EFFECTS OF PATHOGEN ASSOCIATED MOLECULAR PATTERNS ON SKELETAL MUSCLES TO UNDERSTAND THE DEVELOPMENT OF NEUROGENIC HETEROTOPIC OSSIFICATION AFTER SPINAL-CORD INJURY

Selwin Samuel*, Hsu-Wen Tseng*, Whitney Fleming*, Kylie Alexander*, Jean-Pierre Levesque*

***Mater Research Institute, The University of Queensland, Brisbane, Australia**

Introduction

Neurogenic Heterotopic Ossifications (NHO) are incapacitating complication after damage to the central nervous system such as spinal cord injury (SCI) and traumatic brain injury (TBI). They manifest as heterotopic bones growing in periarticular muscles. The pathophysiology is not completely understood and as a result, there are no prophylactic treatments. In retrospective studies, NHO prevalence was significantly higher in SCI and TBI patients with infections. So, our aim was to test whether infections influence the osteogenic potential of muscle progenitor cells in vitro and test their effect in vivo in our mouse model of SDCI-induced NHO. The innate immune system identifies pathogens via conserved motifs called pathogen associated molecular patterns (PAMPs) that are recognized by specific pathogen recognition receptors (PRRs) expressed by immune cells.

Research objectives

To investigate whether PAMPs can enhance the osteogenic potential of muscle progenitor cells

Methods

In-vitro osteogenic assays with PAMPs

Fibro-adipogenic progenitors (FAPs) (CD45-Lin-CD31-Sca1+CD34+) were sorted from mouse muscles and seeded in 96-well plates and treated with PAMPs in different concentrations in osteogenic conditions (β-glycerophosphate and phosphoascorbic acid, $CaCl_2$ and dexamethasone). After 10 –14 days of culture, cells were fixed and stained with Alizarin Red, followed by destaining with 10% cetyl pyridinium chloride. The destained solutions were transferred into a fresh non-treated 96-well plate and the absorbance was read at 562nm.

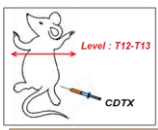
Testing cell-cultures for cross contamination

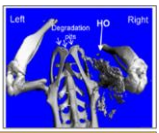
The FAPs were subject to surface cell staining and checked for macrophage/leucocyte contamination. The results are tabulated below.

Antigen	Expression
CD 45	-
F4/80	-
CD11b	-
Sca1	+
CD 31	+
CD 34	+

In-vivo experiments with PAMPs

In our established NHO models, spinal cord of mice were severed at T12 – T13 level and injected with cardiotoxin (0.3125mg/kg) in right hamstring muscles and bone volumes were measured via µCT at days 7 and 21.





Pathology	SCI alone	CDTX alone	SCI + CDTX
HO	0%	0%	99.1%

In these experiments PAMPs were co-administered in different dosages with cardiotoxin to determine their effects.

Results

In vitro experiments:

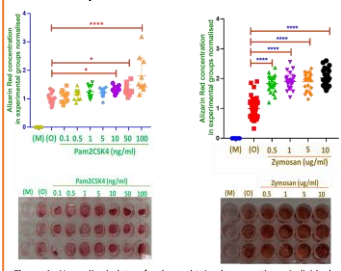
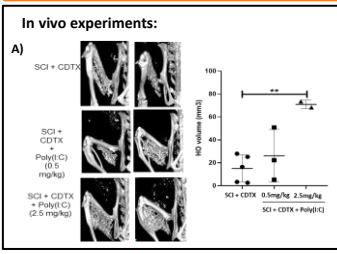


Figure 1: Normalised data of values obtained across three individual experiments with n = 6 wells per condition per experiment. All experimental wells were in osteogenic with indicated concentrations of Pam2CSK4 (A) and Zymosan (B). Controls were cells cultured in medium (M) without addition of osteogenic factors (O). Data represent the absorbance at 562 nm to quantify Alizarin Red deposition. Data are normalised to average absorbance of cells in osteogenic medium without PAMPs (value = 1). Each dot represents a well.

PAMPs	Source	Associated PRRs	Effect on FAP mineralisation
Gardiquimod	Viral ssRNA	TLR7/8 dimer	+
P.aeruginosa flagellin	Flagellated bacteria	TLR5	+
mTriDAP	Gram + and - bacteria	NOD1	+
Furfurman	Skin Fungus	Dectin-2	+
Gluconyl - 6 - tetradecyl octadecanoate	Mycobacteria	Mincle	+
Pam2CSK4 lipopeptide	Gram + and - bacteria	TLR2/6 dimer	+
Pam3CSK4 lipopeptide	Gram + and - bacteria	TLR1/2 dimer	+
CpG oligodeoxynucleotide (ODN) #1668	Bacterial DNA	TLR9	+
PGN – SA (Peptidoglycan)	S. aureus	TLR2	+
Zymosan (β-glucan)	Fungi, yeast	Dectin-1 & TLR2/6 dimer	+
Poly IC	Viral dsRNA	TLR3, MDA-5, PKR and RIG- 1	+

Table 1: Indirect effects of PAMPs on FAP mineralisation when incubated with conditioned media from macrophages treated with the above PAMPs. '+' refers to increased mineralisation of FAPs in comparison to osteogenic environment.

In vivo experiments:



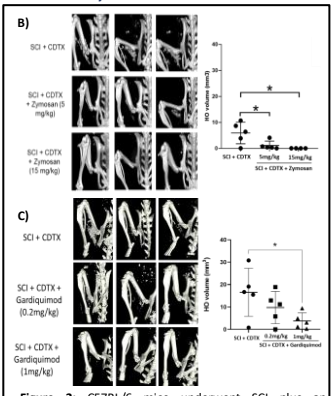


Figure 2: C57BL/6 mice underwent SCI plus an intramuscular injection of CDTX (0.3125mg/kg) alone, or CDTX together with (A) Poly(I:C), (B) Zymosan or (C) Gardiquimod. Representative MicroCT images for each group and total NHO volume quantified by µCT at 7 days post-surgery are displayed as graphs. All dots represent an individual mouse, data represented as mean ± SD, two sided Mann-Whitney test (Group A) and one way ANOVA tests (Groups B and C).

Conclusion

Our observations show that Pam2CSK4 and Zymosan are capable of enhancing mineralization of muscle FAPs in an osteogenic environment, in vitro. All PAMPs had an indirect effect on FAP mineralization. Intramuscular PAMP administration of Poly (I:C) in injured muscles of mice with SCI increased NHO size while PAMPs such as Zymosan and Gardiquimod reduced NHO development. Further investigations need to be carried out to determine whether some PAMPs deplete macrophages in the injured muscles. Experiments with instead systemic intraperitoneal administration of PAMPs will be performed to determine whether the route of administration of PAMPs leads to different effects on NHO development.

References

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