrounding cells (e.g. neurons) upon coculture and 2) that after adoptive transfer, transfected MØs can deliver transgenes to the distal sites of inflammation, specifically to the brain in the mouse model of Parkinsonism (22, 23). These studies suggested that transfected MØs can repackage pDNA and deliver across cell membranes to neuron cells via exosomes and other extracellular vesicles. The present study reinforces the idea that Pluronic may assist muscle transfection through the effects on immune cells and specifically MØs that are recruited in the inflamed muscles, pick up the pDNA and express the transgene. MØs are known to internalize bacterial derived pDNA by a very specific transport mechanism resulting in gene expression both *in vitro* (39) and *in vivo* (40). Here we demonstrate that treatment with Pluronic increases the transfection of MØs with naked pDNA and boosts gene expression up to 4 orders of magnitude. Moreover, after adoptive transfer in MHLIM the transfected MØs are shown to deliver transgene to the ischemic but

not the healthy muscles. These observations suggested that MØs can transfer transgene across the muscle cell membrane, and led to specific experiments using *in vitro* coculture models.

In these experiments we demonstrated that MØs can horizontally transfer pDNA to muscle cells and this process is greatly enhanced in the presence of Pluronic. Interestingly, we observed a unidirectional exchange of MØs cell surface marker (CD11b) to muscle cells but not *vice versa*. This phenomenon may be in common with the evolutionary process, by which MØs recruited to the site of muscle damage deliver growth factors required for muscle regeneration. Recent studies have also reported a novel mechanism of vesicular exchange of protein/genetic material between APCs during cell-to-cell communication (41), which justifies our observations. Using horizontal gene transfer with Pluronic we were able to transfect MTs that are terminally differentiated cells and therefore notoriously difficult to transfect *in vitro*. That is why researchers use transiently transfected mitotically active MBs and differentiate them to form transfected MTs. This procedure limits the skeletal muscle biology studies to early stages of muscle development. Our finding provides a novel approach for gene delivery to MTs at later stages (1-3 weeks) after differentiation and can find application in research labs.

Skeletal muscles are known for complex muscle biology, and recent studies have shown that MØs play a significant role in helping muscle regeneration and preventing muscle atrophy (9, 42-44) via cell-to-cell contacts (14). The role of stem and hematopoietic cells in muscle regeneration has been also extensively studied (45, 46). Evidence points to involvement of cell-to-cell fusion in muscle regeneration, whereby cells with stem cells' properties fuse with damaged muscle fibers or satellite cells to form new muscle fibers. Technologies to deplete and repopulate specific cells including transgenic mice were developed and showed that the stem cells with myeloid cell properties are responsible for

muscle regeneration (13, 47, 48). Similar mechanisms were reported for regeneration of liver by MØs fusion (49-51). Therefore, we posit a mechanism in which MØs are recruited to the sites of naked pDNA injections as a result of physiological insult caused by needle injury. These MØs phagocytose extracellular naked pDNA and then fuse with the existing muscles as a part of regeneration process thus, delivering pDNA into otherwise hard to transfect skeletal muscles. Based on our observations Pluronic can enhance both the transfection of the MØs with pDNA and the horizontal gene transfer from the transfected MØs to muscle fibers. Previously polyethylene glycol (PEG) mediated cell-to-cell fusion has been extensively studied to generate somatic cell hybrids for cloning, gene mapping, gene expression and antibody production 1970s (12, 52, 53). The proposed model involves exclusion of water molecules between lipid bilayers and changing the orientation of molecular dipoles that results in membrane fusion (54). Since Pluronics contain PEG as well as polypropylene glycol chains capable of incorporating into lipid membranes (55), we believe that they can enhance the MØs to muscle cell fusion events eventually delivering pDNA to muscle fibers and increasing transfection and gene expression both in healthy and inflamed muscles.

It is known that after direct *i.m.* injection in skeletal muscle naked pDNA is rapidly degraded in extracellular matrix (56, 57). Therefore, is noteworthy that we observed a block copolymer-dependent increase in pDNA copies in both healthy and ischemic muscles. Unlike cationic lipids or polycations, Pluronics are non-ionic molecules and do not form complexes with pDNA that can protect it from the degradation in the extracellular matrix similar to lipoplexes/polyplexes (16, 58). However, Pluronics can act as adjuvants that activate NF-kB cell signaling and increase pDNA uptake (59), pDNA nuclear import (60) and transcriptional activation of gene expression of pDNA delivered into cells *in vitro* with polyplexes (61, 62). It is well-known that inflammation results in

increased blood neutrophils counts and secretion of pro-inflammatory cytokines like TNF alpha and IL-1β (63). The overall activation of inflammatory signaling can also influence cmv promoter driven gene expression and may explain somewhat increased gene expression of naked pDNA in non-ischemic muscle of MHLIM. It is important to point out in this regard that the pDNA/Pluronic expression in normal muscles was shown to be promoter selective and dependent on the NF-kB signaling pathway (8, 64). Moreover, systemic inflammation caused by ischemia resulted in increased number of circulatory MØs, which is consistent with previous studies (65, 66). This may result in increased MØs recruitment at the site of pDNA injection due to inflammatory response triggered by physical injury and exposure to bacterial CpG motif and ultimately increase muscle transfection with pDNA/Pluronic. We also observed increased transgene expression in draining lymph nodes, which was in agreement with our earlier studies using *i.m.* pDNA/Pluronic suggesting co-localization of transgene expression with APCs in lymphoid organs (16).

Altogether, MØs have gained recent attention for their promiscuity or plasticity to perform central roles in development, homeostasis and disease and have emerged as novel therapeutics target. Here we resolved a key issue of low muscle transfection in ischemic muscles by simple mixing of naked pDNA with Pluronic and showed how MØs assist gene transfer to otherwise hard to transfect skeletal muscle fibers. Since Pluronics are listed in U.S. Pharmacopoeia and have been demonstrated to be safe in clinical trials (67) such formulations have promise for human use.

3.5. Acknowledgments

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CHAPTER 3

SUPPLEMENTARY DATA

Horizontal Gene Transfer from Macrophages to Ischemic Muscles upon Delivery of Naked DNA with Pluronic Block Copolymers

S3.1. Supplementary Methods

S3.1.1. IHC

Fresh muscle tissues were embedded in Tissue-Tek OCT (Sakura Finetec Inc, Torrance, CA), rapidly cooled to -80^{o}C and sectioned with cryostat microtome. 10 µm thick sections were attached to Superfrost® microscope slides (Fisher Scientific, Bellefonte, PA), dried for 1 h at RT and stored at -80^{o}C for subsequent use. Double staining immunofluorescence was performed in the frozen muscle tissue sections to determine cell types expressing GFP. The sections were sequentially treated with (a) polyclonal rabbit anti-desmin antibody (Abcam, Cambridge, MA) 1:100, Rat anti-CD11b (eBioscience, San Diego, CA) 1:100 antibodies and then (b) with fluorophore-conjugated secondary anti-species antibodies (Goat anti rat-Alexa 594/Goat anti rabbit-Alexa 633) 1:1000. Specifically, frozen sections were incubated at RT for 10-15 min and fixed/permeabilized in ice cold methanol for 5 min, followed by ice cold PBS rinse (twice). Slides were incubated with 10% normal goat serum in 1x PBS (blocking solution) for 1h at 4^{0}C, rinsed with PBS (thrice) and incubated with primary antibody in 2% blocking solution o/n at 4^{0}C. After rinsing with PBS (thrice), the slides were incubated with secondary antibodies in 2% blocking solution for 1 h at RT. Finally, the slides were counterstained with DAPI using wet mounting system (Vectashield, Burlingame, CA), stored in 4^{0}C until examined under

microscope. Negative control specimens (treated with secondary antibody alone) were used for setting confocal lasers.

S3.1.2. ICC.

The (MØ+DNA)+MB and (MØ+DNA)+MT cocultures were rinsed twice with PBS at RT and fixed with fresh 2% formaldehyde solution from 16% stock (TED PELLA, Redding, CA) for 20 min at RT. Samples were washed, blocked and labeled with primary and secondary antibodies as explained above in IHC.

S3.1.3. Confocal Imaging.

The samples were analyzed by Zeiss 710 Confocal Laser Scanning Microscope equipped with a blue diode 405 nm (nucleus), argon laser 488 nm (GFP expression), DPSS 594 nm (cell marker) and HeNe 647 nm (cell marker) using 10 x or 20 x objective.

S3.1.4. Exosome isolation.

Briefly, MØs were transfected using genePORTER300 transfection reagent for 4 h in SFM and cells were rinsed (twice) with HBSS to remove any pDNA transfection reagent. Cells were grown in exosome free CM for another 24 h and 72 h and supernatant was collected and exosome isolated by ultracentrifugation method as described in (1).

S3.2. Supplementary Discussion

Since some studies provide evidence for role of extracellular vesicles (microvesicles and exosomes) in cell-to-cell communication and DNA exchange (2-4), we also studied that possibility behind the pDNA exchange mechanism. MBs and MTs were incubated with exosomes, (isolated from pDNA transfected MØ 24 h and 72 h post transfection), with and without 1% P85 but the resulting levels of gene expression were

below the detection limits (data not shown). We believe, that exosomes at 24 h or 72 h represents a snapshot of vesicles. Infact, a more gradual exchange of exosomes from pDNA from MØ to MT may explain the horizontal exchange of DNA resulting in muscle specific increase in gene expression.

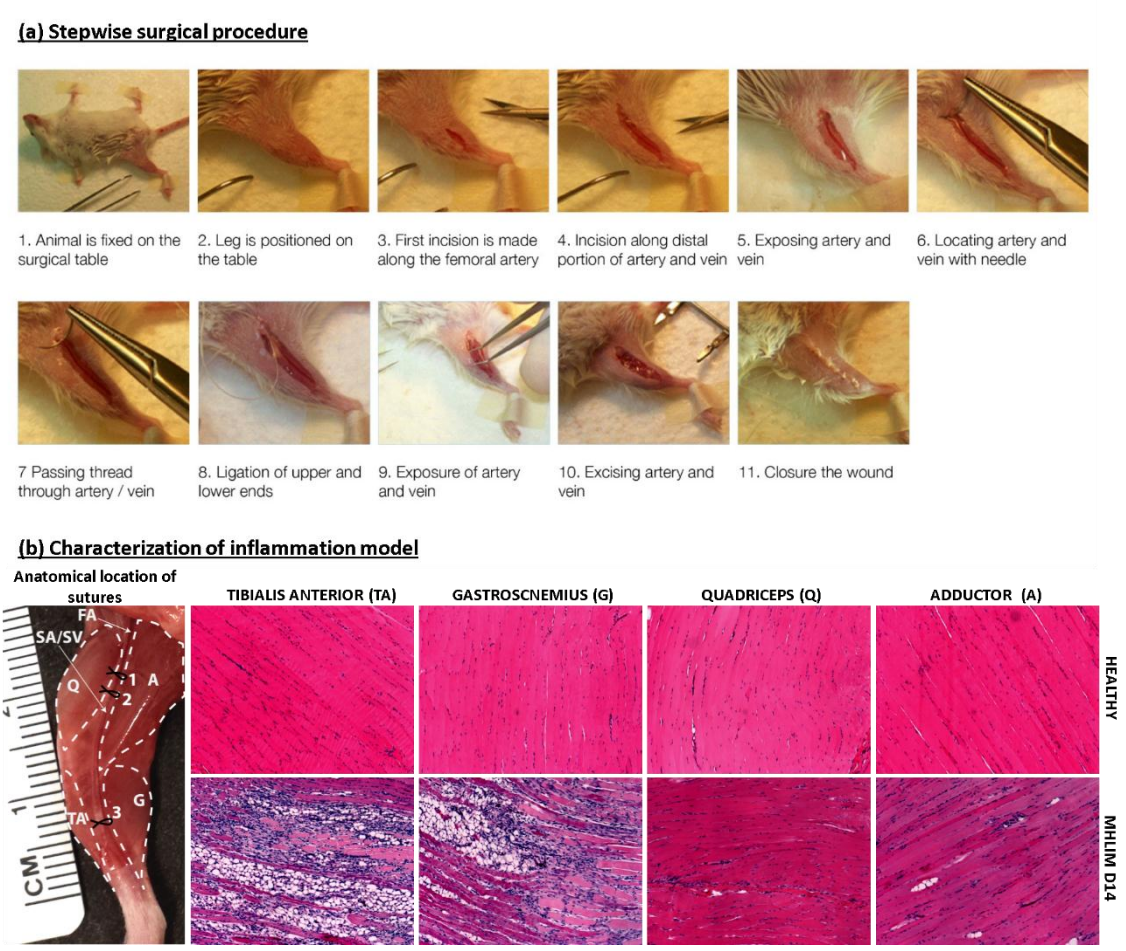

Figure S3.1. Stepwise surgical procedure and characterization of inflammation model (MHLIM). (a) Animals were anesthetized and surgical removal of artery and vein was performed as shown in (b –left) on right leg. **(b)** Anatomical and histological representation of ischemic hind limb muscle tissues and sections. Skin and fascia were removed to show the right hind limb muscles of Balb/c mice and the sites of ligation during surgical procedure. Hind limb ischemia was generated by excision of *FA* before femoral bifurcation (between sutures marked **1** and **2**) and excision of both saphenous artery and saphenous vein (between sutures **2** and **3**). ***TA***, **G**, **Q** and **A:** designate different muscles analyzed. H&E stained 5µm tissue sections of healthy muscles (top row) and ischemic muscles at 14th day post ischemia surgery (bottom row) show histopathology of ischemia

with a typical inflammatory response (cellular infiltrate) during an inflammation. Tissue sections were imaged using 10x objective magnification and images are representative of 5 slides per tissue and 3 animals per group. Consistent with previous reports (1), the effect of ischemia was more pronounced on lower hind limb muscles (**TA** and **G**) compared to upper hind limb muscles (**Q** and **A**). Similar result was obtained H&E sections of ischemic muscles at 3rd day post ischemia surgery (not shown).

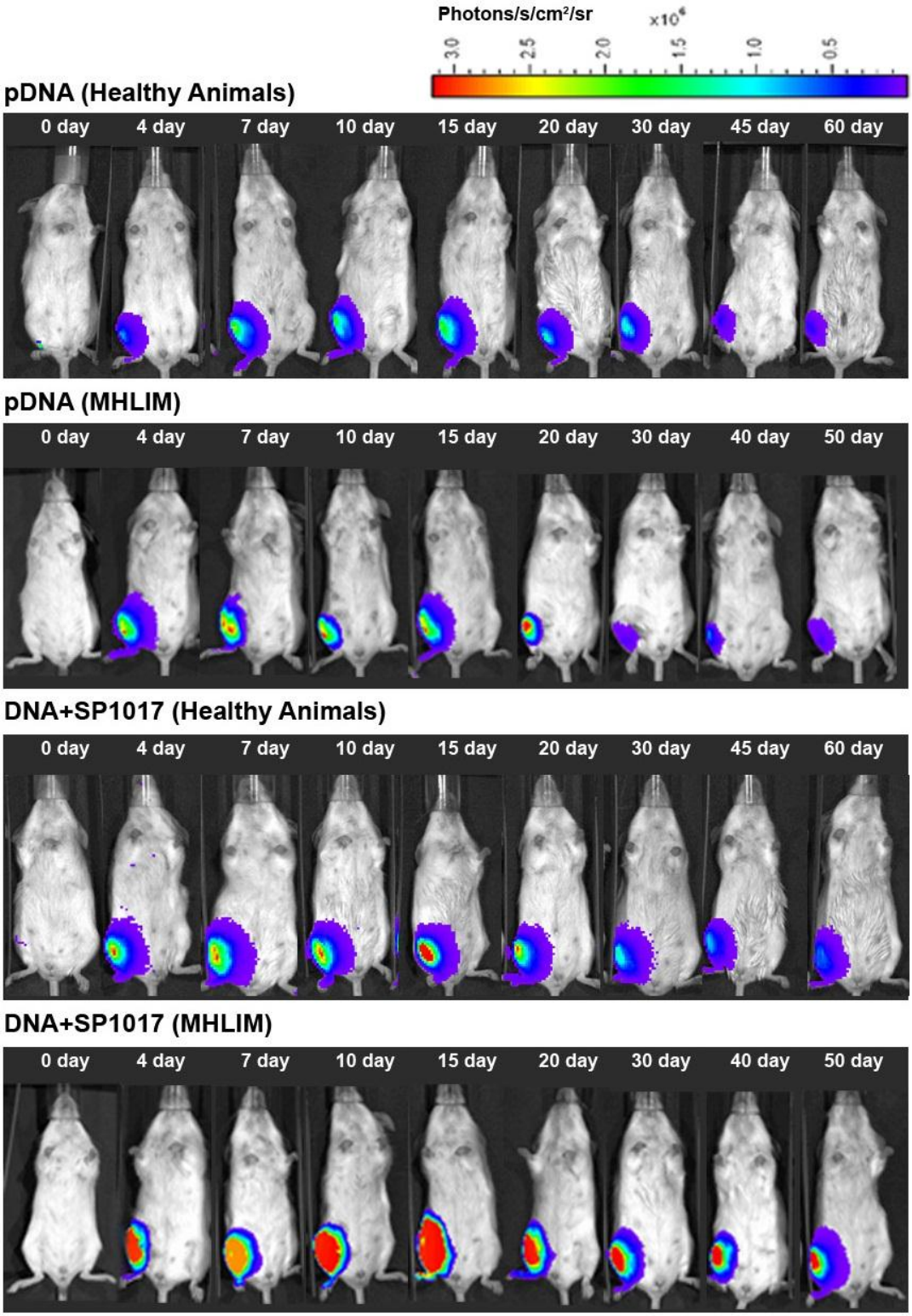

Figure S3.2. Kinetics of gene expression. Representative *in vivo* images (IVIS 200) for healthy and MHLIM mouse after a single injection of 10 µg naked pDNA (gWIZ-Luc, 1st

and 2nd row) and pDNA formulated with 0.1% w/v SP1017 (3rd and 4th row) in 50 µl HBSS solution. The experiment was carried out in quadruplicate for each treatment group and quantified as presented in main text **Fig. 1e**.

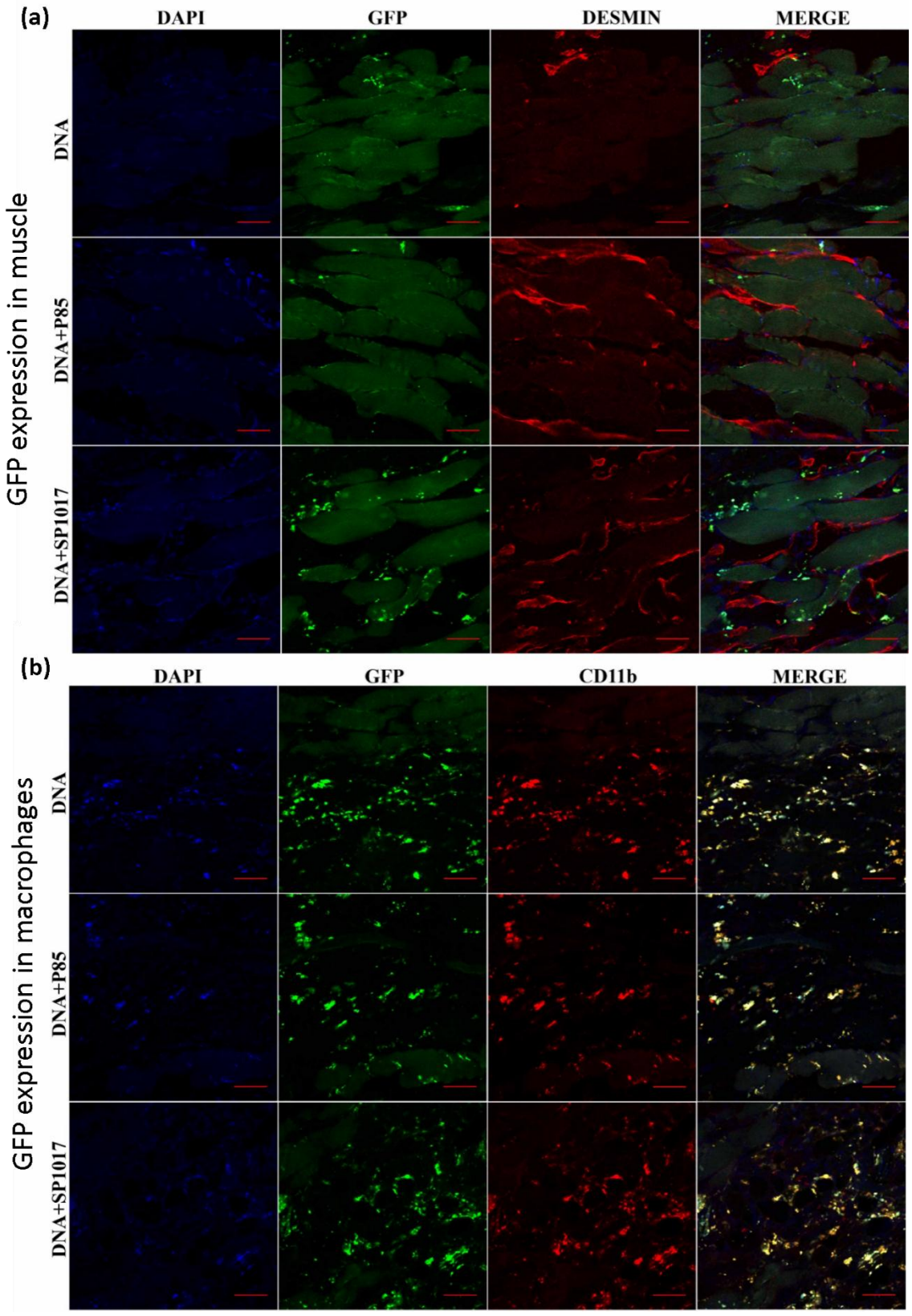
(a)
DAPI
GFP
DESMIN
MERGE
GFP expression in muscle
DNA
DNA+P85
DNA+SP1017
(b)
DAPI
GFP
CD11b
MERGE
GFP expression in macrophages
DNA
DNA+P85
DNA+SP1017

Figure S3.3. GFP expression and colocalization with cellular markers. (a, b) Colocalization of GFP expression with muscle **(a)** and MØ **(b)** markers visualized in longitudinal sections of ischemic *TA* muscles 4 days after gWIZ™ GFP injections. The color staining corresponds to nucleus (blue); MØs ($CD11b^+$; red); myocytes ($desmin^+$; red); and GFP (green). The last panels in each row present digitally superimposed images of preceding panels to visualize the colocalization (yellow). The images were taken with Zeiss 710 confocal laser scanning microscope using 20x objective, scale bar 50µm.

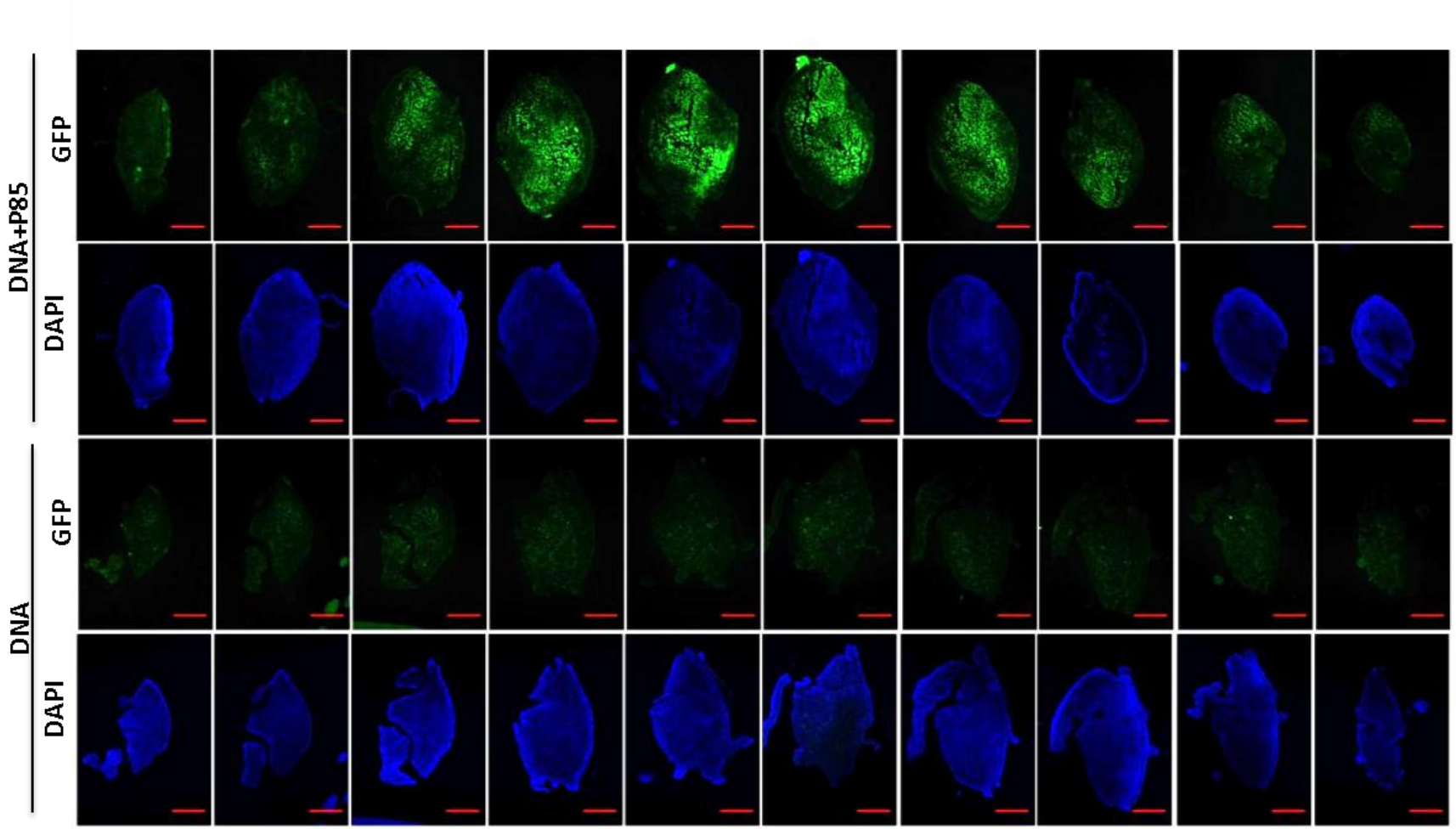

Figure S3.4. GFP expression through ischemic muscle tissue. Tile scanning confocal microscope images (10 x) of 20 µm thick cross-sections at every 500 µm throughout the whole *TA* muscle tissue 4 days after injections of gWIZ™ GFP pDNA alone

or pDNA with 0.6 % w/v P85 in ischemic muscles in MHLIM. Representative images from each treatment group with n = 3 are shown. Scale bar = 1mm.

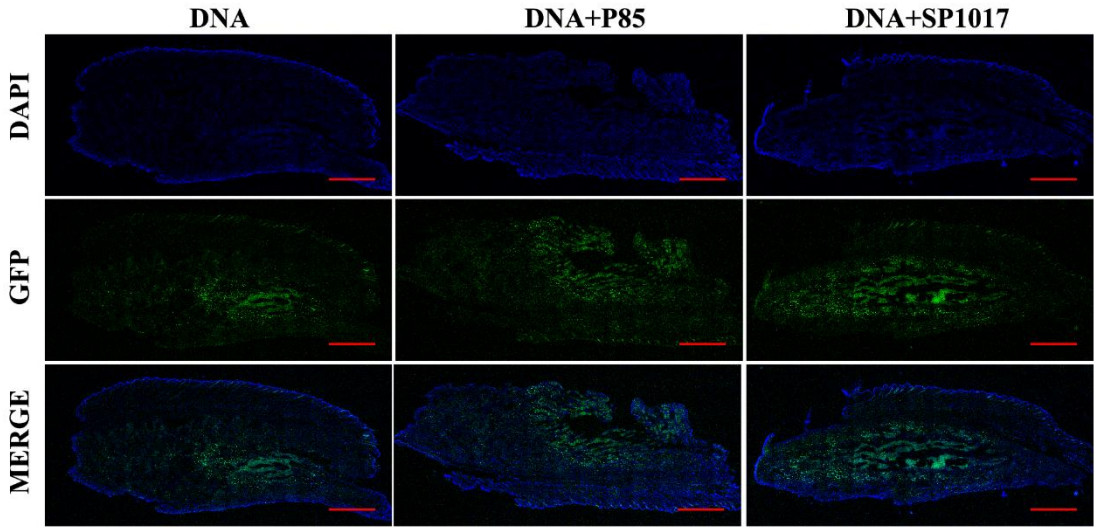

Figure S3.5. Spread of GFP expression in ischemic muscles. 4 days after pDNA injections in ischemic muscles, the spread of GFP expression in 20 µm thick longitudinal tissue section was visualized by tile scanning using confocal imaging (10 x). Scale bar = 1mm. The muscle specimens were harvested after injection and individually processed for IHC. Whole muscle longitudinal sections were imaged by confocal tile scanning to view the overall GFP expression. Pictures are representative of 3 slides per group.

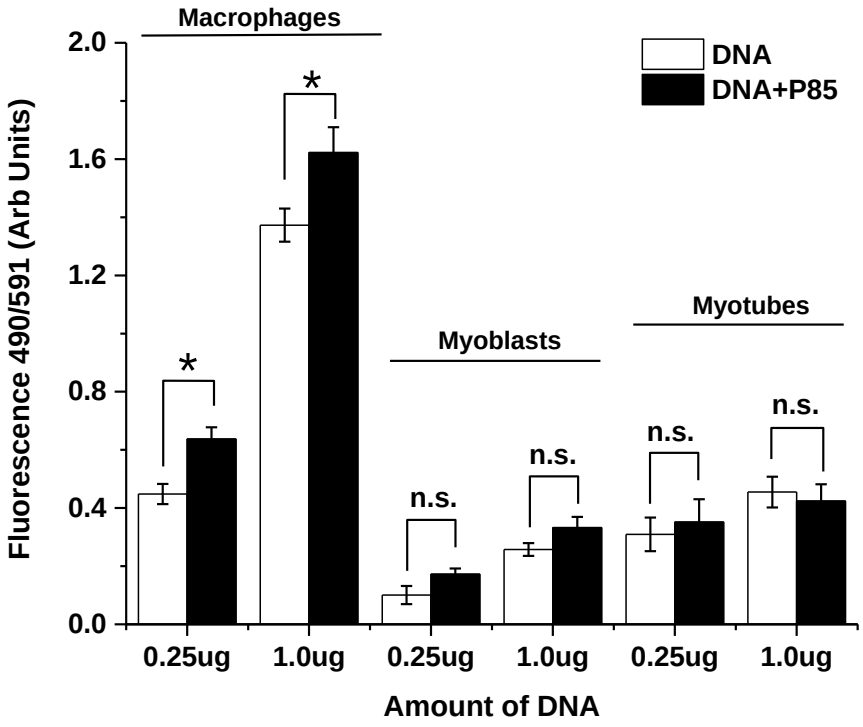

Figure S3.6. Effect of Pluronic on pDNA uptake in various cell types *in vitro*. RAW 264.7 MØ, C2C12 MBs and C2C12 derived D7 MTs (50,000 cells/well in 96 well plates, 24 h after plating) cells were exposed to YOYO-1 labeled gWIZ™ Luc pDNA (0.25 µg and 1 µg) in absence (white bars) or presence of 1% w/v P85 (black bars) in SFM for 2 h. After that cells were rinsed thrice with PBS and lysed using 50 µl M-PER® cell lysis reagent (ThermoFisher Scientific, Vernon Hills, IL) for 5-10 min at 4 ^{0}C. Total fluorescence was quantified in cell lysates using Spectramax M5 plate reader (Molecular devices, Sunnyvale, CA) at λ Ex/Em 490/591. Data are mean ± SEM (n=6) *p<0.05, n.s. – non significant.

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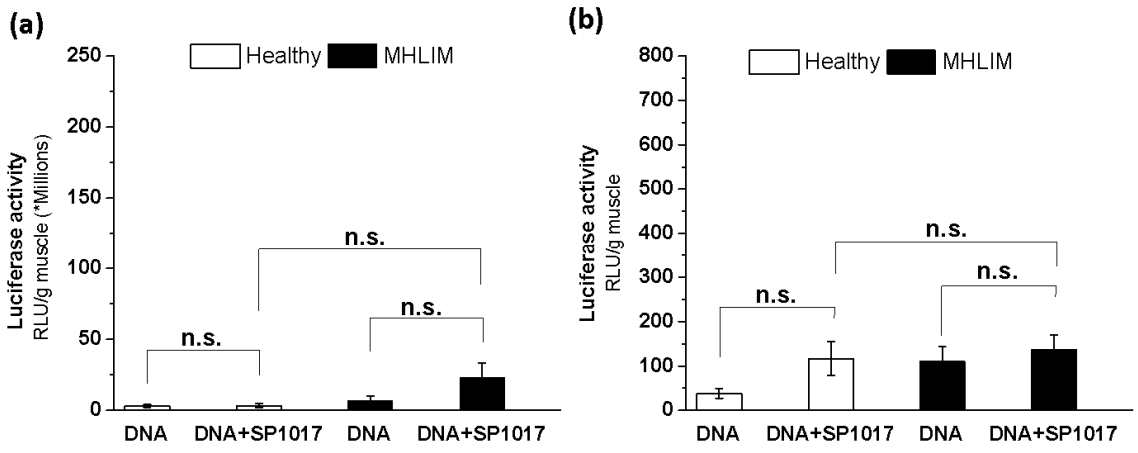

Figure S3.7. Effect of skeletal muscle ischemia on transgene expression in contralateral muscles. As per scheme in Fig. 6b the test articles were injected in *TA* muscles in healthy mice or in contralateral *TA* muscles simultaneously with the ipsilateral ischemia surgery in MHLIM. Luciferase gene expression in the **(a)** *TA* muscles and **(b)** draining lymph nodes of MHLIM (black) and healthy mice (white) was determined 3 days after single administration of 10 µg gWIZ™ Luc with or without 2.25% w/v SP1017. pDNA injections were performed in *TA* muscle of healthy mice (white bars) and healthy or non-ischemic muscles of MHLIM (black bars). Data are mean ± SEM (n = 10), n.s. – non significant. Statistical comparisons were made between MHLIM and healthy mice.

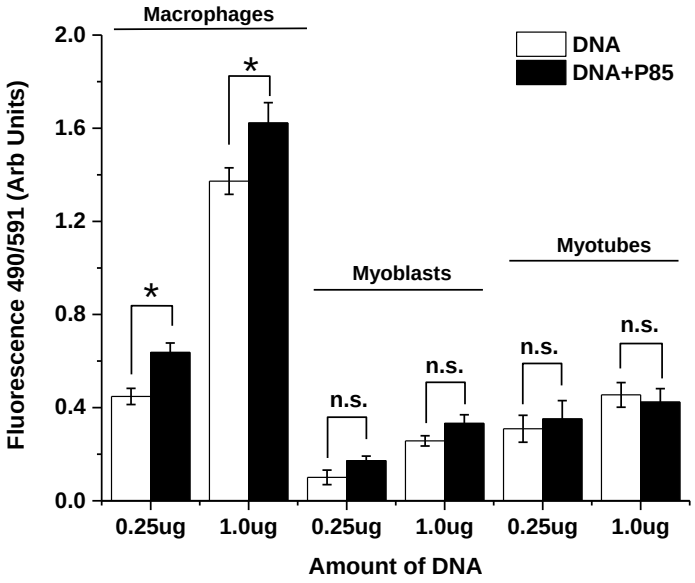

Figure S3.8. Effect of Pluronic on pDNA uptake in various cell types *in vitro*. RAW 264.7 MØ, C2C12 MBs and C2C12 derived D7 MTs (50,000 cells/well in 96 well plates, 24 h after plating) cells were exposed to YOYO-1 labeled gWIZ™ Luc pDNA (0.25 µg and 1 µg) in absence (white bars) or presence of 1% w/v P85 (black bars) in SFM for 2 h. After that cells were rinsed thrice with PBS and lysed using 50 µl M-PER® cell lysis reagent (ThermoFisher Scientific, Vernon Hills, IL) for 5-10 min at 4 ^{0}C. Total fluorescence was quantified in cell lysates using Spectramax M5 plate reader (Molecular devices, Sunnyvale, CA) at λ Ex/Em 490/591. Data are mean ± SEM (n=6) *$p<0.05$, n.s. – non significant.

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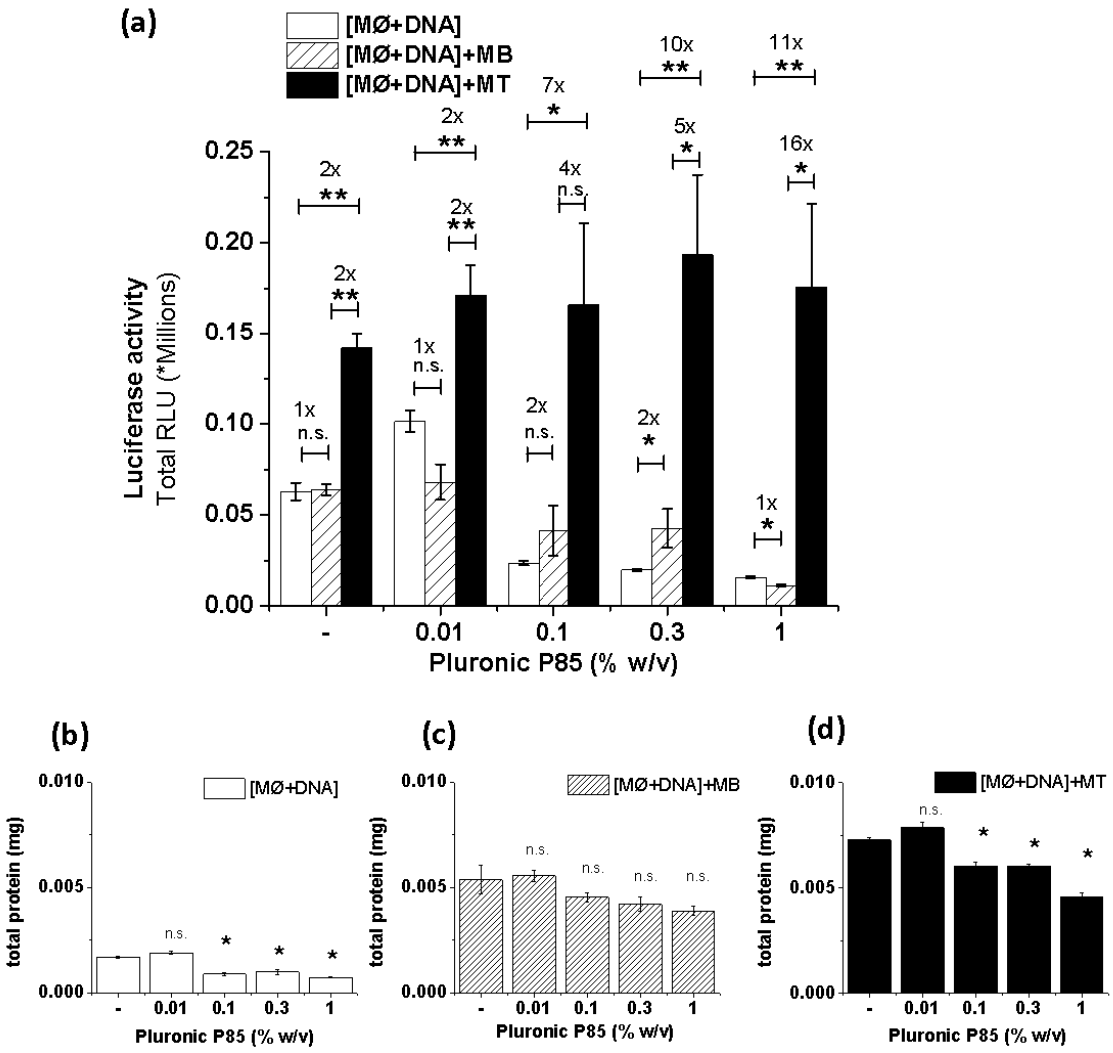

Figure S3.9. Effect of Pluronic on total gene expression and protein levels in the transfected MØs and their co-culture with muscle cells. (a) pDRIVE5Lucia-mDesmin transfected MØs were plated alone ([MØ+DNA]), or cocultured on top of the monolayer of MBs ([MØ+DNA]+MB) or MTs ([MØ+DNA]+MT). After 2 h, when MØs attach to the MBs or MTs, the groups were treated with increasing concentrations of P85 (0.01, 0.1, 0.3 and 1.0 % w/v) or fresh media for 2 h, washed, further incubated. The total secreted luciferase expression was analyzed after 24 h in cell culture media**.** Total protein content was determined in cell lysates after 24 h for **(b)** [MØ+DNA]**, (c)** ([MØ+DNA]+MB and **(d)** MØ+DNA]+MT groups with and without P85 treatment. Data are mean ± SEM (n=6),

Statistical comparisons were made for treated vs. untreated groups. * $p<0.05$, ** $p<0.005$, n.s. – non significant.

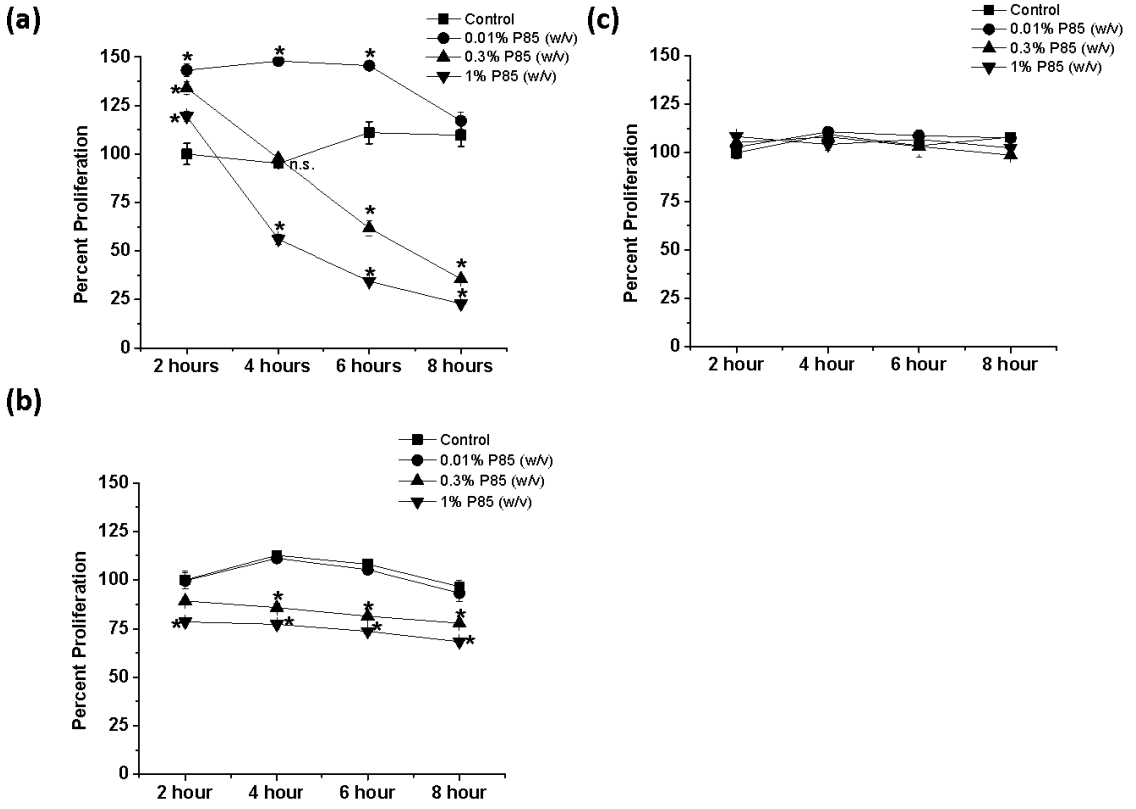

Figure S3.10. Cytotoxicity of P85 on MØs (a), MBs (b) and terminally differentiated MTs (c) was determined after exposure of the cells to different concentrations of P85 (0.01%, 0.3% and 1.0%) for different durations (2, 4, 6, and 8 h) in SFM. Cells were further cultured for total 24 h if fresh media and the percent cell proliferation, i.e. treated cells compared to untreated controls, was determined by MTS assay. Data are mean ± SEM (n=6); statistical comparisons were made for P85 treated and untreated groups: * $p < 0.05$, n.s. – non significant. At all-time points examined the proliferation of MØs exposed to lower concentrations of P85 (0.01% w/v) was significantly increased compared to untreated controls. Likewise MØs exposed to higher concentrations of P85 (0.3 and 1.0% w/v) for 2 h exhibited greater proliferation compared to untreated controls. However, at longer exposures (4, 6 and 8 h) these relatively high concentrations of P85 induced cytotoxic effect in MØs. Exposure of MBs to P85 at higher concentrations of (0.3 and 1.0% w/v)

induced some limited cytotoxicity. MTs were the most resistant cells with respect to P85 as no toxicity was observed at any concentration of P85 at any time point studied.

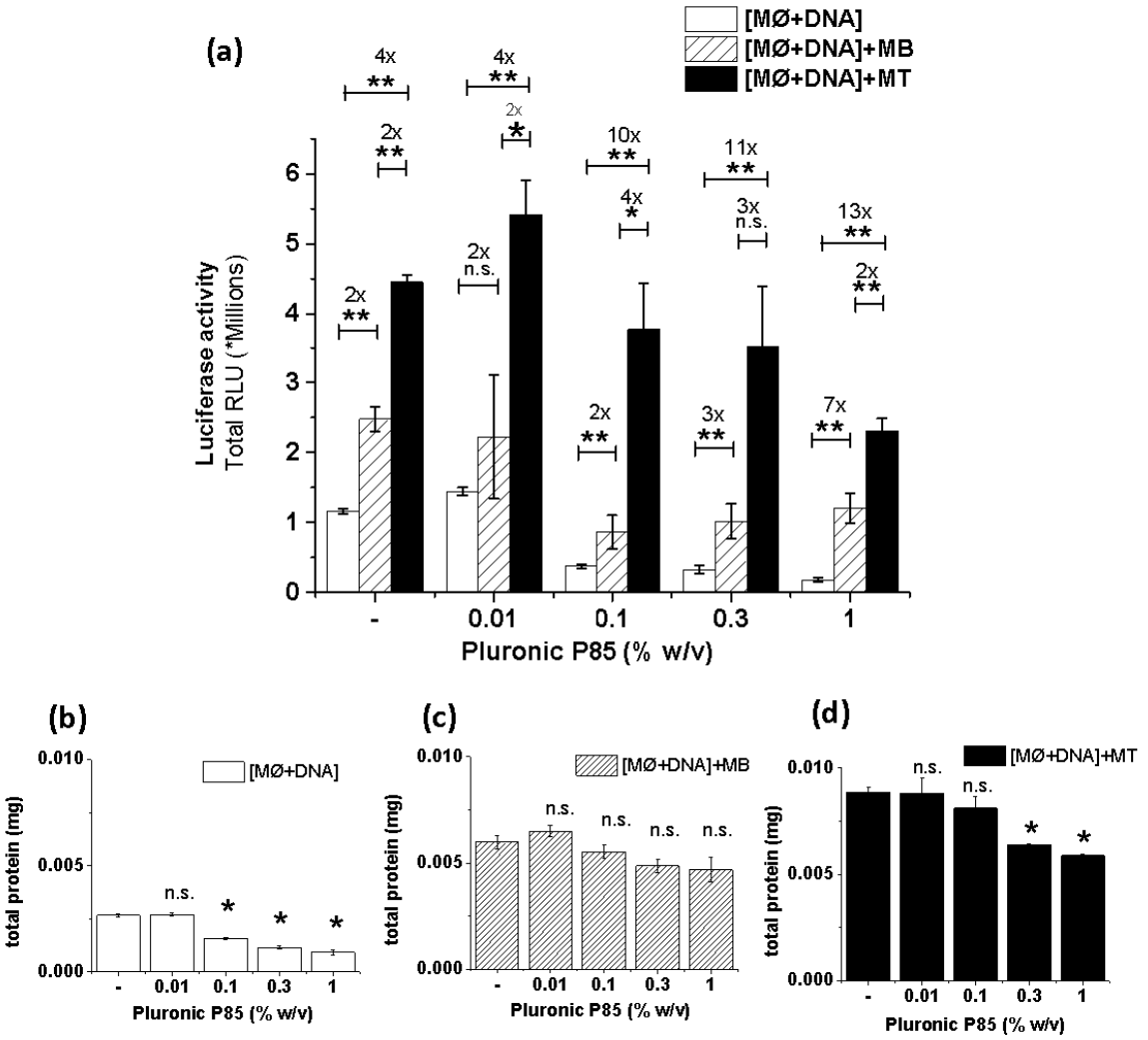

Figure S3.11. Effect of Pluronic on total gene expression and protein levels in the transfected MØs and their co-culture with muscle cells. (a) gWIZ™ Luc pDNA transfected MØs were plated alone, [MØ+DNA], or cocultured on top of the monolayer of MBs, [MØ+DNA]+MB, or MTs, [MØ+DNA]+MT. After 2 h, when MØs attach to the MBs or MTs, the groups were treated with increasing concentrations of P85 (0.01, 0.1, 0.3 and 1.0 % w/v) or fresh media for 2 h, washed, further incubated. The total luciferase expression analyzed after 24 h in cell lysates**.** Total protein content was determined in cell lysates after 24 h for **(b)** [MØ+DNA]**, (c)** [MØ+DNA]+MB and **(d)** MØ+DNA]+MT groups

with and without P85 treatment. Data are mean ± SEM (n=6), Statistical comparisons were made for treated vs. untreated groups. * $p<0.05$, ** $p<0.005$, n.s. – non significant.

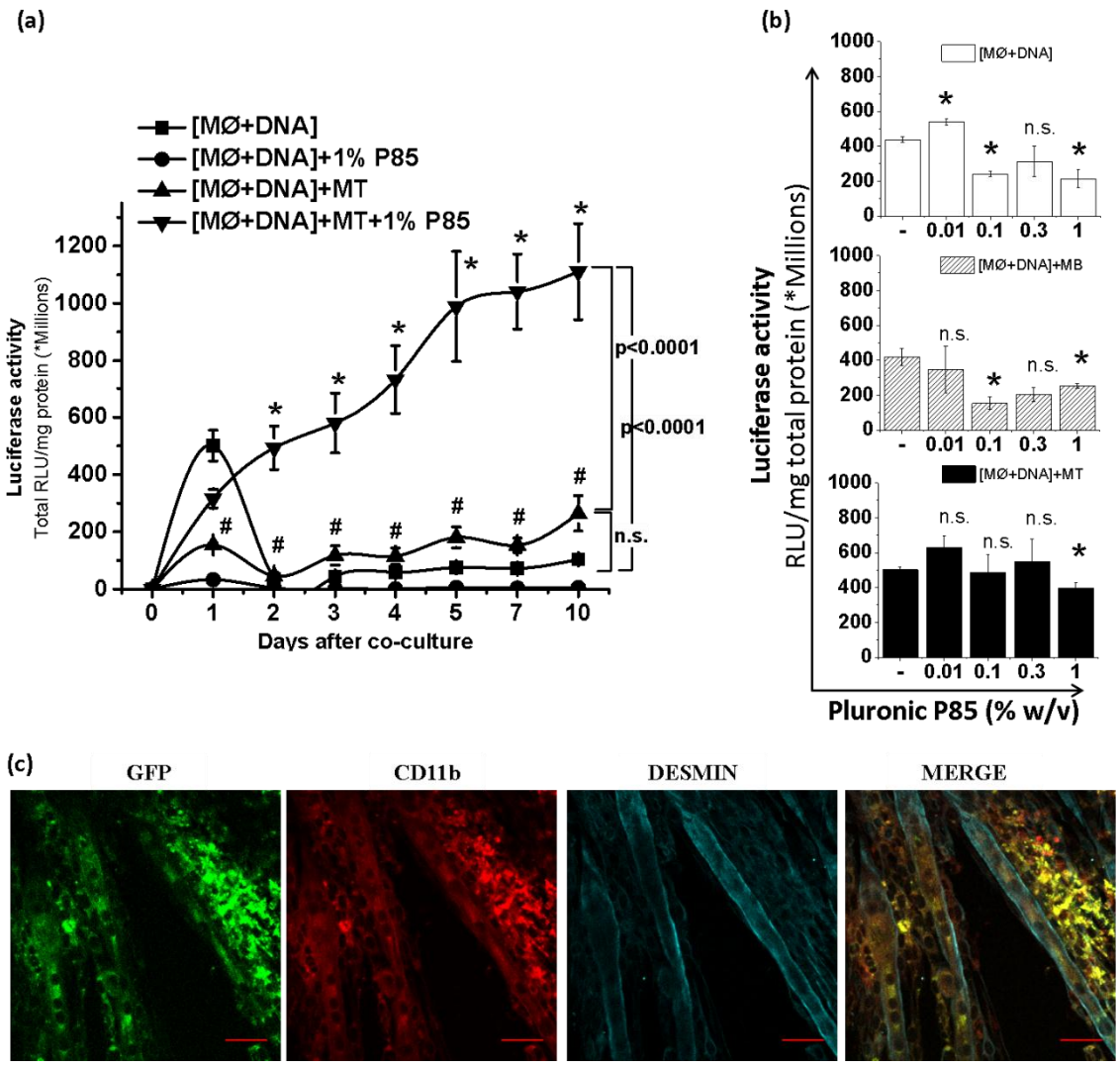

Figure S3.12. Effect of P85 on horizontal gene transfer from transfected MØs to muscle cells upon co-culture. (a, b) gWIZ™ Luc pDNA transfected MØs were plated alone, [MØ+DNA], or cocultured with MBs, [MØ+DNA]+MB, or MTs, [MØ+DNA]+MT, and exposed to P85 (0.01, 0.1, 0.3 and 1.0 % **(b)** or 1 % w/v **(c)**) for 2 h. The total luciferase in cell lysates was determined (a) daily for 10 days or (b) after 24 h and normalized for the cell protein**.** A significant decrease in gene expression in [MØ+DNA] *v.s.* [MØ+DNA]+P85 groups at day 1 can be explained by detachment of freshly plated MØs upon 1% P85 treatment, which also explains the decrease in gene expression in upon treatment with increasing concentration of P85. **(a, b)** Data represents mean ± SEM, * $p<0.05$, n.s. – non

significant, **(a)** (n=12), statistical comparisons were done for coculture with and without P85 treatment vs. MØ+DNA groups alone and the curves were compared using AUC and one-way ANOVA analysis **(b)** (n=6). **(c)** GFP expression (green) in desmin^{+} MTs (cyan) was validated 3 days after their coculture with MØs CD11b^{+} (red) transfected with gWIZ™ GFP pDNA. The last panels in each row present digitally superimposed images (20 x) of preceding panels to visualize the co-localization (yellow). Scale bar = 50 µm.

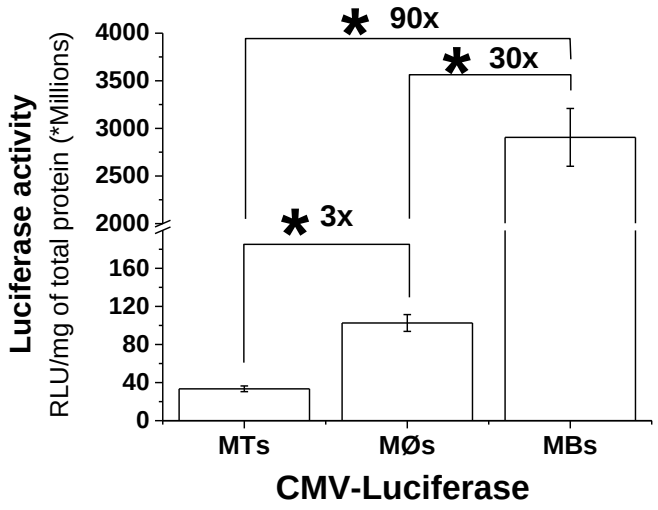

Figure S3.13. Relative transfection efficiencies in MØ, MB and MT *in vitro.* Terminally differentiated C2C12 derived MT, precursor C2C12 MB and RAW264.7 MØ were transfected with gWIZ™ Luc (cmv-luciferase) using genePORTER300 and gene expression was determined in cell lysates after 24 h. Data are mean ± SEM (n=4); statistical significance was calculated using student's t test at $p<0.05$. MTs in general were the most difficult to transfect cells. The transfection of these cells using cmv- and desmin-luciferase pDNA as 90 and 40 times less respectively compared to their precursor MBs cells. Consistent with the literature MØs were in general more difficult to transfect cells than undifferentiated proliferating cells (MBs).

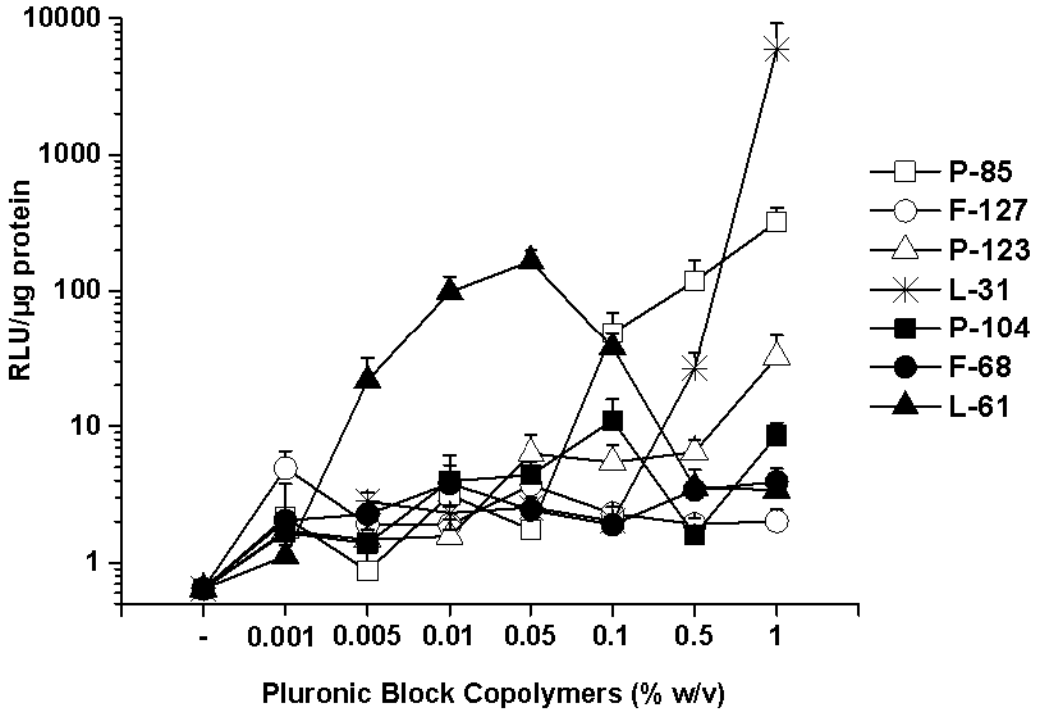

Figure S3.14. Effect of Pluronic block copolymers on the gene expression in RAW 264.7 MØs. Cells were exposed to gWIZ-Luc pDNA with or without P85 in SFM for 2 h using 1 µg pDNA and various concentrations of P85 and replaced with CM. After 24 h, luciferase gene expression in 10 µl cell lysates was quantified using Glomax20/20 luminometer with an integration period of 10 s. Data are mean ± SEM (n=4).

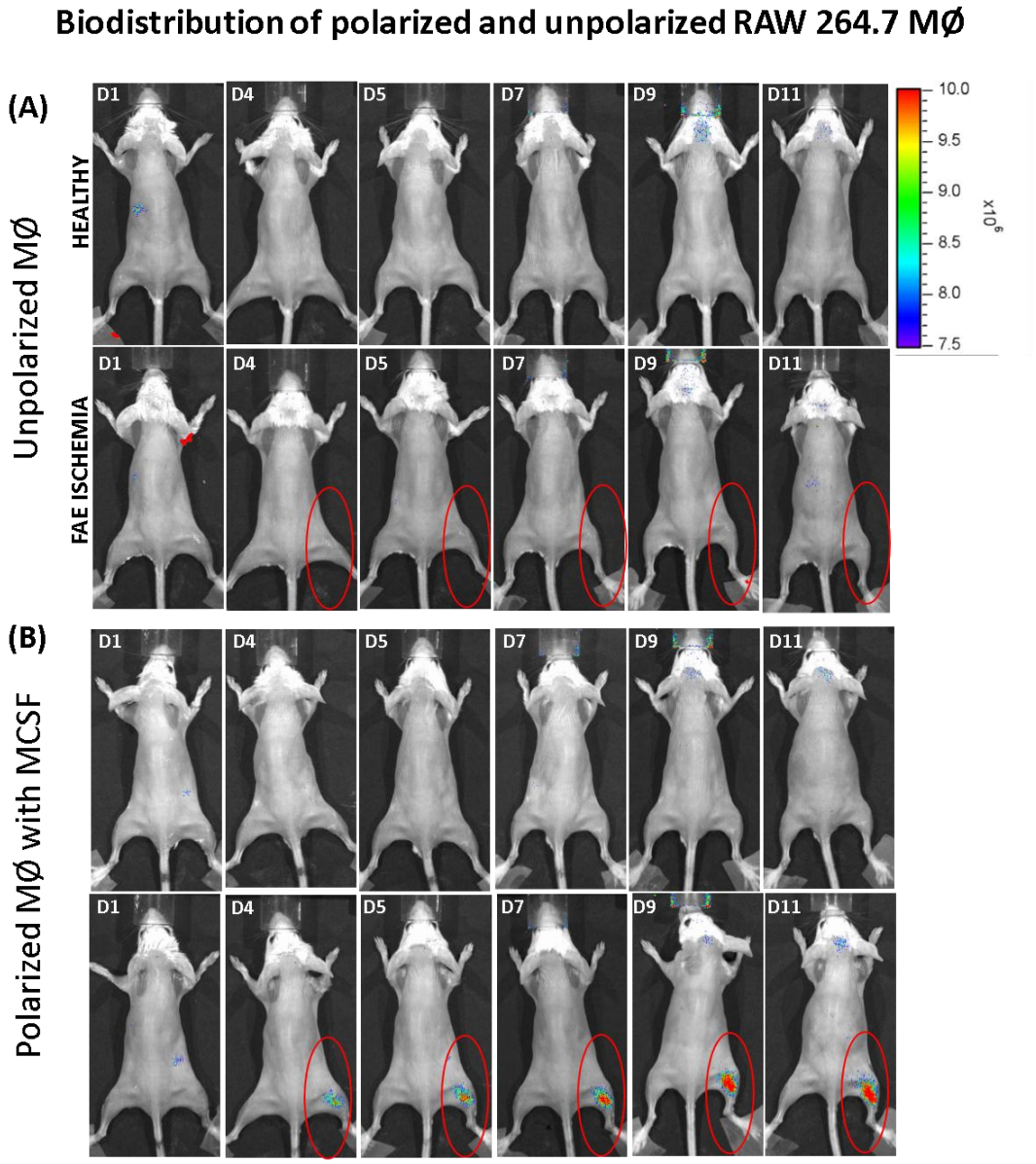

Figure S3.15. Bio-distribution of RAW264.7 Macrophages loaded with alexa fluor-680 labeled nanozyme in healthy and MHLIM. 5X10^6 MØ/200ul PBS were injected via intra jugular in healthy (A) and ischemic (B) of mice, 48 hours post ischemia surgery (Day

0). Representative images of n=3 per group showed no trafficking of unpolarized macrophages to ischemic tissues unless polarized with MCSF for 48 h.

S3.1.4. References

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CHAPTER 4

Harnessing Macrophages Response to Increase Gene Delivery to Muscle Using pDNA Formulated with Pluronic Block Copolymers

ABSTRACT

Previously Pluronic block copolymers were shown to drastically increase the level and duration of reporter gene expression after intramuscular (*i.m.*) administration of plasmid DNA (pDNA) in immunocompetent animals. This was not seen in athymic nude mice. Therefore, this study explores possible involvement of innate immune response in Pluronic enhanced gene transfer. After intraperitoneal (i.p.) administration of gWIZ™Luc plasmid DNA (pDNA), formulated with Pluronic P85 (P85) there was a profound increase in the peritoneal exudate cells (PECs) characterized by enhanced recruitment of anti-inflammatory monocytes (MOs) and neutrophils in euthymic Balb/c, but not in the athymic nude mice. The anti-inflammatory MOs response in Balb/c mice was observed to both pDNA/P85 and P85 copolymer alone and peaked about 1 day after their administration. This was very different from responses to conventional adjuvants such as alum and lipopolysaccharide (LPS) that increased PECs in both mouse strains (except for LPS in Balb/c), and enhanced recruitment of neutrophils, while having relatively little or no effect on anti-inflammatory MOs. In contrast to P85 neither alum nor LPS increased pDNA expression in the muscle of Balb/c mice. The depletion of MOs / macrophages (MØs) in Balb/c mice by clodronate-liposomes resulted in abolishment of P85 enhanced pDNA expression, which reinforced the role of these cells in the muscle transfection. To increase MOs / MØs recruitment the muscles were pre-injected with P85 1.5 days prior to subsequent pDNA/P85 injections in the same muscles. This strategy resulted in further

significant enhancement of the muscle transfection. Overall, the results suggest that by harnessing the MOs / MØs responses using Pluronic block copolymers one can enhance the gene transfer to the muscle using pDNA.

4.1. Introduction

The transfection of the skeletal muscle after direct intramuscular (*i.m.*) injection of naked plasmid DNA (pDNA) was discovered in 1990s by John Wolf and coworkers (1). Since then this simple methodology has been used in numerous clinical trials (2, 3). However, development of a successful gene therapy on its basis has been hindered due to low levels and short duration of gene expression after *i.m.* injections of naked pDNA. Therefore, improvements of the muscle transfection efficiency have been actively sought for nearly quarter of century (4-10). Early on it became obvious that conventional non-viral transfection approaches involving condensation of pDNA with cationic lipids or polymers that have been developed for *in vitro* gene delivery are not applicable for direct *i.m* injection (11, 12). Incorporation of pDNA in lipoplexes or polyplexes instead of increasing gene expression in the muscle abolished it in comparison to the naked pDNA.

In contrast, some progress in gene delivery to the muscle was achieved with physical methods in combination with pDNA, such as electroporation that produces limited injury to the muscle and increases gene expression (13, 14). The electroporation-mediated gene transfer (EGT) involves application of electric current that generates transient aqueous pores in the membranes of muscle cells and induces electrophoretic movement and entry of pDNA through these pores (12, 15). The electrical injury also results in intensification of recruitment of immune response cells (16, 17) in particular CD11b+ cells (18), which may affect gene expression.

Another, successful approach reported by us and others involves co-administration of pDNA with Pluronics (poloxamers), a series of nonionic block copolymers that do not condense pDNA but nevertheless drastically increase the level and duration of gene expression in the muscle (19-21). The efficiency of this method is comparable with that of EGT (20). Moreover, pDNA/Pluonic formulation applied in combination with EGT results in further increase in gene expression in the muscle compared to the naked pDNA with EGT (22). This effect was previously explained by ability of Pluronic to facilitate sealing of the electroporation-damaged membranes and increase survival of the transfected cells (23).

However, until recently the mechanism of gene delivery using pDNA/Pluronic formulations was not entirely clear. Kabanov's group has discovered that Pluronics can increase gene transfer in the cells *in vitro* using pDNA-condensed polyplexes (pDNA/polycation interpolyelectrolyte complexes) (24-26). They explained this by increased intracellular uptake of pDNA, enhanced translocation of pDNA to the nucleus and activation of transcription of the delivered gene (24-26).The two latter processes appeared to correlate with the ability of Pluronic to activate NF-κB signaling in the cells and the presence of NF-κB response elements in the pDNA, suggesting that the copolymer can engage innate mechanisms for cellular transport and processing of pDNA (21, 26). Similar factors may actually contribute to the pDNA gene expression in the muscle since the effect of Pluronic on pDNA expression in the muscle strongly depends on the presence of the NF-κB in the promoter region of the plasmid (21).

In the meantime it became clear that the muscle transfection is a complex process that involves multiple cells types and depends on specific and non-specific immune responses initiated by the needle tissue damage, presence of CpG sequences in pDNA backbone and the expression of the encoded protein (27-29). Additionally, the

immune response cells such as macrophages (MØs) can capture pDNA in the cytoplasmic vesicles and express transgenes not only in the injected muscles but also in the distal lymphoid organs (30, 31). It is generally believed that immune responses can negate the gene expression of pDNA in the nucleus by inducing premature silencing of episomal pDNA due to methylation of the CMV promoter (16, 27-29, 32). Moreover, a specific cytotoxic T-lymphocyte response against antigen expressed by the transfected muscle fibers can also damage host tissue and thereby further decrease gene expression (33). Therefore, inflammatory responses have been generally visualized as an adverse factor for a successful gene delivery (16, 27-29, 32, 33).

We did report, however, that Pluronic effect on gene expression in the muscle was strain-dependent (absent in immunodeficient athymic nude mice) suggesting strong correlation of immune response and gene expression enhancement (21). Moreover, following *i.m.* administration of pDNA/Pluronic formulation in euthymic mice the levels of DNA, RNA and encoded protein were greatly increased in draining lymph nodes and spleen, where the expressed protein co-localized with MØs and dendritic cells (34). Most recently we demonstrated that the *in vitro* transfected MØs can horizontally transfer the transgene *in vitro* to the new host cells and after adoptive transfer to animal models carry the transgenes to the distal sites of inflammation (35, 36). Therefore, we hypothesize that monocytes (MOs) and MØs can participate in the Pluronic enhanced gene transfer of the naked pDNA to the muscle. To test this hypothesis in this paper we 1) examined the effect of Pluronic on MOs recruitment in healthy euthymic and athymic mice; 2) depleted MOs/MØs in euthymic mice and examined the effect of the depletion on the Pluronic enhanced pDNA expression in the muscle, and 3) attempted to boost MOs recruitment by Pluronic pre-injections followed by *i.m* administration of pDNA/Pluronic formulation to further increase gene expression in the muscle. The results suggest that 1) the innate

immune responses and in particular recruitment of MOs/MØs play essential role in Pluronic enhanced gene expression and 2) MOs/MØs can be harnessed to further increase *i.m.* expression of pDNA/Pluronic formulations.

4.2. Material and Methods

4.2.1. Plasmids

The gWIZ™ high expression vector encoding the reporter genes, luciferase (gWIZ™ Luc) under control of an optimized human cytomegalovirus (CMV) promoter followed by intron A from the CMV immediate-early gene (Genlantis, San Diego, CA) was used for *i.m.* injections throughout the study. All plasmids were expanded in *E. coli* DH5α host and purified using endotoxin-free Qiagen Plasmid Giga Kit (Qiagen, Valencia, CA) according to the supplier's protocol. Final pDNA pellet was reconstituted in endotoxin free Dulbecco's phosphate buffered saline (DPBS) and characterized by restriction endonuclease digestion followed by agarose gel electrophoresis. pDNA was stored at -20^{o}C until use. Plasmid solution endotoxin levels quantified using ToxinSensor^{TM} Chromogenic LAL Endotoxin Assay Kit were negligible (13E U/mg the FDA allowed limit ≤40EU/mg pDNA).

4.2.2. Animals

All animal experiments were carried out with approval of the University of Nebraska Medical Center (UNMC) and University of North Carolina at Chapel Hill (UNC-CH) Institutional Animal Care and Use Committee (IACUC) and in accordance with the NIH Guide for Laboratory Animal Use. Female Balb/c or female athymic nu/nu (Charles River Laboratories, Wilmington, MA) mice of 6 to 8 weeks age were used throughout this study. The animals were kept in groups of five and fed *ad libitum*.

4.2.3. pDNA/Pluronic formulations and i.m. injections

Pluronic L61 (batch # WPNT-511B), P85 (batch # WPNT-511B), F127 (batch # WPNT-511B) were a kind gift from BASF Co. (Mount Olive, NJ). The pDNA formulations were prepared as described (34) and used immediately for *i.m.* injections using 1 c.c. insulin syringes. Specifically, mice were anesthetized using ketamine and xylazine mixture (100 mg/kg and 5 mg/kg respectively) and pDNA/Pluronic mixtures were injected in *tibialis anterior* (*TA*) muscle as 50 µl sterile solution. Injections were performed with needle direction parallel to *TA* muscle and a flow rate of 0.6-0.7 ml/min. In select experiments P85 at a given concentration alone was injected in *TA* muscle followed by injection of naked pDNA alone or pDNA/0.3% or 0.6% P85 in the same *TA* after 1.5 day or 5 days.

The sample of SP1017 used in this work by V.M. behaved very different compared to SP1017 samples previously described in literature and other laboratories. In particular SP1017 as tested was not increasing pDNA expression in the muscle as was previously documented by many investigators. We suspect that the components used for preparation of this sample were subjected to chemical degradation. Unfortunately, prior to completion of this dissertation we were not able to obtain new sample of SP1017 and therefore present the results obtained with the old samples with understanding that in the future our laboratory will carry out studies as the new sample is available.

4.2.4. Tissue Histology

In one experiment the *TA* muscles were injected with P85 (0.3% w/v, 3% w/v and 10% w/v) and then either 1.5 or 5 days after injections removed en bloc and processed as described earlier (14). Five µm thick, 3 sections per muscle were processed for Hematoxylin & Eosin (HE) staining. In another experiment, the spleen tissues from MO/MØ depleted and normal mice were isolated en bloc, embedded using Tissue-Tek

O.C.T. compound and frozen at -80^{0}C. 10 µm thick sections were stained with F4/80$^+$ antibody-HRP conjugate (Clone BM8; ebiosciences, San Diego, CA) followed by HIGHDEF red IHC chromogen staining as per supplier's recommendation (Enzo Life Sciences, Farmingdale, NY).

4.2.5. Luciferase gene expression

Unless indicated otherwise mice were euthanized at the indicated time points in the figure legends and tissues were processed as described in (34). The luciferase gene expression in 10 µl tissue homogenates was quantified using Glomax 20/20 luminometer (Promega, Fitchburg, WI) for an integration period of 10 s respectively as described before (34). Alternatively, luciferase activity in live animals was measured for the indicated time using *in vivo* imaging system IVIS-200 (Xenogen Corporation, Alameda, CA) 5 min after *i.p* injection of D-luciferin and the imaging data were quantified as described before using 1 min integration time (34). The signal intensity was quantified as the sum of the photons/sec/cm^2/sr detected and the results were analyzed using Living Image® v2.50 software.

4.2.6. Peritoneal lavage and multi-color flow cytometry

Each euthymic Balb/c and athymic nude mouse was injected intraperitoneally (*i.p.*) with 250 µl of DPBS, 50 µg naked pDNA alone, 0.3% w/v P85/pDNA, 0.01% w/v SP1017/pDNA, 25 µg lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) or 500 µg alum (2% Alhydrogel; InvivoGen, San Diego, CA). All mice were sacrificed by cervical dislocation at the indicated time points and the peritoneal cavities were lavaged with cold 3 ml RPMI media (without phenol red) supplemented with 10% fetal bovine serum using 25G needle, massaged for 1 min and cells extracted using 22 G needle. The procedure was repeated thrice. Finally, peritoneal cavity was opened to collect any residual fluid and

a total ~9 ml peritoneal exudate cells (PECs) were centrifuged at 500 g for 10 min, washed twice with DPBS, and resuspended in 100 µl 1x FACS buffer (BD Biosciences, San Jose, CA). Cell viability was determined by trypan blue exclusion test using Countess® automated cell counter (Life Technologies, Grand Island, NY) before processing the cells for flow cytometry. 10^6 live cells in 100 µl 1x FACS buffer were treated with Mouse BD Fc Block™ (Rat anti-mouse CD16/CD32), washed twice with 100 µl DPBS and stained with LIVE/DEAD fixable violet stain (Life Technologies, Grand Island, NY) as per supplier's recommendations. Excess LIVE/DEAD stain was removed by washing cells twice with 100 µl DPBS and finally resuspended in 50 µl 1x FACS buffer. The PECs were then mixed with 50 µl mixture of various fluorescently labeled monoclonal antibodies against murine cell surface markers (Supplementary **Tables S1, S2**) and incubated for 30 min at 4^0C in dark. Finally, after washing the cells in cold 1x FACS buffer, stained samples were fixed with BD stabilizing fixative, filtered using 35 µm nylon mesh and kept in the dark at 4^0C until data was acquired using LSR-II flow cytometer (Becton Dickinson, San Jose, CA) at UNC flow cytometry core facility (within 24 h of surface labeling). Data was acquired with Forward (FSC) and side (SSC) scatter on a linear scale, while fluorescent signals were collected on a 4-decade log scale with a minimum of 50,000 events per sample. Flow samples were compensated with single color compensation data collected using AbC™ anti-mouse bead kit (Life Technologies, Grand Island, NY) and the frequency distribution of different cells types (MOs and neutrophils) was determined by applying gates derived from the FMO (fluorescence minus one) controls on cells. Finally, the data analysis was performed using FlowJo_V10 (FLOWJO LLC, Ashland, OR).

4.2.7. *In vivo* MØs/MOs depletion

MØ/MO depletion protocol using clodronate-liposomes was adopted from a previous study (37). Specifically, 200 µl clodronate- or clodronate free-liposome

(Encapsula NanoScience LLC, Brentwood, TN) were injected twice via lateral tail vein injections either a) 20 or 2 h before or b) on and 18 h after pDNA injections. According to the manufacturer the liposomes were composed of L-alpha-phosphatidylcholine (5mg/ml) and cholesterol (18.8 mg/ml) with and without clodronate (5mg/ml) suspended in DPBS saline at pH 7.4. In each administration scheme, luciferase gene expression was analyzed in tissue homogenates 24 h after pDNA injections.

4.2.8. Statistical analysis

Student's t-test was used to calculate statistical differences between groups at $p < 0.05$ in all *in vitro* and *in vivo* experiments unless indicated otherwise.

4.3. Results

4.3.1. PEC responses to pDNA/Pluronic, LPS and Alum in immunocompetent and nude mice

Previously, we reported that Pluronics increase muscle transfection of naked pDNA in immunocompetent (euthymic C57Bl/6 and Balb/c) but not in immunodeficient (athymic nude) mice (21). To better understand the strain-dependency of muscle transfection, we compared the immune responses in euthymic Balb/c and athymic nude mice to pDNA alone and pDNA formulated with copolymers (pDNA/P85 and pDNA/SP1017). Along with the pDNA and pDNA/Pluronic groups the treatment groups also included mice administered with alum or LPS to illicit a conventional adjuvant (alum) and non-specific pathogen-derived inflammatory (LPS) responses. A standard peritoneal lavage assay was used for immunological profiling of innate immune cells in different treatment groups as explained in (38, 39). In Balb/c mice the pDNA and pDNA/P85 injections increased PEC counts by ~2- and ~6-fold respectively, while pDNA/SP1017 injections did not produce significant changes compared to DPBS controls (**Fig. 4.1a**). In contrast, in nude mice changes in PEC were not significant in pDNA- or any of the pDNA/Pluronic groups. On the contrary, alum- and LPS that considerable responses in both in Balb/c and nude mice. Interestingly, the responses to alum and LPS in the nude mice were even greater than those in Balb/c mice (~7 and ~8 times vs. ~4 and ~1.4 times, respectively).

Next we delineated the MOs response. It was reported that upon co-administration with pDNA in the muscle P85 increases expression of the transgene in distal lymphoid organs, such as draining lymph nodes and spleen, where the transgene co-localizes with antigen presenting cells (APCs), specifically dendritic cells and MØs (34).

Moreover, our recent work implicated MØs in the horizontal gene transfer to muscle (submitted) and brain cells (35, 36) during inflammation. Therefore total PECs were labeled with antibodies against cell surface markers to characterize the MOs recruitment. Flow cytometric dot plots showed that all treatment groups except for LPS displayed recruitment of mainly anti-inflammatory MOs (M2-MOs, $SSC^{int\text{-}hi}FSC^{int\text{-}hi}CD11b^{+}Ly6G^{lo}F4/80^{lo/int}Ly6C^{lo}$). However, LPS recruited both inflammatory MOs (M1-MOs, $SSC^{int\text{-}hi}FSC^{int\text{-}hi}CD11b^{+}Ly6G^{lo}F4/80^{lo/int}Ly6C^{hi}$) and anti-inflammatory M2-MOs in both euthymic and athymic mice (red and black arrows in **Fig. 4.1b** and gating scheme in Supplementary **Fig. S4.1**). Still the MOs recruitment by alum or LPS in athymic mice was relatively small and the considerable spikes in the overall PEC counts in these groups (**Fig. 4.1a**) were explained by the recruitment of other cell types. In particular, there was an increase in the percent (and total counts) of neutrophils both in alum and LPS treated athymic mice to 73% and 50% compared to only 2% and 10 % M2-MOs in each of these groups, respectively (Supplementary **Fig. S4.2**).

Of all the compositions pDNA/P85 elicited the greatest and significant increase in both frequency (~5 times) and counts (~27 times) of M2-MOs compared to DPBS control in the Balb/c mice (**Fig. 4.1c, d**). The M2-MOs counts in this group were also significantly higher than those in pDNA alone group (~13 times) suggesting a strong adjuvant effect of P85 resulting in recruitment of M2-MOs. This effect was only observed in euthymic Balb/c mice and was attenuated in nude mice where the recruitment of M2-MOs in response to pDNA/P85 was much less than that in Balb/c mice. Still it remained significantly higher than the recruitment of M2-MOs in pDNA alone and DPBS control groups. Interestingly, in these two groups in nude mice the levels of M2-MOs have also shown a tendency to decrease compared to the respective groups in Balb/c mice.

Along with the M2-MOs pDNA/P85 also elicited recruitment of neutrophils in Balb/c mice (Supplementary **Fig. S4.2**). But in contrast to alum and LPS the neutrophils response in athymic mice treated with pDNA/P85 was low and significantly less than that in euthymic mice. Interestingly, a considerable increase in the T-lymphocyte recruitment was also observed in euthymic Balb/c after pDNA/P85 injection (Supplementary **Fig. S4.3**).

Finally, we studied the kinetics of Pluronic-induced MOs recruitment. In this case along with the pDNA alone and pDNA formulated with copolymers the treatment groups also included the copolymers alone. Both P85 and pDNA/P85 produced a considerable increase in M2-MOs frequency and total counts compared to DPBS control or pDNA, which peaked at 24 h after *i.p.* administration (Supplementary **Fig. S4.4**). Interestingly, at this time point the response to P85 alone was even higher than that to pDNA/P85 i.e. vs. 5.3 $x10^6$ *vs.* $3.5x10^6$ total M2-MOs counts. The MOs recruitment response subsided by day 5 or 10 post injection (Supplementary **Fig. S4.4**). In this experiment neither SP1017 nor pDNA/SP1017 increased MOs recruitment compared to DPBS or pDNA groups. Therefore, in subsequent experiments we focused only on formulations containing P85.

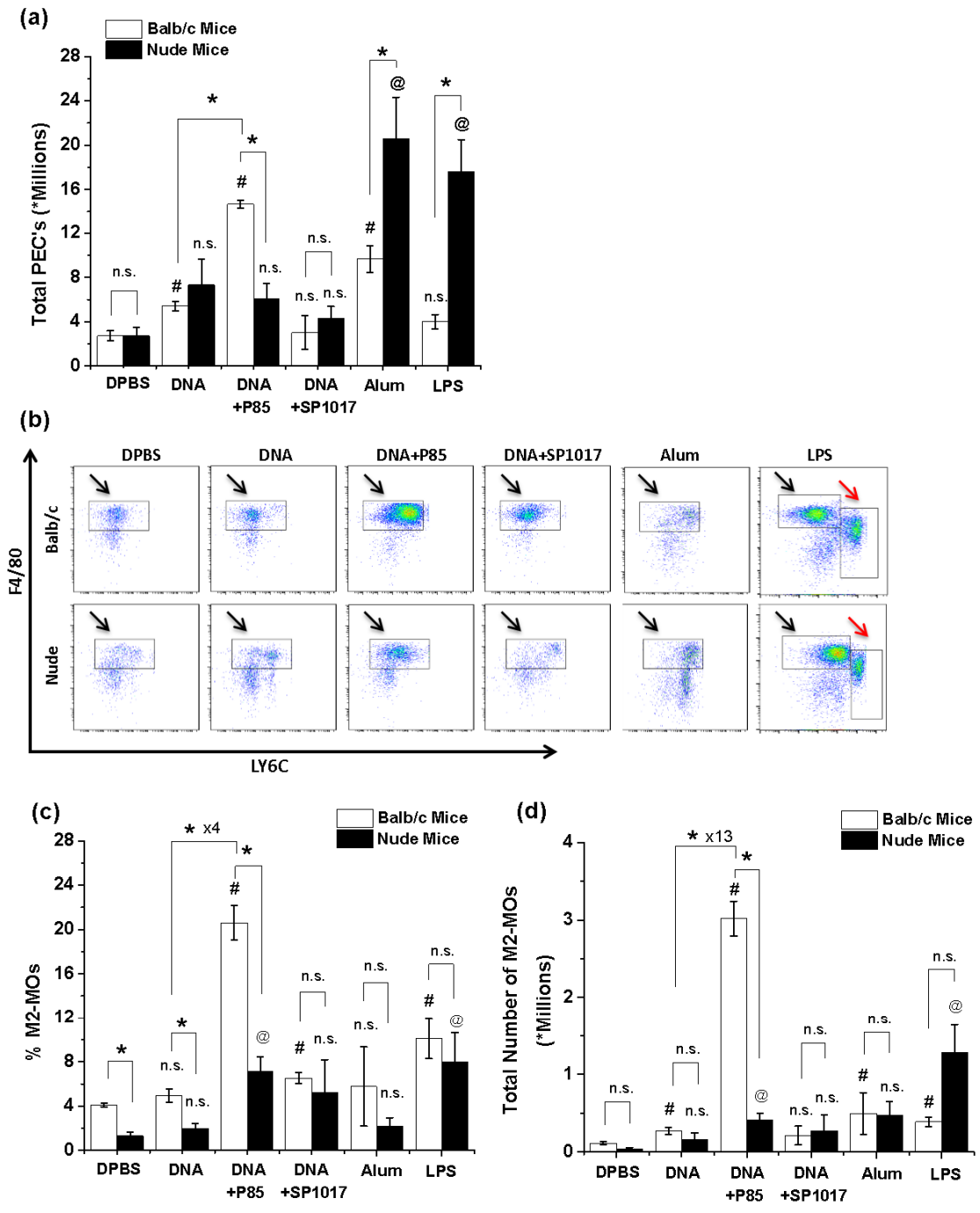


Figure 4.1. Innate immune response to adjuvant formulations quantified using peritoneal lavage. The panels present **(a)** total PEC response, **(b)** qualitative dot plots, **(c)** frequency (%) and **(d)** total numbers of peritoneal M2-MOs in Balb/c (white) and nude (black) mice. Each mouse was injected *i.p.* with 250 µl of the following solutions: saline (DPBS), 50 µg naked pDNA, 50 µg pDNA/0.3% P85, 50 µg pDNA/0.01% SP1017, 500 µg Alum or 25 µg LPS. The PECs were isolated 24 h after injections by the lavage procedure and **(a)** counted or **(b-d)** processed for flow-cytometry. **(b)** The inflammatory ($SSC^{int\text{-}hi}FSC^{int\text{-}hi}CD11b^{+}Ly6G^{lo}F4/80^{lo/int}Ly6C^{hi}$) and anti-inflammatory ($SSC^{int\text{-}hi}FSC^{int\text{-}hi}CD11b^{+}Ly6G^{lo}F4/80^{lo/int}Ly6C^{lo}$) MOs are marked by red and black arrows, respectively (Gating scheme shown in Supplementary **Fig. S1**). **(a, c, d).** Data are mean ± SEM of n = 3 to 6. Statistical comparisons were made between treatment group and DPBS control for each

mouse strain (# Balb/c, $^{\#}p<0.05$ and @ nude, $^{@}p<0.05$) and between treatment groups of different or same strain (*$p<0.05$). In all cases n.s. stands for non-significant

4.3.2. *In vivo* MOs and MØs depletion abolishes Pluronic-induced increase in muscle transfection

Intravenous (*i.v.*) clodronate-liposomes have been used to deplete MOs in circulation, as well as MØs in liver and spleen (40). The maximum depletion of circulatory MOs is observed ~18 h to 24 h post administration of clodronate-liposomes. In this work to elucidate the role of circulatory MOs in gene transfer we used two doses of clodronate-liposomes administered in two different schedules: either a) prior to or b) on and after pDNA injections (**Fig. 4.2a, b**). The control groups were injected with DPBS as well as empty liposomes of the same composition. The transgene expression in the muscle was quantified 24 h after pDNA injections.

In the first schedule pDNA/P85 displayed significant increase in gene expression compared to naked pDNA in DPBS or empty liposomes groups, but not in clodronate-liposome groups where there was a trend for an increase (**Fig. 4.2c**). The gene expression with pDNA/P85 in this latter group was attenuated compared to animals pretreated with empty liposomes suggesting that MOs depletion inhibited gene delivery with pDNA/P85. (Interestingly, empty liposomes boosted transgene expression compared to DPBS controls in pDNA/P85 but not the naked pDNA groups.)

In the second schedule clodronate-liposome produced even more pronounced decrease in the gene expression of pDNA/P85 compared to both DPBS and empty liposome treated animals (**Fig. 4.2d**). This scheme probably provided a better timing for the MOs depletion, since the recruitment of anti-inflammatory MOs by pDNA/P85 injections (which takes about 24 h, see Supplementary **Fig. S4.4**) was probably offset by MOs depletion due to the second injection of the clodronate-liposomes. Notably the clodronate-liposomes treatment led to a nearly complete reversal of gene expression of

pDNA/P85 to the naked pDNA levels. No decrease in gene expression was observed in naked pDNA groups, suggesting that MOs play a much lesser role in the muscle transfection with the naked pDNA. Our interpretations were further confirmed by the analysis of the spleen sections suggesting that MØs depletion was greater in mice treated with clodronate-liposome on and after pDNA/P85 injection compared to those treated with clodronate-liposome before pDNA injections (**Fig. 4.2e**).

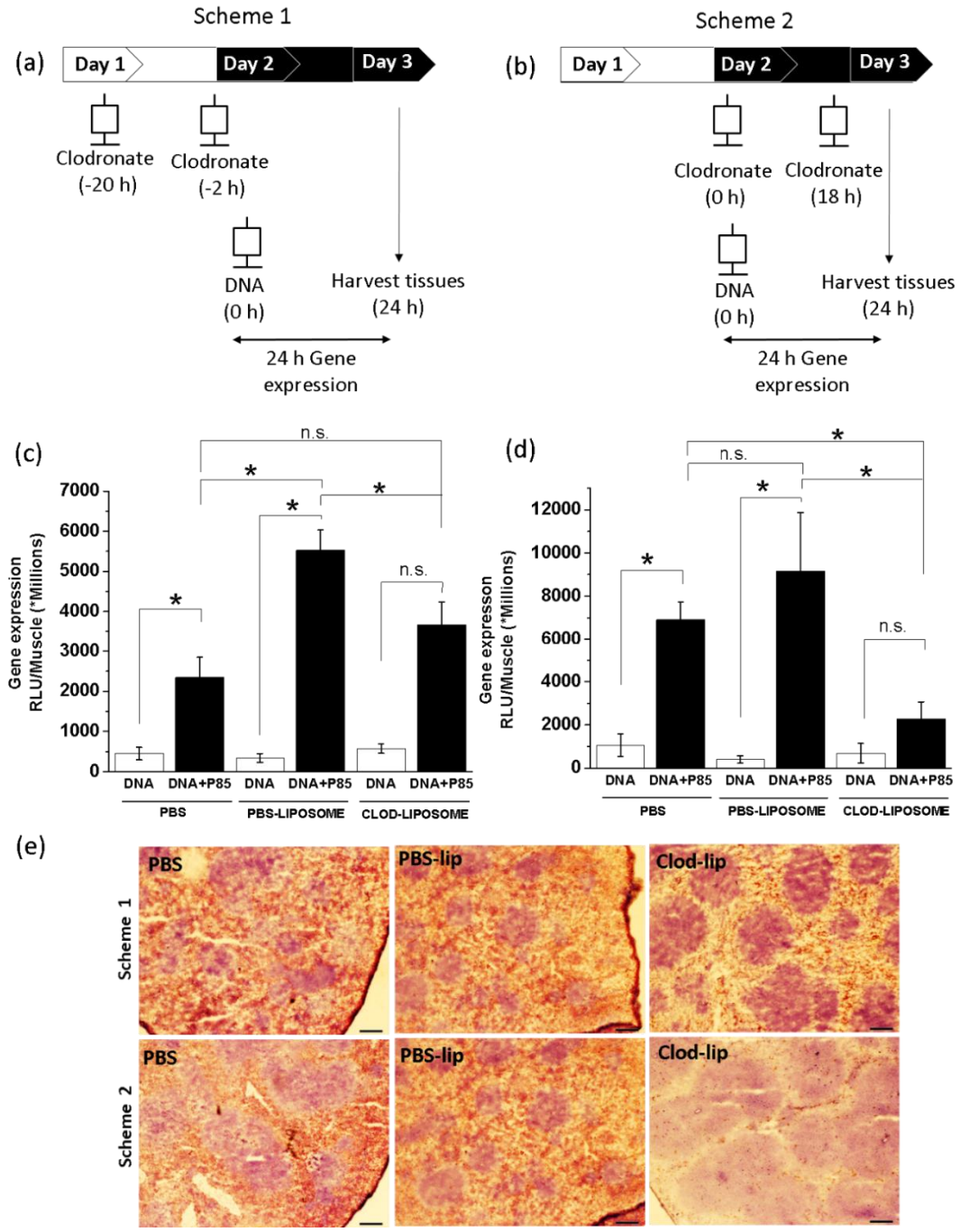


Figure 4.2. Effect of *in vivo* MOs and MØs depletion on muscle transfection. (a-d) MOs/MØs were depleted by *i.v.* administration of clodronate-liposomes (two injections of 0.2 ml liposome/mouse) either **(a, c)** before (scheme 1) or **(b, d)** on/after (scheme 2) pDNA administration. The mice injected with DPBS or clodronate free liposomes are shown for comparison. In all test groups 10 µg / 50 µl naked gWIZ™ Luc pDNA and pDNA/P85 were injected in left *TA* muscle and luciferase gene expression was quantified in muscle tissue homogenates after 24 h. Data represent means ± SEM, n = 5, and was analyzed using Student's t-test at *p<0.05. In all cases n.s. stands for non-significant. **(e)** Immunohistochemical staining of F4/80 MØ marker (red) on frozen spleen

sections obtained from mice injected *i.v.* with DPBS, PBS-liposome or Clodronate liposome in scheme 1 and scheme 2, 24 h after pDNA/P85 injection. Scale bar=200 µm.

4.3.3. Effects of Alum and LPS on muscle transfection with pDNA

Since LPS was shown to increase MOs frequency in both euthymic and athymic mice, while alum did not have such effects in any of the strains we compared the effects of Alum and LPS on the pDNA expression with that of P85. In this study pDNA or pDNA/P85, LPS or alum were injected into *TA* muscles and the transgene expression was quantified as described above. As expected and published earlier, pDNA/P85 failed to increase transfection of the muscles in nude mice compared to the naked pDNA (21). In addition, we did not observe any increase in transgene expression using pDNA/LPS or pDNA/alum instead of the naked pDNA (**Fig. 4.3**). In fact, the transgene expression after administering pDNA/alum was 10 and 68 times lower than that of the naked pDNA in Balb/c and nude mice, respectively. This could be explained by decreased muscle transfection upon condensation of naked pDNA by cationic alum (41) and strong alum-induced neutrophil infiltration response (73% of total cells) resulting in muscle damage (42). Moreover, pDNA/LPS did not increase transgene expression in either Balb/c or nude mice. This suggests that either MOs recruitment by LPS is not sufficient to achieve the desired effect or both MOs recruitment and co-delivery of P85 with pDNA are necessary to produce high transgene expression.

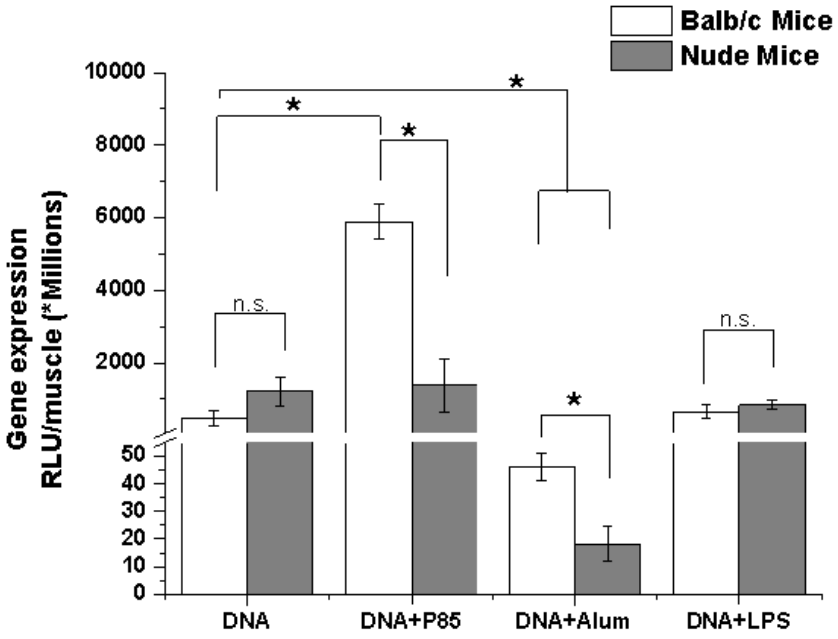


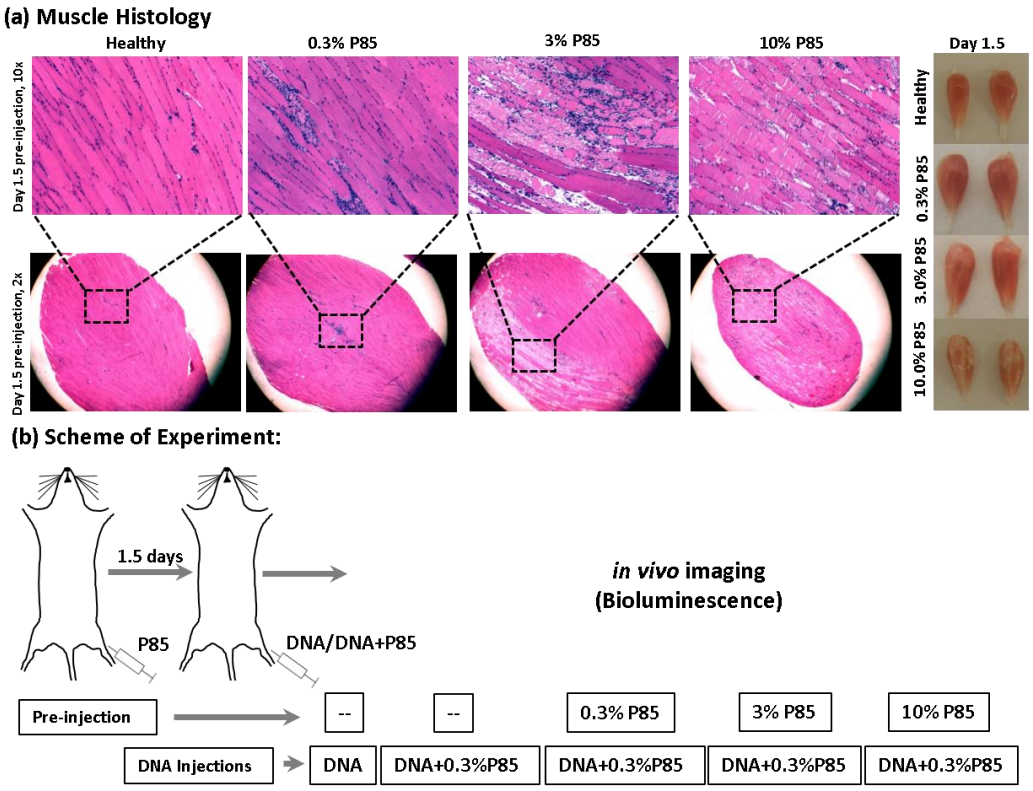
Figure 4.3. Differential effects of P85, Alum and LPS on muscle transfection. 10 µg / 50 µl gWIZ™ Luc pDNA alone or formulated with adjuvants (0.3% P85, 5 µg LPS and 100 µg Alum) were injected in *TA* muscle of euthymic Balb/c and athymic nude mice. Gene expression analysis 24 h after DNA injection demonstrated a strain- and adjuvant-dependent effect on muscle transfection. Data represents mean ± SEM of n = 5 and statistical significance was calculated by using student's t-test at *$p<0.05$. In all cases n.s. stands for non-significant.

4.3.4. Preinjection of P85 further increases muscle transfection levels

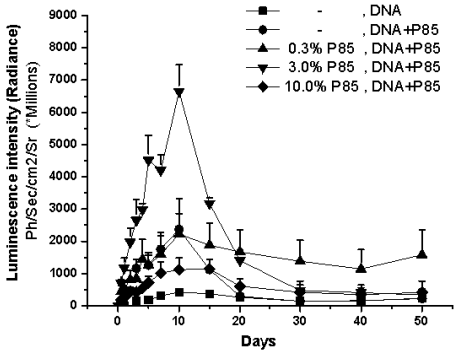
After determining that MOs and P85 have mutually inclusive role in assisting gene transfer of pDNA in the muscle we evaluated whether MOs can be harnessed to further increase the transgene expression. To this end we examined the cell recruitment in the *TA* muscles injected with P85 (0.3%, 3% and 10% w/v) by tissue histology. There was a typical cellular recruitment response characterized by purple hematoxylin nuclear staining in the muscle tissue along with the weak pink eosin staining suggesting tissue regeneration on day 1.5 after injection of the copolymer (**Fig. 4.4a**). This response subsided on day 5 in 0.3% and 3% P85 groups (Supplementary **Fig. S4.5a**). However, at higher concentration of 10% P85 the muscle injury was beyond repair resulting in excessive cellular recruitment and necrosis.

To synergize MOs recruitment response with the pDNA delivery we first injected P85 in the *TA* muscles, and then after 1.5 days (right after the peak MOs recruitment) administered pDNA/P85 to the same muscles (**Fig. 4.4b**). The time course of luciferase gene expression was quantified by IVIS imaging (Supplementary information **Fig. S4.6**). The results suggested that preinjection of P85 affects the transgene expression in a concentration dependent fashion (**Fig. 4.4c-e**). Specifically, the 0.3% P85 injected 1.5 days before pDNA/P85 did not change the peak expression on day 10 but considerably prolonged the transgene expression for nearly 60 days compared to expression in pDNA/P85 groups without preinjection that faded after 20 days (**Fig. 4.4d-e**). The 3% P85 increased the peak gene expression on day 10 by ~2.5 and ~16 times compared to pDNA/P85 and pDNA without the preinjection (**Fig. 4.4c,d**). Interestingly, preinjection of 3% P85 also showed some effect on expression of naked pDNA (Supplementary **Fig. S4.7**). The highest concentration of the copolymer (10% P85), which produced sustained muscle injury and necrosis decreased the gene expression level of pDNA/P85. In a

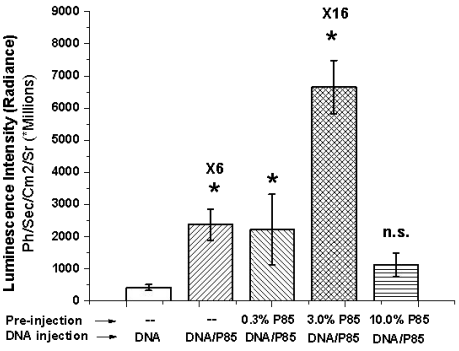
parallel experiment, we administered pDNA/P85 5 days after P85 injection when the MOs recruitment response subsided (**Fig. S4.4b**). This had either negative or no effect on the transgene expression (**Figs. S4.4c-e**). Only with 0.3% P85 preinjection some prolongation of gene expression was observed similar to the effect observed at the same dose with short preinjection schedule.



(c) Time course of gene expression in 1.5 Day pre-injection model



(d) Day 10 gene expression



(e) Day 40 gene expression

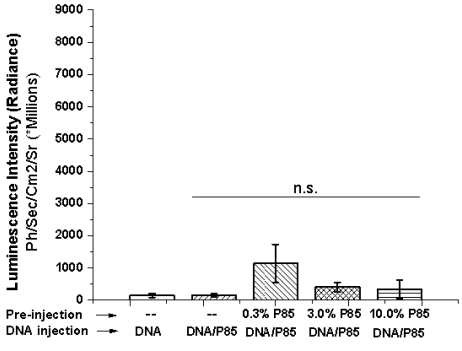


Figure 4.4. Effect of Pluronic preinjection 1.5 days before before administration of pDNA/P85 on the gene expression in the muscle. (a) Muscle histology (H&E staining) at 1.5 days after P85 preinjection. (Left) Longitudinal sections (2x and 10x) showed a typical inflammatory cellular infiltrate (purple; hematoxylin nuclear staining) accompanied by muscle regeneration (light pink; weak eosin staining). (Right) Representative images of freshly isolated muscles en bloc of n = 3. **(b)** Scheme of the experiment involving preinjection of *TA* muscles with P85 (0.3%, 3% and 10%) followed 1.5 days after that by injection of 10 µg pDNA/0.3% P85. **(c-e)** Luciferase expression at different time points after pDNA or pDNA/P85 injections quantified by IVIS imaging 5 min after *i.p* injection of D-luciferin: **(c)** time course **(d)** Day 10 and **(e)** Day 40. Data represents mean ± SEM, n = 3. Statistical comparisons were made between naked pDNA alone (without P85 preinjection) and pDNA/P85 groups (with and without preinjection), *p<0.05, n.s. stands for non-significant.

4.4. Discussion

Coordinated sequence of leukocyte recruitment is the hallmark of innate immune response (43-45). Adjuvants intensify and modulate the leukocyte infiltrate (magnitude, quality and kinetics) resulting in a specific adaptive immune response (38). Accordingly, adjuvants that assist in cell mediated or humoral immune response are defined as Th1 (e.g., dimethyldioctadecylammonium liposomes with monophosphoryl lipid-A or DDA/MPL) and Th2 (e.g., Aluminium hydroxide or $Al(OH)_3$), respectively. Development of new customized adjuvants capable of recruiting specific cell populations is a promising strategy to elicit a unique adaptive immune response (38) or other downstream effects such as muscle transfection.

Pluronic block copolymers have gained considerable interest as adjuvants in protein (46, 47) and DNA based vaccination approaches (48, 49). Their effects on immunity are known to involve both Th1 and Th2 signaling (50). We and others have shown that they can also act as very potent enhancers of gene expression in the muscle (19-21) and that their effect on gene expression is mediated by the APCs, that can carry the gene from the site of injection in the muscle to spleen and distal lymph nodes (34). Moreover, previously published data suggested that Pluronics do not enhance muscle transfection in immunedeficient athymic nude mice (21, 51), which implied a mutually inclusive role of both immune cells and copolymers in the muscle transfection.

In this study we conducted profiling of the innate immune responses to pDNA/Pluronic and compared them with the responses to common adjuvants, alum and LPS. This study resulted in two important findings. The first finding was the different responses to pDNA/Pluronic observed in euthymic Balb/c and athymic nude mice. Specifically, pDNA/P85 in euthymic Balb/c mice greatly increased the recruitment of M2-MOs, as well as neutrophils and T-cells at 24 h after administration of pDNA/P85. In

contrast, in athymic nude mice that lack a normally developed T-cell system the recruitment of M2-MOs and neutrophils was attenuated, suggesting T-cell dependence of the response. Since T cells may enhance the innate immune cell recruitment at the site of pDNA injection (52), T cell absence in nude mice is likely to explain the loss of MOs and neutrophils recruitment in pDNA/P85 injected nude mice.

The second finding was the very different profiles of the responses to pDNA/Pluronic compared to alum and LPS. Although the alum and LPS appeared to somewhat increase the recruitment of M2-MOs in Balb/c mice, their effect was marginal compared to that of pDNA/P85. Most importantly the major component in the responses to these compositions in Balb/c mice was the neutrophil recruitment. And these responses in contrast to pDNA/Pluronic were not attenuated but rather increased in the nude mice. The latter finding was consistent to earlier studies, which reported an abnormally high "compensatory" neutrophil infiltration in nude mice resulting in their protection against otherwise lethal bacterial and fungal infections (53, 54). Our results suggested that pDNA/P85 was recognized by a mechanism different than pathogen recognition and the T-cell system dependent recruitment of MOs was a major difference compared to the common adjuvants.

Interestingly, pDNA co-formulated with alum or LPS did not show any increase in the gene expression in TA muscle compared to the naked pDNA. We speculate that this might be due to the lack of the MOs response. Some grounds for this speculation was already contained in our previous publications. In particular, we reported co-localization of expressed genes with MØs as well as dendritic cell markers in muscle, spleen and lymph nodes, along with the increased systemic and local expansion of the MØs, dendritic cell and natural killers after *i.m.* injection of pDNA/P85 (34). We also reported the co-culture experiments demonstrating horizontal gene transfer from *in vitro* transfected MOs/MØs to

brain cells (35, 36) and most recently to muscle cells (submitted). Finally, we demonstrated that the transfected MOs/MØs can be adoptively transferred to a mouse model and carry the gene to the site of injury and inflammation where this gene is expressed (35, 36). In this study we directly demonstrate that the depletion of MOs/MØs essentially abolished the effect of Pluronic on pDNA expression in the muscle. We speculate that the needle injury upon injection produces the inflammation response and MOs/MØs infiltration in the muscle (16), where the MOs/MØs capture the pDNA and promote transfection. The copolymer, which does not bind or interact with pDNA (21), boosts the innate immune response and recruitment of MOs as described above, thereby enhancing the MOs/MØs dependent gene transfer to the surrounding cells. The initiation of innate immune response in the muscle is still being investigated. Reports question the possibility of resident MØs during steady state in muscle parenchyma and instead showed MØs in muscle epimysium (connective tissue surrounding muscle tissue) and perimysium (connective tissue surrounding muscle fibers), where they may help orchestrate the initial immune recognition recruiting more cells from the nearby blood vessels (55, 56). Since the block copolymer enhancement of muscle transfection appears to be dependent on innate immune response, additional MOs recruitment can be triggered by resident MØs in epimysium and perimysium.

In this case the proper timing of recruitment of the MOs could be a very important factor in gene transfer. Since the copolymer alone was shown to activate the MOs recruitment 24 h after *i.m.* injection the pre-injections of the copolymer can be used to harness the MOs to the sites of subsequent pDNA injections resulting in further improvement of muscle transfection. Specifically, the level of gene expression was greatly increased when the muscle was preinjected with P85 followed by administering pDNA/P85. Interestingly, the peak levels as well as the duration of the gene expression

depended on the concentration of the copolymer in the preinjection solution as well as in the pDNA/P85 formulation. The timing of preinjection was also critical as the separation of the copolymer and pDNA/P85 by 5 days when the MOs recruitment subsided did not result in the increased gene expression. Therefore, by optimizing the copolymer doses as well as the treatment time schedule one can possibly fine-tune the immune response and the gene expression profiles, which can result in interesting opportunities in gene delivery strategies.

4.5. Conclusions

Gene therapy approaches based on *i.m*. injection of naked pDNA are hindered by low and transient gene expression of the pDNA in the muscle. Pluronic block copolymers co-injected with the pDNA have been shown to increase the level and duration of the gene expression thereby potentially expanding the use such approaches. This study provides a new mechanistic insight in the effect of Pluronics, using P85 as example. The findings strongly suggest that the copolymer activates the innate immune response, in particular recruitment of M2-MOs, which is instrumental in assisting Pluronic/DNA transfection. This is the first report that demonstrates that 1) by depleting MOs/MØs the effects of P85 were abolished and 2) by harnessing MOs/MØs response prior to administration of pDNA/P85 the muscle tissue transfection can be further enhanced. These findings are of significant theoretical and practical interest and can be used to further optimize the use of Pluronics in *i.m.* gene delivery applications.

4.6. Acknowledgements

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CHAPTER 4

SUPPLIMENTARY DATA

Harnessing Macrophage Response to Increase Gene Delivery to Muscle Using pDNA Formulated With Pluronic Block Copolymers

Table S4.1. Fluorescently labeled monoclonal antibodies used for cell surface staining of PEC.

Panel for MOs and Neutrophils	Excitation (nm)	Emission (nm)	Amount used (μg/10^6 cells)
Rat Anti-Mouse CD11b-APC-Cy7	650	785	0.25 μg
Rat anti-mouse Ly-6C-APC	650	660	0.25 μg
Rat Anti-Mouse Ly-6G PE-Cy7	496	667	0.25 μg
Rat Anti Mouse F4/80 PE	496	578	0.25 μg

Table S4.2. Fluorescently labeled monoclonal antibodies used for cell surface staining of lymphoid cells.

Panel for T cells	Excitation (nm)	Emission (nm)	Amount used (μg/10^6 cells)
Rat Anti-Mouse CD19b-PE	650	785	0.25 μg
Rat Anti-Mouse CD45-PE-Cy7	496	667	0.25 μg
Hamster Anti-Mouse CD3e-PE	496	578	0.25 μg

S4.1. Materials and Methods

S4.1.1. TLR screening

P85 solutions were made fresh and shipped overnight to InvivoGen laboratories (InvivoGen, San Diego, CA) to test TLR binding to P85 on individual TLR expressing HEK cells. Seven different HEK cell lines stably transfected with individual mTLR (2, 3, 4, 5, 7, 8 and 9) along with secreted alkaline phosphatase reporter gene under the control of NF-kB inducible promoter were incubated with P85 (0.1% and 1%) and positive controls (TLR2: HKLM (Heat killed *listeria monocytogenes*) at 10^8 cells/ml, TLR3: poly (I:C) at 1ug/ml, TLR4: E.Coli K12 LPS at 100ng/ml, TLR7: CL097 at 1ug/ml, TLR8: CL075 at 10ug/ml+Poly(dT) at 10uM, TLR9: CpG ODN 1826 at 1ug/ml) for 16-20 h in a 96 well plate. The activity of P85 solutions were tested at 1/10 of stock conc. (10% and 1%) by adding 20ul of sample in 180 µl of media and TLR activation was quantified by measuring secreted alkaline phosphatase (SEAP) activity in cell culture media using QUANTI-Blue substrate and colorimetric detection of SEAP product at 622-655 nm using spectramax (Molecular Devices, Sunnyvale, CA).

S4.1.2. Endotoxin assay

The ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ) was used to detect endotoxin levels in formulations as per supplier‘s recommendation. The observed levels were negligible (same as endotoxin free DPBS) and 13EU/mg respectively which were less than the FDA allowed limit (≤40EU/mg pDNA).

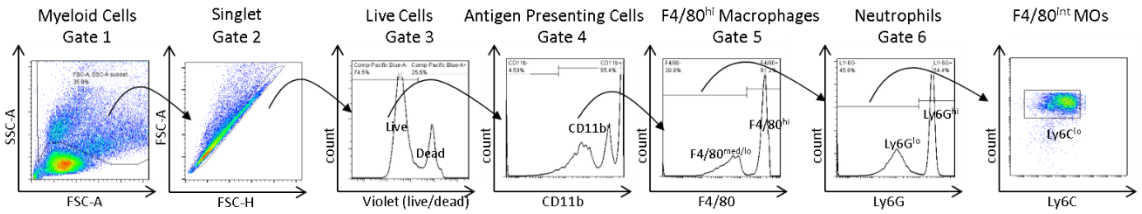
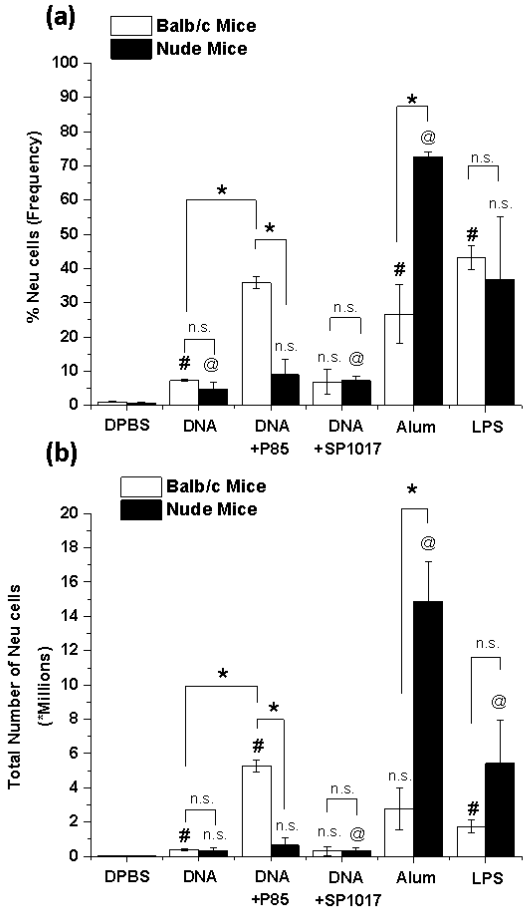


Figure S4.1. Gating strategy used for discriminating MOs ($SSC^{int-hi}FSC^{int-hi}CD11b^{+}Ly6G^{lo}F4/80^{lo/int}Ly6C^{lo/hi}$) and neutrophil ($SSC^{int-hi}FSC^{int-hi}CD11b^{+}F4/80^{int-lo}Ly6G^{hi}$) from total PECs. Debris and lymphocytes (SSC-A vs. FSC-A) and doublets (FSC-H vs. FSC-A) were excluded. Live/dead cells were determined using the amine reactive fixable violet stain. CD11b+ myeloid lineage cells were subgated on $SSC^{int-hi}FSC^{int-hi}CD11b^{+}Ly6G^{lo}F4/80^{lo/int}Ly6C^{lo/hi}$ MOs by excluding $CD11b^{-}$, $F4/80^{hi}$ (MØs) and $Ly6G^{hi}$ (Neutrophil) cells.



(c) **Table 1 : % cell infiltration**

	Euthymic mice		Athymic mice	
	Neu	M2-MOs	Neu	M2-MOs
DNA+ P85	36%	21%	9%	7%
Alum	27%	5%	73%	2%
LPS	43%	8%	37%	8%

Figure S4.2. Neutrophil (Neu) recruitment induced by alum and LPS in athymic mice resulted in spike in PEC response. Each euthymic Balb/c (open bars) and athymic nude (solid bars) mouse was injected *i.p.* with 250 µl of DPBS, 50 µg naked pDNA alone, 50 µg pDNA/0.3% P85, 50 µg pDNA/0.01% SP1017, 500 µg Alum or 25 µg LPS. The PEC's were isolated 24 h later by lavage procedure and processed for flow-cytometry. **(a, b)** Peritoneal neutrophils were characterized as $SSC^{int\text{-}hi}FSC^{int\text{-}hi}CD11b^{+}F4/80^{int\text{-}lo}Ly6G^{hi}$cells in lavage fluids of Balb/c (white) and nude (black) mice (Gating scheme shown in **Fig. S1**). Data represent means ± SEM of n = 3 to 6. Statistical comparisons were made between treatment group and DPBS control for each mouse strain (# Balb/c, $^{\#}p<0.05$ and @ nude, $^{@}p<0.05$) and between treatment groups of different or same strain, *p<0.05. In all cases n.s. stands for non-significant.

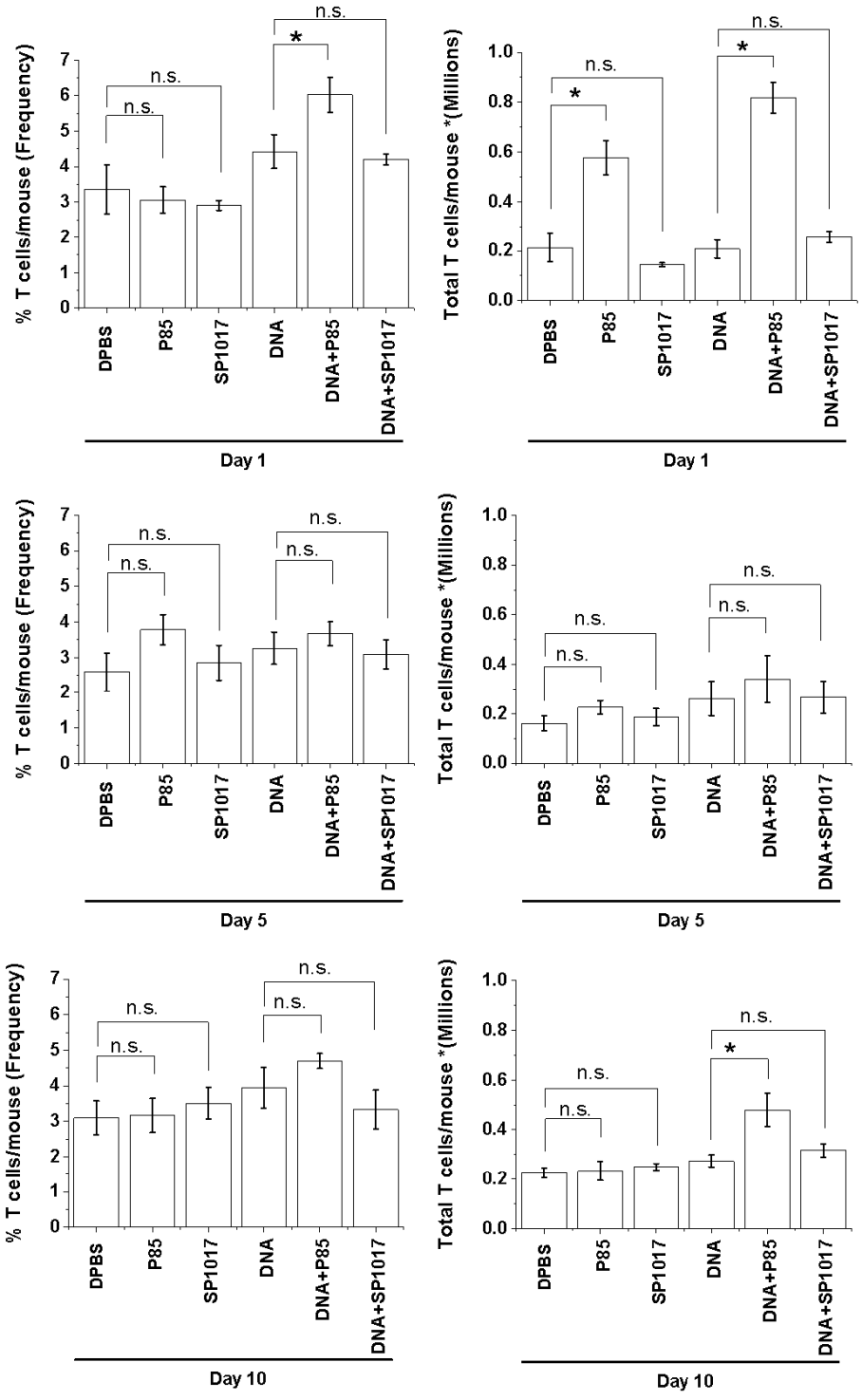


Figure S4.3. Effect of Pluronic and pDNA/Pluronic on the recruitment of T cells ($CD45^+CD19^-CD3e^+$) in the euthymic mice. Balb/c mice were injected *i.p.* with 250 µl of DPBS, 0.3% P85, 0.01% SP1017 alone or these solutions containing 50 µg pDNA. PECs

were isolated at various time points (day 1, day 5 and day 10) and labeled with respective cell surface markers to identify $CD45^{+}CD19^{-}Cd3e^{+}$ T cells and analyzed by FlowJo. (Left) T cells frequency (%) and (Right) total T cells are presented. Data are mean ± SEM of n = 3. Statistical comparisons between groups were done using student's t-test at $*p<0.05$. In all cases n.s. stands for non-significant.

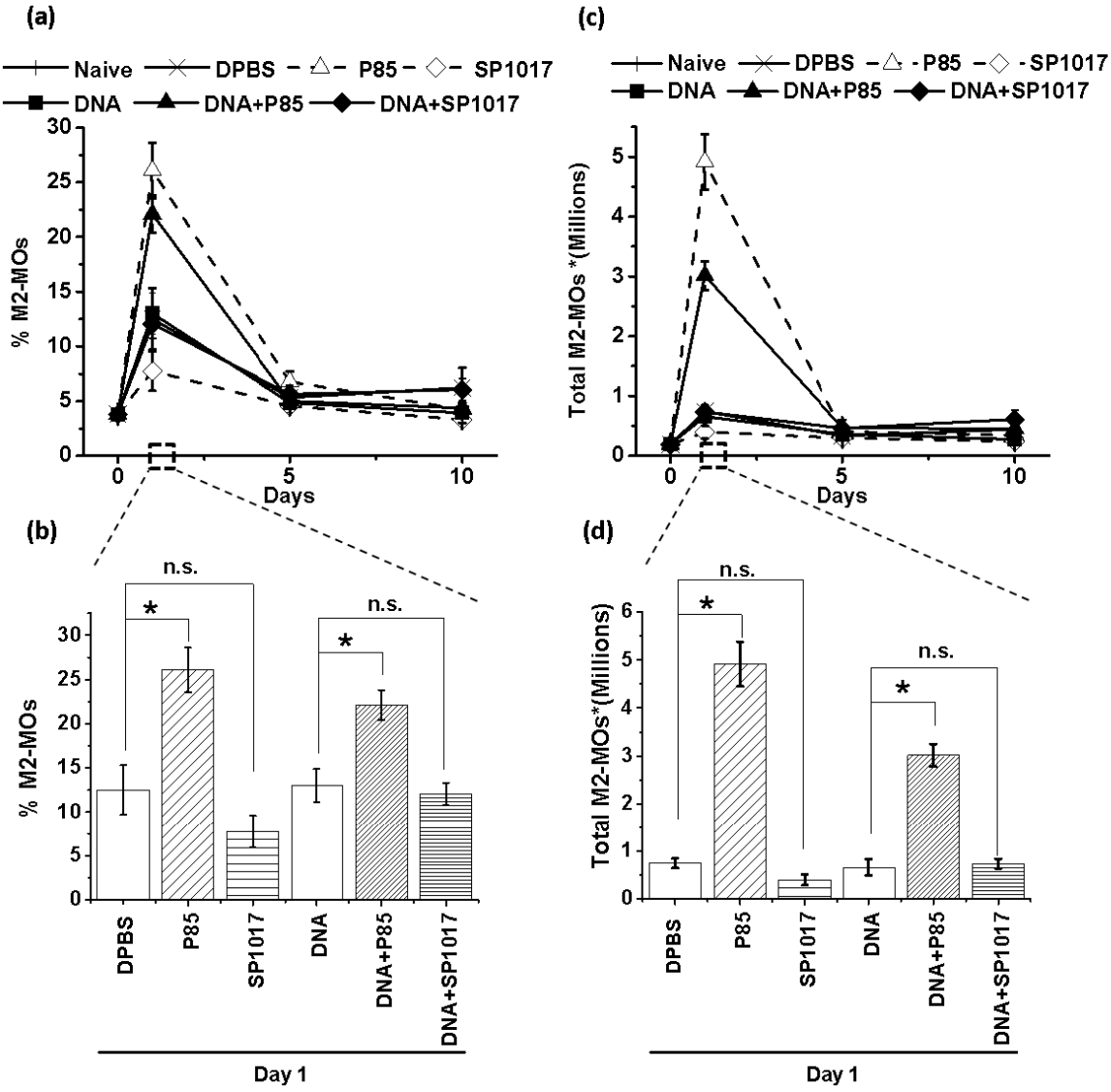


Figure S4.4. Time course of the Pluronic induced M2-MOs recruitment in euthymic mice. Balb/c mice were injected *i.p.* with 250 µl of DPBS, 0.3% P85, 0.01% SP1017 alone or mixed with 50 µg pDNA. PECs were isolated at various time points (day 1, day 5 and day 10) and labeled with respective cell surface markers to characterize and quantify **(a, b)** % M2-MOs frequency (%) and **(c, d)** total M2-MOs by flow cytometry. Data represent means ± SEM of n = 3 and statistical significance was calculated by using student's t-test at *p<0.05. In all cases n.s. stands for non-significant.

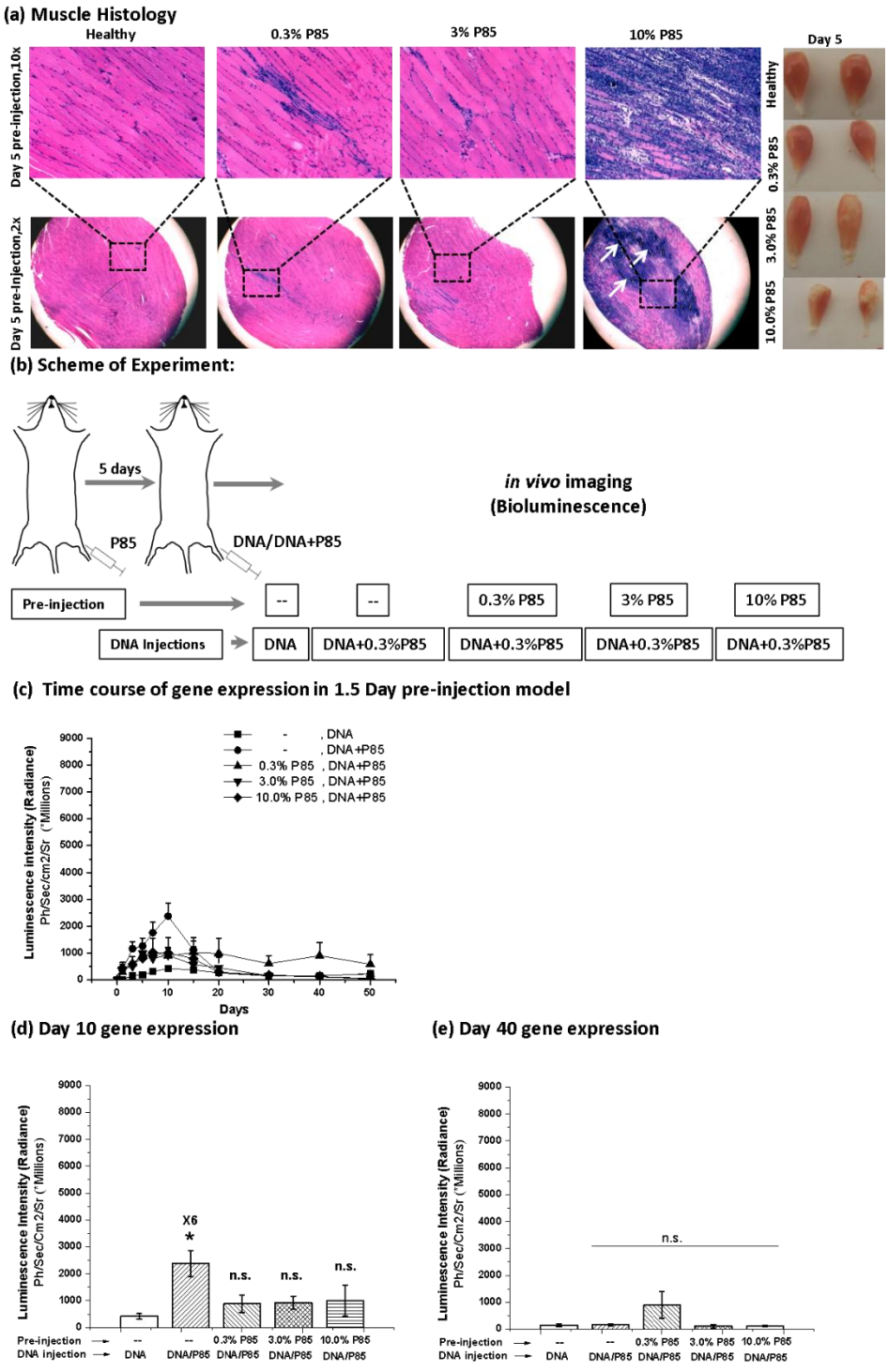


Figure S4.5. Effect of Pluronic preinjection 5 days before administration of pDNA/P85 on the gene expression in the muscle. (a) Muscle histology (H&E staining) at 1.5 days after P85 preinjection. (Left) Longitudinal sections (2x and 10x) showed no

cellular recruitment at 0.3% and 3% but tissue necrosis at 10% P85 injection. (Right) Representative images of freshly isolated muscles en bloc of n = 3. **(b)** Scheme of the experiment involving preinjection of *TA* muscles with P85 (0.3%, 3% and 10%) followed 5 days after that by injection of 10 µg pDNA/0.3% P85. **(c-e)** Luciferase expression at different time points after pDNA or pDNA/P85 injections quantified by IVIS imaging 5 min after *i.p* injection of D-luciferin: **(c)** time course **(d)** Day 10 and **(e)** Day 40. Data represents mean ± SEM, n = 3. Statistical comparisons were made between naked pDNA alone (without P85 preinjection) and pDNA/P85 groups (with and without preinjection), *$p<0.05$, n.s. stands for non-significant.

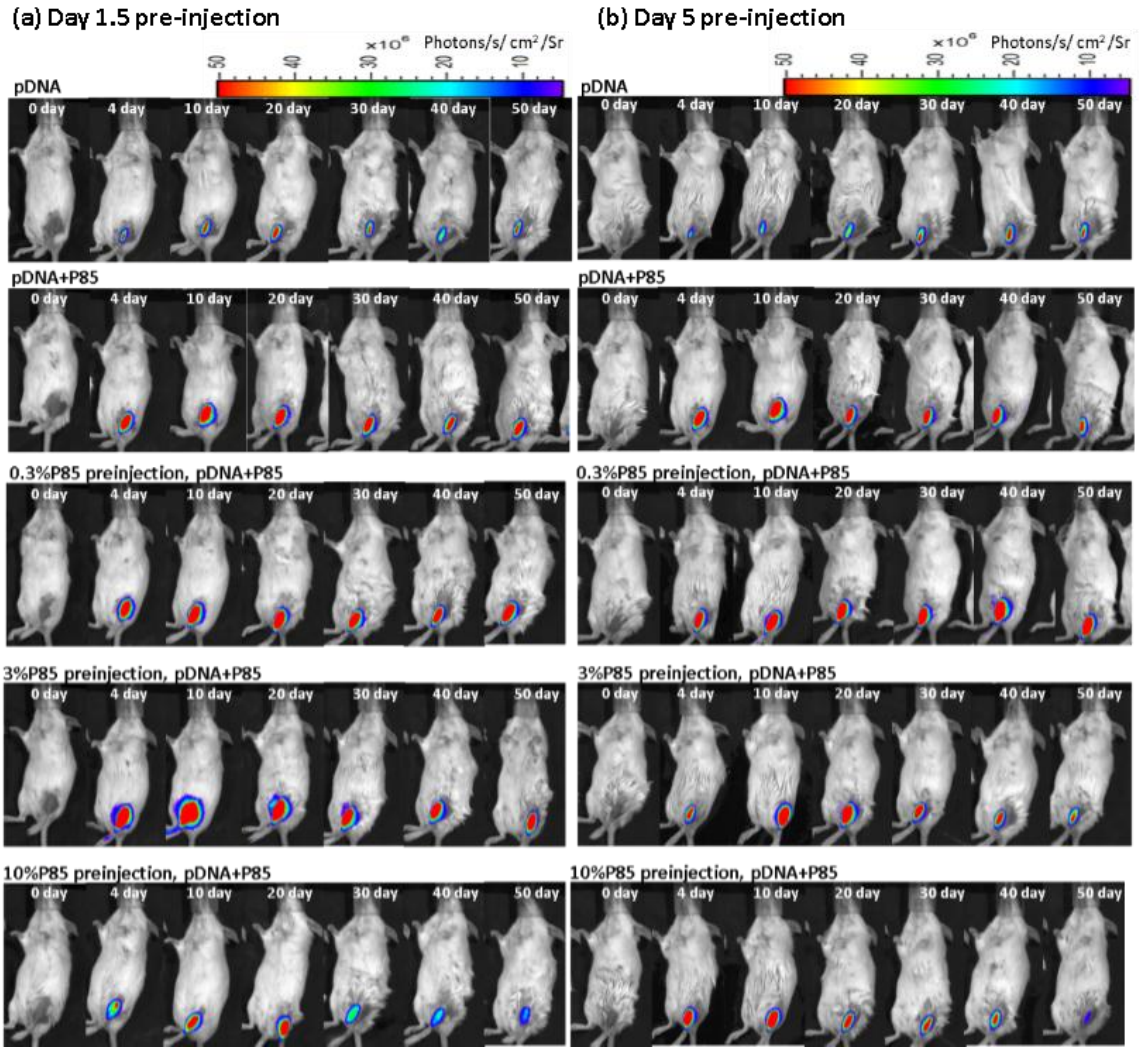


Figure S4.6. Day 1.5 and Day 5 Preinjection imaging. Representative ventral images of the transgene expression in Balb/c mice taken at different time points after *i.m.* injections of 10 µg gWIZ-Luc pDNA without or with 0.3% P85 in 50 µl DPBS. In select experiments **(a)** 1.5 days or **(b)** 5 days before gene transfer the animals were preinjected in the same muscles P85 solutions of different concentrations (0.3%, 3 % and 10 %) of P85 in 50 µl DPBS. Luciferase gene expression was quantified by IVIS imaging 5 min after *i.p* injection of D-luciferin substrate.

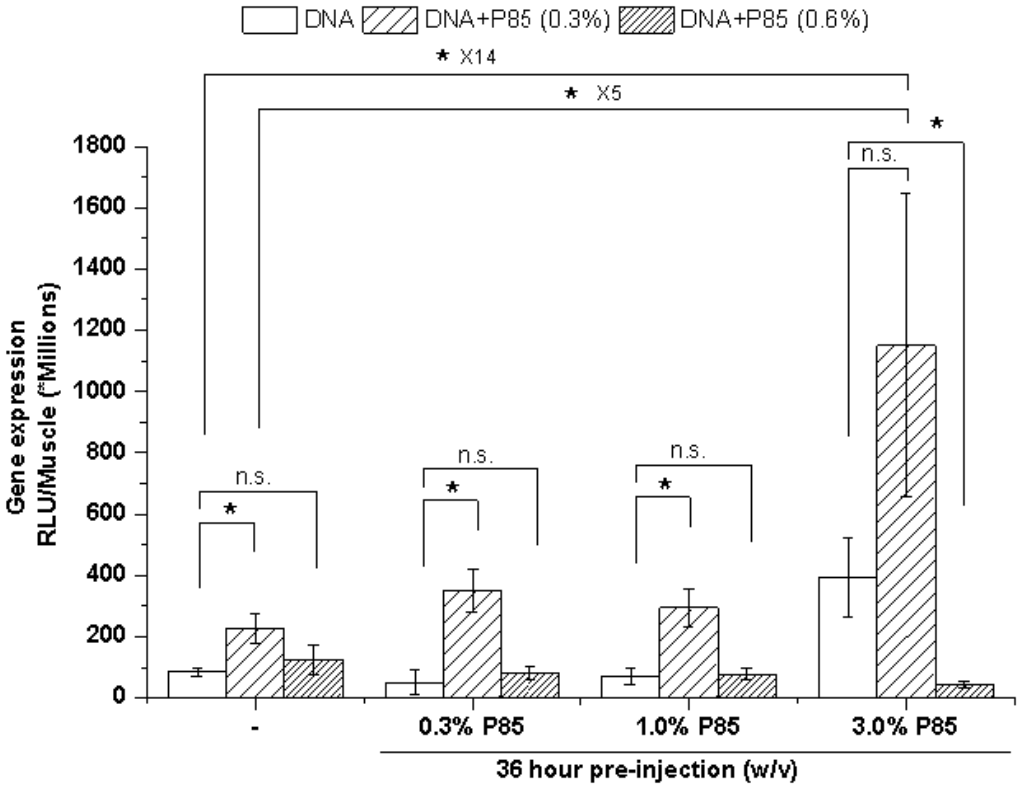


Figure S4.7. Effect of P85 preinjection 1.5 days before administration of naked pDNA, pDNA/0.3% P85 or pDNA/0.6% P85 on the gene expression in the muscle. Bilateral *TA* muscles were first injected with 50 µl P85 solution (0.3 %, 1.0 % and 3.0 %) and then after 1.5 days with 50 µl formulation of 10 µg naked pDNA, pDNA/0.3% P85 or pDNA/0.6% P85. Luciferase gene expression was quantified in tissue homogenates 4 days after gene transfer. Data represent mean ± SEM (n=6-8). Statistical comparisons: * $p<0.05$; n.s. stands for non-significant.

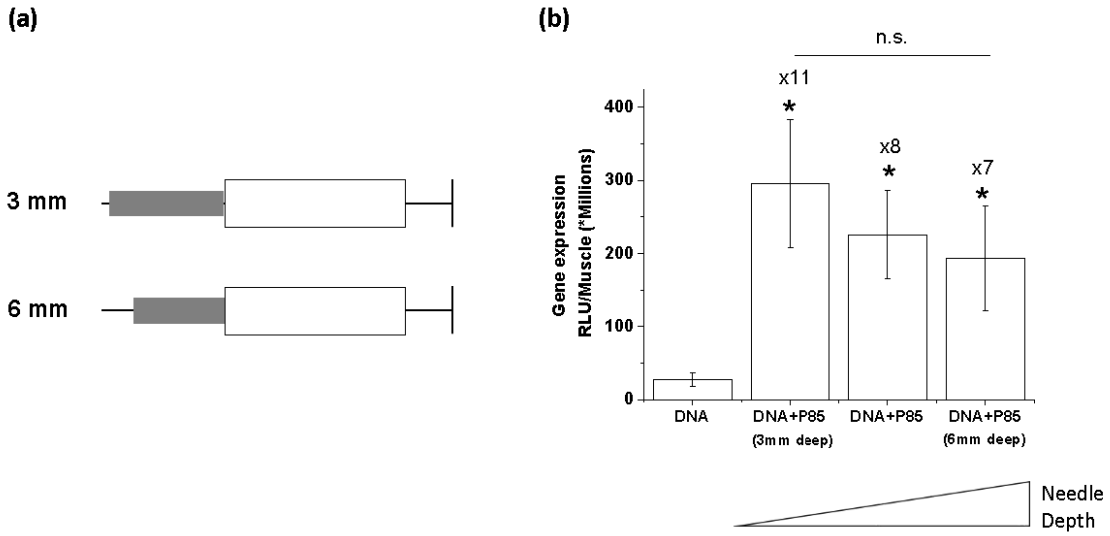
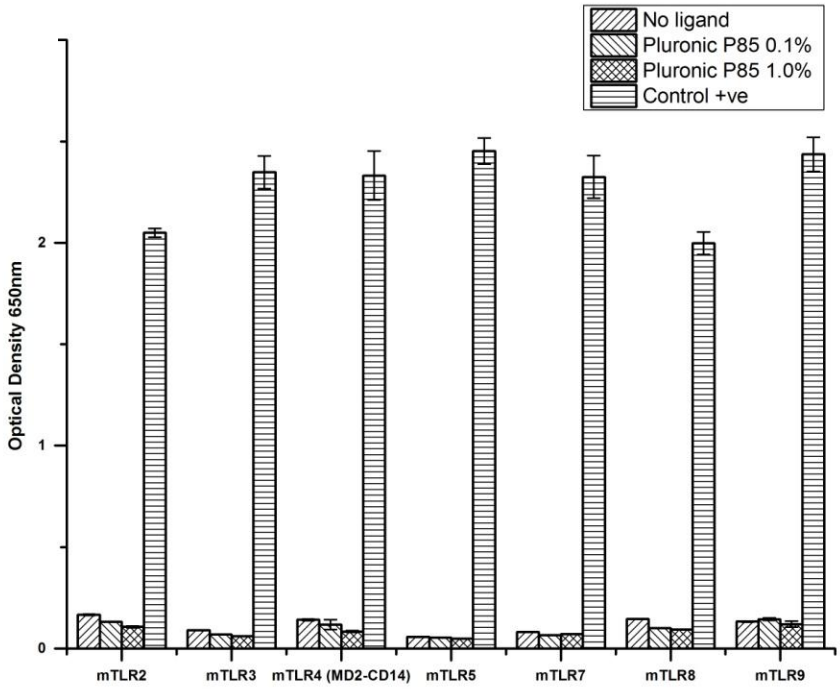


Figure S4.8. Effect of the size of needle injury on pDNA/P85 gene transfer. (a) 18 G metallic needles were trimmed to appropriate size to make two different sized collars that exposed only 3 mm or 6 mm (including bevel length of ca. ~1mm) of 25 G needle tip during *i.m*. injections. **(b)** Gene expression in muscles injected 3mm and 6mm deep were compared with muscles injected with pDNA and pDNA/0.3% P85 without needle collars. The gene expression increased inversely with needle depth, marginally higher levels in 3 mm deep, intermediate levels in randomly deep and marginally lower in 6 mm deep injections. Data represents mean ± SEM of n = 5 and statistical comparisons were made between pDNA/P85 and pDNA alone using student's t-test at * $p<0.05$.

(a)


No ligand
Pluronic P85 0.1%
Pluronic P85 1.0%
Control +ve
Optical Density 650nm
0
1
2
mTLR2
mTLR3
mTLR4 (MD2-CD14)
mTLR5
mTLR7
mTLR8
mTLR9

(b)

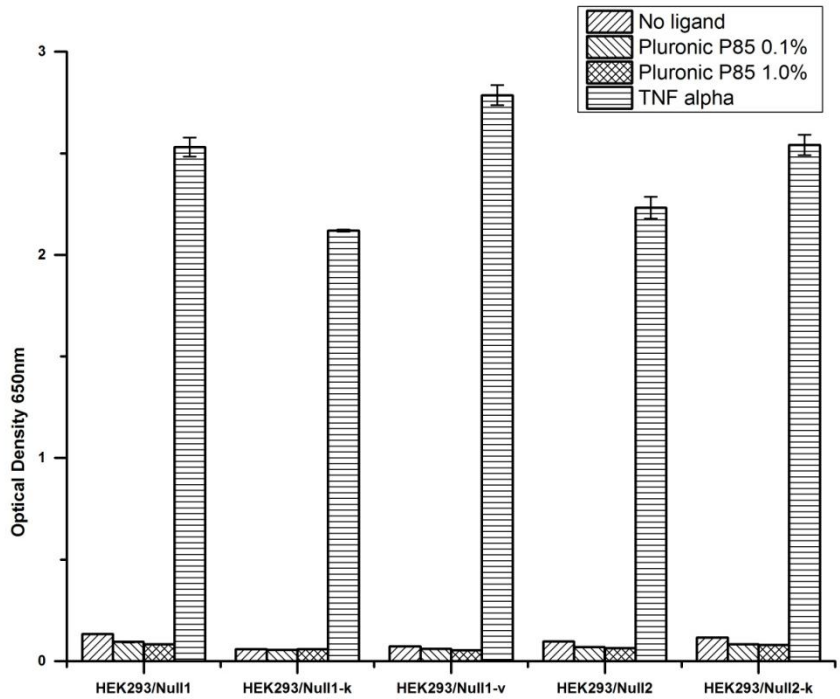

No ligand
Pluronic P85 0.1%
Pluronic P85 1.0%
TNF alpha
Optical Density 650nm
0
1
2
3
HEK293/Null1
HEK293/Null1-k
HEK293/Null1-v
HEK293/Null2
HEK293/Null2-k

Figure S4.9. Pluronic do not activate Toll Like Receptors (TLR's). Pluronic P85 (0.01% and 1.0% w/v) and control ligand were exposed to each stably transfected HEK-293 cell line with a given mouse TLR **(a)** and NFKB negative control HEK-293 cells **(b)** for 16-20 h and tested for reporter gene expression by Molecular Devices Spectramax340PC. Data are mean ± SEM (n=3).

CHAPTER 5

OVERALL CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

In this dissertation work, we used a pragmatic approach to understand the key questions related to gene delivery in skeletal muscles. Direct i.m. injection of naked pDNA to transfect skeletal muscles for the purpose of therapeutic protein expression is a well-established approach in preclinical and clinical studies. We were the first to show that muscle transfection can be further enhanced upon co-injection of non-ionic block copolymer with pDNA. However, this effect is strain-dependent and not observed in immune-deficient athymic mice. Also, coadministration of block copolymer with pDNA resulted in enhanced transgene expression not only in injected muscles but also in draining lymph nodes and spleen. Therefore, we studied the role of immune cells in gene transfer and gene expression. The current work examined **1) the role of inflammation and inflammatory cells, specifically, MØs on gene transfer upon delivery of naked DNA with pluronic block copolymers; 2) the immunological profiling of innate immune response against pDNA/copolymer to identify key cells involved in gene transfer and evaluate the potential of engineering the immune response to further enhance muscle transfection of pDNA/copolymer.**

In chapter 3 we investigated the effects of local inflammation in murine hind limb ischemia model (MHLIM) on gene expression and found that inflammation drastically increased DNA, RNA and protein levels in muscles injected with pDNA/Pluronic. Ischemic muscles showed high number of GFP^+ muscle fibers with GFP expression co-localized with $desmin^+$ muscle fibers and $CD11b^+$ MØs. This suggested that Pluronic assist muscle transfection through MØs. Therefore, using *in vitro* cell culture model we showed that P85

enhanced (~4 orders) transfection of MØs with naked pDNA. Moreover, adoptively transferred DNA transfected MØs were shown to traffic to the sites of inflammation and pass the transgenes to inflamed muscle cells. We observed a clear colocalization of CD11b marker along the sides of desmin suggesting cell-to-cell contact dependent exchange of genes from MØs to muscle cells. However, we cannot rule out the possibility of other immune cell recruitment to ischemia muscles like neutrophils contributing to the colocalization of $CD11b^{+}$ with desmin. Nevertheless, we observed a significant colocalization of GFP with CD11b confirming that transfected MØs homed to the ischemic muscles.

Therefore, we developed an *in vitro* model by co-culturing transfected MØs with MTs and used constitutive (cmv-luciferase) or muscle specific (desmin-luciferase) reporter gene expression to show that P85 enhances horizontal pDNA transfer from MØ to MTs. This study was particularly interesting because it recapitulated inflammatory microenvironment similar to *in vivo* setting. Also, we understand that the concentrations of Pluronic treatment in coculture was around 3.3 times higher (1% w/v) than used for i.m. injection (0.3% w/v), but we limited our treatment to only 2 h duration which is relatively very short duration of treatment to observe drastic increase in muscle specific gene expression. Finally, significant increase in transgene expression was seen upon systemic inflammation (MHLIM or peritonitis) with pDNA/Pluronic but not pDNA alone. The findings of this chapter indicate that MØs present in inflammation play a significant role in muscle transfection with pDNA/Pluronic by assisting horizontal exchange of pDNA. We conclude that pDNA/Pluronic has potential for therapeutic gene delivery in muscle pathologies that involve inflammation.

In this study we propose that in pluronic gene delivery approach, MØs can uptake the pDNA and as a part of evolutionary process of regeneration, can fuse with

muscle fibers resulting in gene delivery to other wise hard to transfect skeletal muscles (discussed in chapter 3 and shown in **Fig. 2.2**). However, the frequency of MØ to muscle fusion is very low (<0.1%) and Pluronic may enhance these events as a function of PEG motifs (Poly-ethylene oxide). Therefore a follow-up study may require analysis of MØ to muscle cell fusion with and without Pluronic treatment using live imaging to quantify the absolute effect.

In chapter 4, we carefully dissected innate immune response to the naked pDNA alone or when formulated with Pluronic. Immunophenotyping of the PECs from both euthymic Balb/c and athymic nude mice showed that P85 evokes a response unlike pathogen recognition response of bacterially derived LPS. Specifically, each adjuvant (alum/LPS/P85) helped recruit a characteristic PEC response which was both qualitatively and quantitatively different in Balb/c mice. The overall response further increased in athymic nude mice in response to LPS/alum, but an opposite effect was observed to P85. Increase in PEC response in P85 Balb/c and LPS/alum nude group was composed majorly of MØs and neutrophils, respectively. These results suggested that P85 induced MØ recruitment may explain the reason for increase in gene expression upon i.m. injection of pDNA/Pluronic in healthy mice. This effect was confirmed when pDNA/P85 was unable to transfect MØ depleted Balb/c mice upon pDNA/P85 injection. These results confirmed that lack of MØ recruitment in nude mice was the reason for strain-dependent response in pDNA/P85. Moreover, we applied this knowledge to engineer a MØ recruitment response to the subsequent site of pDNA/P85 injection to further increase the muscle transfection. Specifically, pre-injection with P85 1.5 day prior to subsequent pDNA/P85 injections in the same muscles resulted in further improvement in pDNA/P85 muscle transfection. We understand that preinjection with P85 induced an inflammatory response, which can be a concern, however a detailed pathological and histological examination can help optimize

the scheme based on the disease pathology under investigation. Furthermore, gene expression with of pDNA/P85 was inversely correlated with the depth of needle injection, suggesting that survival of muscle fibers along the needle track may be critical for gene delivery in healthy muscles.

It was striking that Pluronic, alum and LPS adjuvants did not evoke a typical inflammatory response but rather a very specific cellular recruitment composed of different proportions of MØs and neutrophils. Since Pluronic resulted in a response different from alum/LPS both qualitatively and quantitatively, it can be used to design a custom adjuvant formulation to modulate immune responses in a specific and controlled manner. Therefore, we believe that more immunological profiling studies can help derive structure function relationships amongst a range of Pluronics aimed at designing “smart adjuvants” for gene and protein delivery applications.

During the studies, many open ended questions arose that need to be addressed carefully. Specifically, activation of NF-kB and other inflammatory signaling by Pluronic can be a concern for gene delivery applications in diseased muscles with chronic inflammation e.g. muscular dystrophy where the aim is to rescue muscle death. Also, there is a direct correlation of hydrophobicity or adjuvancy of pluronic with muscle transfection efficiency (L61>P85>F127) which might raise similar concerns. Also, there is a dose dependent response of Pluronic on muscle transfection with a typical bell shaped curve for each Pluronic with an optimal concentration. This could be explained by membrane damage to the transfected muscle fiber beyond repair after a certain concentration (optimal) resulting in eventual decrease in muscle transfection. However, we still did not understand the rationale for the right shift in the bell shaped curve in ischemic muscle compared to healthy muscles resulting in increased optimal concentrations of both P85 and SP1017.

Overall, we thoroughly investigated the effects of Pluronic on gene expression using various *in vitro* and *in vivo* models.

Future directions.

We showed that Pluronic block copolymers enhance gene transfer from MØ to muscle cells is a pragmatic approach. However, it will be interesting to study the effects of Pluronic on muscle regeneration. A body of evidence in EGT and regenerative medicine suggests that Pluronics (Poloxamer 188) help reduce the traumatic injury caused by electric pulses during EGT (1) and also help regeneration in wound healing. Specifically, we observed an increase in desmin expression when MBs were cocultured with MØ, this may imply a MØ dependent satellite cell maturation to form new muscle cells. Since Pluronic modulates cellular recruitment to MØ predominated response, this may further enhance the differentiation of precursor muscle cells in to muscle fibers. Alternatively, since Pluronic may assist MØ to muscle cell fusion as explained in our proposed model of gene transfer (**Fig 2.2**), this response may also enhance overall muscle regeneration.

Pre-injection scheme for improved muscle transfection results in artificial APCs/MØ recruitment which can potentially intensify the antigen presentation in muscle and draining lymph nodes in otherwise non-immunogenic nature of DNA vaccines. It will be very interesting to utilize this scheme (pDNA/P85 injection 1.5 day after 3% P85 preinjection) and compare it to pDNA/P85 and naked pDNA alone to evaluate a potential prophylactic or therapeutic efficacy in a relevant disease mouse model.

Drastic increase in transgene expression in pDNA/P85 in response to local or distal inflammation can be very effective regime to enhance the gene expression levels in human clinical trials specifically because most of the disease pathologies often involve

inflammation. Currently upto 2.4 mg of pDNA of the course (Neovasculogen; plasmid genetic construction containing human gene VEGF165) (2) is injected in the patients suffering from lower limb ischemia in Phase –III clinical trials for achieving enough VEGF expression for therapeutic efficacy. With pDNA/Pluronic approach, there is great potential to decrease the potential amount of pDNA required for same or even enhanced protein expression and simultaneously decreasing the potential inflammatory response caused by high dose of pDNA injections (3).

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