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Immodulin peptides influence musculoskeletal homeostasis by linking extracellular cues to macrophage and myoblast nuclear receptors

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Abstract

Immodulins are synthetic peptides derived from the C-terminal domains of insulin-like growth factor binding proteins (IGFBPs). Immodulins from the 3/5/6 (but not 1/2/4) IGFBP evolutionary clade transduce extracellular matrix (ECM) signals to RXR, NR4A1 and PPAR-alpha nuclear receptors (NRs) to stimulate novel macrophage lineages. The rationale of this study was to reconcile physical associations of immodulins with ECM and NRs, effects of siRNAs and chemical inhibitors in vivo, and immodulin-driven pro-differentiation effects in cell culture. When added to THP1D cells, immodulins stimulate CD169+ Clec9a+ and Clec12a+ macrophage lineages via a EP300/RXRγ/Nur77 transcriptional mechanism. This phenomenon is accompanied by the secretion of CCL22, IL-10 and TGFbeta and the ability to stimulate FoxP3+ T-cells in co-culture. ECM ligands of 3/5/6 immodulins include iron, zinc, glycosaminoglycans, transferrin and phosphatidylinositol-4,5,-biphosphate (PIP2), which can influence their pro-differentiation effects. Remarkably, immodulins also stimulate myogenesis in C2C12 myoblasts, thereby revealing a novel link between immune and musculoskeletal homeostasis. Distinct NR agonists stimulate these companion differentiation processes. Using solution NMR to guide design, immodulins with a tripeptide extension near the iron-binding pocket demonstrated higher iron-binding and improved pro-differentiation activities. Transferrin-bound immodulin shows binding preference for both high-molecular-weight hyaluronan (HMWHA) and HMWHA:CD44 complexes at endosomal pH, and interacts with PIP2 at normal physiological pH, offering intriguing mechanistic insights.

Key Words: Immodulin; CD169; myogenesis; hyaluronan; iron.

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Immodulins are synthetic peptides derived from the C-terminal domains of classical insulin-like growth factor binding proteins (IGFBPs) 1-6, conserved proteins that belong to two ancient (>700 million ybp) evolutionary clades consisting of IGFBPs 1/2/4 and 3/5/6, respectively.[1] Unlike the 1/2/4 clade, members of the 3/5/6 clade of IGFBPs contain putative nuclear translocation sequences and bind members of the retinoid X receptor (RXR), PPAR and NR4A nuclear receptor (NR) families.[2] Some IGFBPs and derived peptides have been implicated in binding to metals, glycosaminoglycans (GAGs), collagen and transferrin, among other ligands.[3-6] The biological significance of this broad range of IGF-independent binding activities has never been fully explained, though they may suggest transduction of environmental cues from the extracellular matrix (EM) to transcriptional programs, possibly in an iron-dependent manner. In a recently described atypical glycan-CD44 mediated iron endocytosis pathway involving high molecular weight hyaluronan (HMWHA), nuclear iron operates as a metal catalyst to demethylate repressive histone marks that govern the expression of mesenchymal genes, while CD44 itself is transcriptionally regulated by nuclear iron.[7] Iron-bound immodulin peptides have demonstrated remarkable biological efficacy in restoring tissue homeostasis after traumatic insult in an animal model.[8] The present study explores a possible homeostatic role for immodulin peptides in the context of the possible role of RXR heterodimers with PPAR-class and Nur77 NRs, as these heterodimers control transcriptional programs in numerous cell types.[9]

In a well-known tissue distribution dendogram for NRs,[10] the three known isoforms of RXR are located in separate clusters: RXRgamma is adjacent to heterodimer partner Nur77, RXRalpha is adjacent to

heterodimer partners PPARalpha and PPARdelta, and RXRbeta is distant from both. Nur77 has been implicated in innate immune cell function,[11] while PPARalpha and PPARdelta are critical to musculoskeletal homeostasis.[12]

This study examines the ability of immodulin class peptides to i) directly bind iron, NRs and GAGs; ii) stimulate THP-1 monocyte differentiation towards tolerizing lineages; and iii) stimulate C2C12 myoblast differentiation into myotubes. The ultimate goal of this study is to elucidate underlying mechanisms of action.

Materials and Methods

Peptide synthesis

All peptides used in this study were synthesized by Lifetein LLC (Hillsborough, NJ) and purified to >80% purity. Identity was confirmed by mass spectroscopy.

Chemical agonists and inhibitors

Unless otherwise specified, chemical reagents were purchased from Cayman Chemical Company (Ann Arbor, MI) or Sigma-Aldrich Chemical Company (St. Louis, MO).

3D-NMR Studies

Details of this experiment will be reported elsewhere. Briefly, C13/N15-labelled imm3 peptide was synthesized (Lifetein LLC, Hillsborough, NJ) and solubilized in the presence of equimolar ferric iron (chloride) at 4-8 mg/ml peptide in 6 mM sodium acetate buffer pH 5.2. Solution NMR spectra were collected and analyzed under contract by Avomeen Analytical Services (Ann Arbor, MI) using a 800 MHz Bruker spectrometer with helium cooled H/C/N CryoProbe at 25 °C to acquire spectra. 2500 random structures were generated and 10000 independent annealings were performed to minimize input constraints. The top 10 PDB structures ranked in terms of least constraint violation are overlaid in Fig. 1A.

Binding Assays

The peptides listed in Table 1 were used in metal-binding assays of two types, involving either an Alexa488-labeled streptavidin protocol (Binding Assay 1) or FITC-NTA assay (Binding Assay 2). Essentially identical results were obtained with both assays, and an average is reported here. Fluorescence was standardized to binding with immX3AVD as the control peptide (=100) and the average of multiple experiments using both protocols is shown. Binding Assay 1 was done as follows: NTA coated 96-well plates (G-Biosciences, St. Louis, MO) were charged with ferric chloride, ferrous sulfate or zinc chloride and contacted with equimolar biotinylated peptide:streptavidin-A488 complex (400 ng peptide per well) for 60 min at room temperature, then washed with phosphate-buffered saline (PBS) and read at 485/525 nm in a Beckman fluorescence counter. Binding Assay 2 was done as follows: Streptavidin coated 96-well plates (G-Biosciences, St. Louis, MO) were contacted with 400 ng peptide per well in PBS buffer for 60 minutes at room temperature, washed in PBS, then contacted with FITC-NTA (TRC Co, Toronto, Canada) complexed with equimolar ferric chloride, ferrous sulfate or zinc chloride (10x molar equivalent per well) for 60 min at room temperature, then washed with PBS and read at 485/525 nm in a fluorescence counter. Recombinant nuclear receptor proteins were purchased from Abcam Inc, Cambridge, MA. 100 ng/well of recombinant RXR-alpha DNA-binding domain (DBD) or 200 ng/well of RXR-alpha ligand-binding domain (LBD), or 400 ng/well full-length Nur77/NR4A1, RXR-alpha, RXR-beta, RXR-gamma, PPAR-alpha or PPAR-gamma was adsorbed to wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate for 60 minutes at room temperature, then blocked with 200 uL 1% bovine serum albumin (BSA) in PBS buffer overnight.

The plate was washed with PBS buffer and 1ug/well Streptavidin-Alexa 488 conjugate-(SA488)-labelled immodulin peptide was added per well. The plate was incubated for 60 min at room temperature, then washed with PBS buffer and read in a fluorometer, as above. FITC-labeled glycosaminoglycans, PIP2 and transferrin ligands were tested as follows: Streptavidin-coated 96-well plates (G-Biosciences, St. Louis, MO) were pre-treated with biotinylated peptides at 400 ng/well, at room temperature for 60 minutes.

The plate was washed with PBS buffer and 1 ug FITC-labelled ligand in 100 uL PBS buffer was added to each well. After 60 minutes incubation at room temperature (with or without the indicated unlabeled competitors) followed by two PBS buffer washes, the plate was read in a fluorometer as above. All tests were done in quadruplicate.

Cell culture

C2C12 myoblast cell line (ATCC, Manassas, VA) was cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. For differentiation, cells were cultured in the same medium, except that 10% FBS was replaced by 2% horse serum. Peptides and inhibitors were added at the indicated concentrations and cells were harvested 96 hours later for assay. Cell extracts were assayed for creatine kinase (CK) per mg protein.

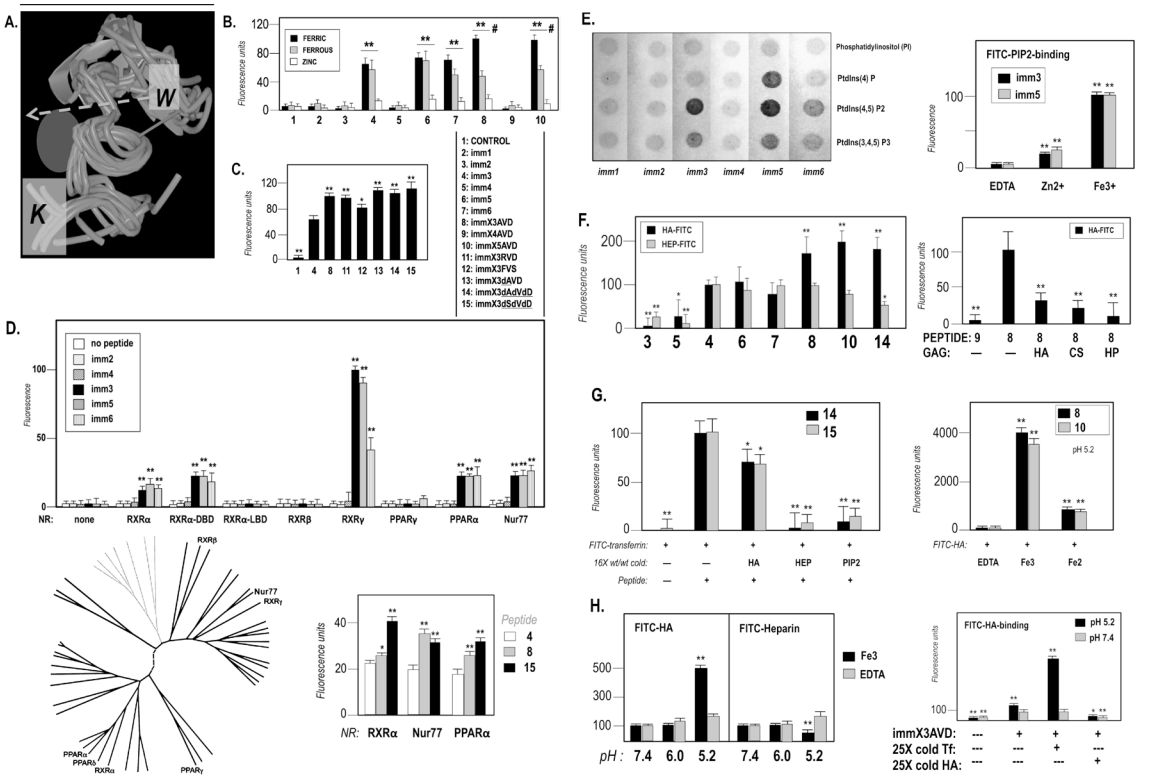

Fig. 1 *C-terminal tripeptide extension increases binding of immodulins to ligands. [A] Overlaid images of the ten lowest energy structures of imm3 peptide acquired by solution 3D-NMR. N-terminal lysine (K) is shown at bottom left, and the predicted orientation of the XVD tripeptide extension to the C-terminal tryptophan (W) is shown by the dashed arrow, top left and the location of the iron-binding pocket (oval) is predicted by analogy to the iron-binding region of IGFBP-1. The bar shows the location of the disulfide bond. [B] Binding to iron and zinc. ** p<0.01 versus buffer control; # p<0.05 vs immX3AVD [C] C-terminal tripeptide improves binding to ferric iron. [D] Top panel: Binding of immodulin 3/5/6 clade, but not 1/2/4 clade to various nuclear receptors in vitro. Lower left: Dendogram using hierarchical, unsupervised clustering of NR tissue expression distribution (from r.10) showing location of NRs relevant to this study. Lower right: C-terminal tripeptide improves binding to NRs. * p<0.05, ** p<0.01 versus imm3 peptide. [E] Left: PIP-binding assay using Echelon Biosciences (Salt Lake City, UY) Membrane Lipid Strip P-6002, following manufacturer's recommendations. 1 ug/ml of biotinylated peptide was applied to each strip, followed by detection with streptavidin-horseradish peroxidase and precipitable color substrate. Right: 400 ng peptide bound by 1 ug FITC-PIP2 (Cayman Chemical, Ann Arbor, MI) per well as described in Methods. ** p<0.01 vs EDTA control. [F] 400 ng/well of indicated peptides bound to HA-FITC or heparin-FITC (left) with or without 10X excess cold GAG indicated (right). [G] Left: Similar to F, except FITC-transferrin was used as ligand, and 16X excess cold HA, heparin or PIP2 were used to compete. Right: Binding of FITC-HA to indicated peptides in 6 mM sodium acetate buffer pH5.2, in the presence of 25 uM EDTA, ferric chloride or ferrous sulfate. **p<0.01 vs EDTA control. [H] Left: Binding to 400 ng/well peptide 8; identical to experimental conditions described for panel G, right, with either FITC-HA or FITC-heparin, except that buffers at pH 7.4 (phosphate), 6.0 (phosphate) or 5.2 (acetate) were used during binding. Right: Competitive binding to 400 ng/well peptide 8 (immX3AVD) as done in panel F (right), except that 25X holotransferrin (Tf) or 25X hyaluronan (HA) were used to compete, and the experiment was done either in acetate (pH 5.2) or phosphate (pH 7.4) buffer. *p<0.05, **p<0.01 versus peptide 8 alone at pH 7.4.*

CK was measured using the ECPK-100 kit from BioAssay Systems (Hayward, CA). Protein was assayed using the BCA protein assay kit from Thermofisher Scientific (Waltham, MA). Macrophage differentiation and polarization assays were done using the THP1-Dual monocyte reporter cell line (Invivogen Inc, San Diego, CA) seeded at 2x10^5 cells per well in 96-well plates and cultured at 37 degrees C in RPMI-1640 growth

Table 1. *Sequences of peptides used in this study.*

PEPTIDE	SEQUENCE
None (buffer)	
imm1	KNGFYHSRQCETSMDGEAGLCW
imm2	KHGLYNLKQCKMSLNGQRGECW
imm3	KKGFYKKKQCRPSKGRKRGFCW
imm4	RNGNFHPKQCHPALDGQRGKCW
imm5	RKGFYKRKQCKPSRGRKRGICW
imm6	HRGFYRKRQCRSSQGQRRGPCW
immX3AVD	KKGFYKKKQCRPSKGRKRGFCW**AVD**
immX4AVD	RNGNFHPKQCHPALDGQRGKCW**AVD**
immX5AVD	RKGFYKRKQCKPSRGRKRGICW**AVD**
immX3dAVD	KKGFYKKKQCRPSKGRKRGFCW**(dA)VD**
immX3dAdVdD	KKGFYKKKQCRPSKGRKRGFCW**(dA)(dV)(dD)**
immX3dSdVdD	KKGFYKKKQCRPSKGRKRGFCW**(dS)(dV)(dD)**
immX3FVS	KKGFYKKKQCRPSKGRKRGFCW**FVS**
immX3RVD	KKGFYKKKQCRPSKGRKRGFCW**RVD**

Tripeptide extensions are shown in bold font and D-amino acid residues are in parentheses

medium plus 10% fetal bovine serum and 1% penicillin/streptomycin, then treated for 24 hours with 100 ng/ml Phorbol 12-myristate 13-acetate (macrophage PMA protocol).

Peptide (330 or 660 nM) was then added, and incubation continued for an additional 24 hours. Culture supernatants were then assayed for C-C motif chemokine 22 (CCL22), interleukin-10 (IL-10) or transforming growth factor beta (TGFbeta) using Duoset ELISA kits (R&D Systems, Minneapolis, MN). Adherent cells were washed twice with PBS and cells were assayed for immunoreactivity of surface markers CD169, Clec9a and Clec12a using biotin-labeled anti-human antibodies for these markers purchased from Miltenyi Biotec (Auburn, CA) and a streptavidin-horseradish peroxidase/TMB secondary detection reagent. Results were expressed as arbitrary ELISA immunoreactivity units relative to the control immX3AVD peptide (=100). Alternative differentiation protocols, including M1 macrophage (25 ng/ml interferon-gamma plus 25 ng/ml lipopolysaccharide) and M2 macrophage (25 ng/ml IL-4) used reagents purchased from Peprotech (Rocky Hill, NJ). All cell culture data were collected in quadruplicate, and the reported observations were representative of at least two independent experiments.

Statistical analysis

Data are presented as means ± standard deviation (SD) unless otherwise indicated.

Probability values (p values) were computed using Student's t-test and expressed relative to control treatment, as indicated.

Results

Tripeptide extension-enhanced binding of immodulins to ferric iron.

Table 1 shows the sequences of immodulin peptides used in this study. 3D-NMR spectra were aquired from C13/N15-double-labeled imm3 peptide in solution, as described in the Methods section.

Fig. 1A shows an overlaid rendering of the ten lowest-energy structures. Notably, the bound iron molecule was not visible in these structures, suggesting a low affinity interaction. In order to strengthen iron-binding, the extended immodulins shown in Table 1 were tested using two types of metal-binding assays, as described in Methods, with binding to ferric iron, ferrous iron, and zinc being compared. Averages were obtained for each peptide from 2-5 independent experiments, each done in quadruplicate.

The results, in Fig. 1 panels B and C, show that an AVD tripeptide extension (e.g. in immX3AVD peptide) significantly increases ferric iron (but not ferrous iron or zinc) binding of 3/5/6 clade (but not 1/2/4 clade) immodulins when compared to imm3 peptide without the extension. Moreover, the substitution of D-amino acids in the tripeptide does not adversely affect iron binding.

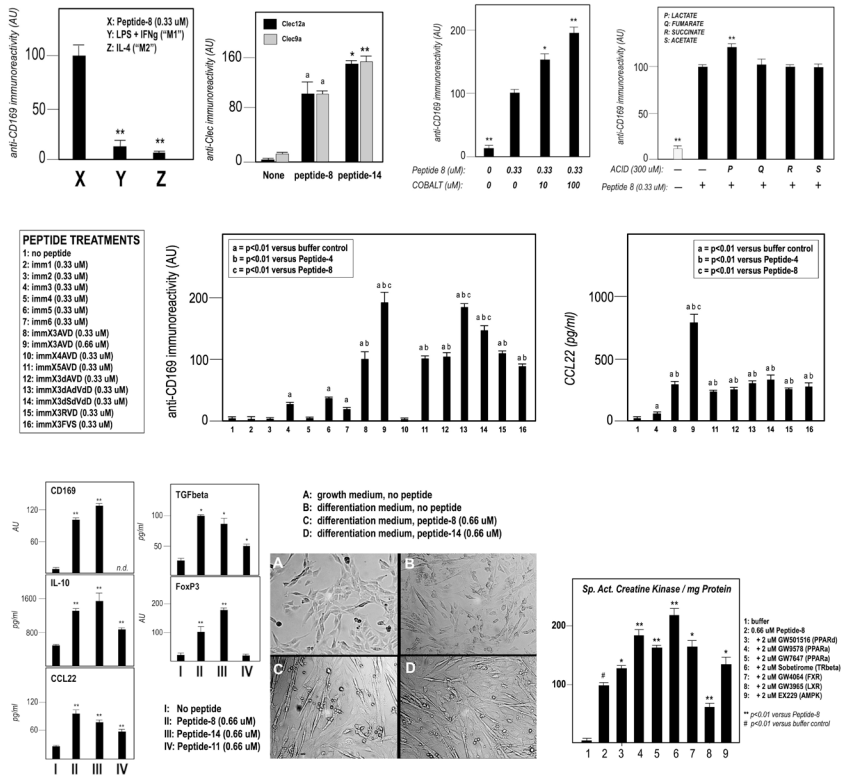

Fig. 2 *Immodulins drive differentiation. TOP PANEL: Generation of CD169, Clec9a and Clec12a positive moAPCs. From left to right: Reduced stimulation of CD169 marker in THP1D cultures (without peptide 8) by alternative polarization protocols for generating M1 (25 ng each IFN-gamma and lipopolysaccharide) or M2 (25 ng IL-4) macrophages. ** p<0.01 vs peptide alone; peptides 8 and 14 increase stimulation of Clec9a and Clec12a markers in THP1D cultures. a=p<0.01 vs no peptide control; * p<0.05, ** p<0.01 vs peptide 8 control; enhanced stimulation of CD169 marker in THP1D cultures by peptide 8 plus cobalt chloride * p<0.05, ** p<0.01 vs peptide alone; enhanced stimulation of CD169 marker in THP1D cultures by peptide 8 plus 300 uM lactate, fumarate, succinate or acetate buffer, final of culture medium was pH 7.4 for all samples. ** p<0.01 vs peptide 8 alone. MIDDLE PANEL: Stimulation of CD169 marker in THP1D cultures (as described in Methods) using the peptides shown; stimulation of CCL22 in THP1D cultures by immodulin peptides. BOTTOM PANEL: cell culture supernatants of THP1D cultures treated with PMA (24 hrs) and then 0.66 uM immodulin peptide plus 1 ug/ml GAD65 antigen primer for an additional 24 hours were assayed for CD169, IL-10, CCL22, TGFbeta, and cell extracts prepared post-96 hour-overlay of ~10e5 GAD-specific T-cells per well assayed for FoxP3 using commercial ELISA assay kits according to the manufacturer's instructions (Duoset, R&D Systems, Minneapolis, MN) ** p<0.01 vs. unprimed control, AU=arbitrary ELISA units; stimulation of myogenesis in C2C12 myoblasts was performed as described in Methods, representative fields were photographed under the microscope; C2C12 cells were treated with 0.66 uM peptide 8 plus 2 uM of various NR ligands (Cayman Chemical, Madison, WI) in differentiation medium and creatine kinase (CK) per mg protein was measured in cell extracts using the ECPK-100 kit from BioAssay Systems (Hayward, CA) while protein was assayed using the BCA protein assay kit from Thermofisher Scientific (Waltham, MA).*

Immodulins bind nuclear receptors in vitro

Immodulins rapidly localize to the nucleus of over 50 cell types tested,[13-24] but whether they can bind RXRs, PPARs or Nur77/NR4A1 is not known. [25,26] As shown in Fig. 1D, top panel, immodulin peptides of the 3/5/6 clade (but neither of the two representatives of the 1/2/4 clade tested) bind recombinant NR proteins RXR-alpha, RXRalpha-DBD domain, RXR-gamma, PPAR-alpha and Nur77/NR4A1 and did not bind RXR-alpha-LBD, RXR-beta or PPAR-gamma in vitro. Among the full-length RXR isoforms (alpha, beta and gamma) immodulins 3/5/6 bound RXR isoforms alpha and gamma, with a marked preference for the latter. These results comport with the proximate positions of these NRs in the dendogram of tissue distribution (Fig. 1D, lower left). Extended immodulins 8 and 15 (Fig. 1D, lower right) bind RXR-alpha, Nur77/NR4A1 and PPAR-alpha more avidly than the parental peptide 4.

Table 2. *Effects of chemical additives on immX3AVD(0.33 uM)-driven generation of CD169+ moAPCs in 24 hr cultured THP1-D macrophages.*

	CLASS	*CAS #*	*[CONC]*	*CD169 (AU)*	
None (buffer)				100.0 ± 4.9	
[A] MODULATORS OF NUCLEAR RECEPTORS					
C-DIM12	NR4A1 agonist	178946-89-9	2 uM	119.0 ± 3.7**	
Cytosporone B	NR4A1 agonist	321661-62-5	2 uM	120.3 ± 6.4**	
Dihydroergotamine	NR4A1 agonist	6190-39-2	2 uM	149.0 ± 11.2**	
TMPA	NR4A1 agonist	1258275-73-8	2 uM	181.3 ± 22.7**	
C-DIM8	NR4A1 antagonist	151358-47-3	2 uM	83.3 ± 6.3**	
Cilostazol	NR4A2 agonist	73963-72-1	2 uM	99.4 ± 27.7	
6-mercaptopurine	NR4A3 agonist	6112-76-1	2 uM	104.9 ± 4.0	
Bexarotene	Rexinoid (pan-RXR)	153559-49-0	2 uM	96.9 ± 6.8	
LG100268	Rexinoid (pan-RXR)	153559-76-3	2 uM	76.8 ± 11.9**	
HX600	Rexinoid (pan-RXR)	172705-89-4	2 uM	92.7 ± 6.6	
Spironolactone	RXRgamma agonist	52-01-7	2 uM	120.1 ± 10.5**	
HX531	RXRgamma inhibitor	188844-34-0	2 uM	82.7 ± 5.7**	
GW7647	PPARalpha agonist	265129-71-3	2 uM	101.8 ± 15.7	
GW501516	PPARdelta agonist	317318-70-0	2 uM	94.1 ± 7.9	
GW3965	LXR agonist	405911-09-3	2 uM	84.4 ± 7.3*	
GW4064	FXR agonist	278779-30-9	2 uM	100.5 ± 15.2	
Sobetirome	TRbeta agonist	211110-63-3	2 uM	87.4 ± 7.4	
[B] SELECTED TRANSCRIPTIONAL REGULATORS					
Cyclosporin A	NFAT inhibitor	59865-13-3	2 uM	129.2 ± 7.6**	
Bisindolylmaleimide IX	PKCbeta inhibitor	138489-18-6	2 uM	130.1 ± 8.4**	
C646	EP300 inhibitor	328968-36-1	2 uM	75.0 ± 4.0**	
IQ-1	EP300 inhibitor	331001-62-8	2 uM	91.2 ± 5.6*	
CPI-637	CBP/EP300 inhibitor	1884712-47-3	2 uM	95.5 ± 1.1*	
PNU74654	B-catenin inhibitor	113906-27-7	2 uM	99.8 ± 11.8	
[C] CYTOKINES, CHEMOKINES, GROWTH FACTORS					
GM-CSF	Cytokine class	*83869-56-1*	0.1 ug/ml	147.0 ± 15.3**	
IL-4	Cytokine class	na	0.1 ug/ml	95.9 ± 9.4	
CSF1	Cytokine class	na	0.1 ug/ml	108.0 ± 32.3	
[D] TLR MODULATORS					
PAM2CSK4	TLR2 agonist	868247-72-7	2 uM	36.8 ± 5.9**	
E.coli LPS	TLR4 agonist	93572-42-0	0.25 ug/ml	78.6 ± 3.1**	
R-848	TLR7/8 agonist	144875-48-9	2 uM	81.8 ± 5.6*	
GIT-27	TLR2/4 inhibitor	6501-72-0	2 uM	152.4 ± 10.3**	
[E] RLR/STING AGONISTS					
tlrl-3prnalv	RIG-I agonist	na	1 ug/ml	135.4 ± 9.1**	
KIN1400	RIG-I agonist	446826-86-4	2 uM	131.6 ± 4.6**	
G10	STING agonist	702662-50-8	2 uM	143.4 ± 17.8**	
2'3'-cGAMP	STING agonist	1441190-66-4	15 uM	168.5 ± 10.9**	

*Control AU (arbitrary units of immunoreactivity by ELISA) set to 100; *p<0.05, **p<0.01.*

In vitro binding of immodulin peptides PIPs, glycosaminoglycans and holotransferrin

In addition to metals, whose binding to IGFBP-3 and its corresponding immodulin sequence have been demonstrated, whether immodulin peptides can bind other known IGFBP-3 ligands such as glycosaminoglycans (GAGs), transferrin and collagen is not known. Figure 1 panel E shows that immodulin peptides imm3/5/6 (but not 1/2/4) bind phosphatidylinositolphosphates (PIPs), in a ferric iron-sensitive manner. Neither full-length IGFBPs nor immodulin peptides have previously been shown to bind PIPs.

Fig. 1F shows that 3/5/6 clade peptides (but not their 1/2/4 clade controls) bind heparin and hyaluronic acid. Interestingly, binding of imm3 and imm5 to hyaluronic acid — but not heparin — is significantly increased by the C-terminal tripeptide XVD. An excess of any of the three unlabelled GAGs tested — hyaluronan (HA), chondroitin sulfate (CS) or heparin (HP) — can compete successfully against immX3AVD immodulin peptide binding to FITC-hyaluronic acid. Fig. 1G shows that tripeptide-extended immodulins bind FITC-labelled human holo-transferrin, and this interaction is disrupted weakly by excess hyaluronic acid, but strongly by excess heparin or PIP2. Moreover, at the low pH (5.2) typically found in late endosomes, peptide binding of

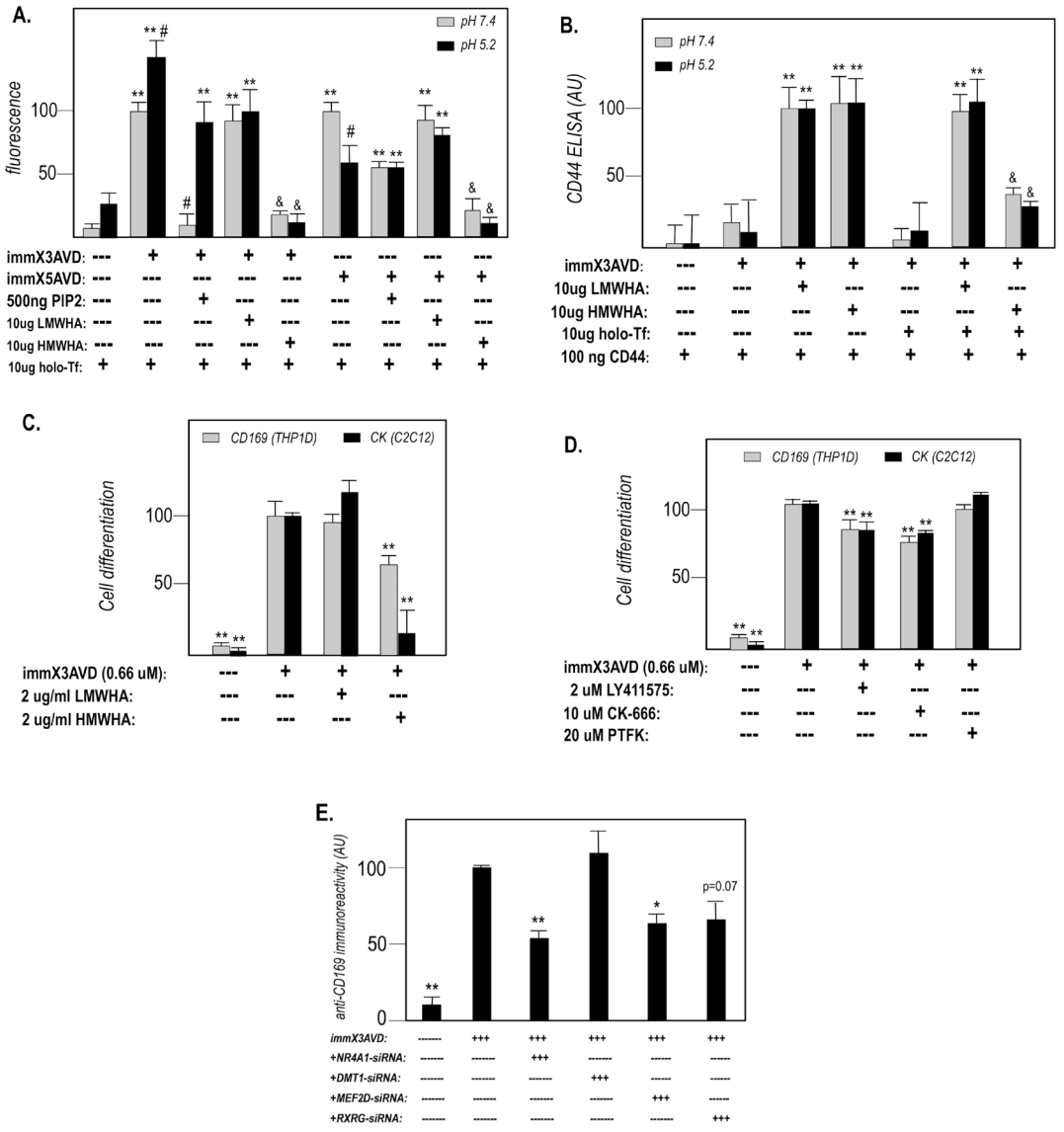

Fig. 3 *Mechanistic aspects of immodulin action. Holo-Tf=holotransferrin; AU=arbitrary ELISA units; [A] FITC-hyaluronan binding to holotransferrin::immodulin peptide complex at pH 7.4 and 5.2. ** p<0.01 vs no peptide control; & p<0.01 for LMWHA-HMWHA comparison; # p<0.01 for pH 7.4-5.2 comparison; [B] CD44 (100 ng/well) binding was detected with an anti-CD44 monoclonal antibody and developed using horseradish peroxidase/TMB detection; peptide+HMWHA pH7.4 control=100; ** p<0.01 vs peptide control; & p<0.01 for LMWHA-HMWHA comparison; [C] and [D] Differentiation markers for polarized THP1D macrophages (CD169 AU) or C2C12 myogenesis (creatine kinase/mg protein). ** p<0.01 vs Peptide+holo-Tf in panel C and peptide alone in panel D (each set to =100). [E] Anti-NR4A1 and anti-MEF2D siRNAs (1 nM) inhibit stimulation of CD169 marker in THP1D cells by 0.33 uM peptide 8. ** p<0.01 vs peptide 8 (immX3AVD) plus 1 nM control siRNA.*

FITC-HA is stronger in the presence of ferric than ferrous iron. Compared to the physiological pH 7.4, binding of immX3AVD to FITC-HA is significantly stimulated at pH 5.2, but FITC-heparin binds less strongly at that pH (panel 1H). Additionally, at pH 5.2 (but not 7.4) 25X excess cold HA competes with FITC-HA binding to peptide, but 25X excess cold holotransferrin stimulates it, suggesting a synergistic effect. Thus, complex interactions control ligand binding to 3/5/6 immodulin peptides, making these peptides sensitive to environmental cues.

Immodulins drive differentiation of THP1D-derived macrophages into novel lineages.

Polarization studies using the human THP1D monocyte reporter cell line were performed as described in

Methods. The results (Fig. 2, top) show that nanomolar concentrations of 3/5/6/ immodulin peptides — and especially their C-extended derivatives — strongly induced novel cell surface markers of differentiation CD169+, Clec9a+ and Clec12a+ as well as CCL22 in cell culture supernatants. As a control, polarization of macrophages with M1 (interferon-gamma + LPS) or M2 (IL-4) protocols does not generate CD169+ macrophages above background levels. Hypoxia-mimic cobalt chloride and L-lactate, a key interstitial signaling molecule (but not fumarate, succinate and acetate at identical concentrations) stimulate CD169+ macrophage formation. C-terminal tripeptide extensions to 3/5/6 (but not 2/4) immodulins increase CD169+ macrophage stimulation (Fig. 2, middle panel).

Effects of chemical modulators on peptide-driven CD169+ differentiation

The THP1-Dual cell differentiation assay was carried out as described above, in the presence of various chemical modulators. The results are shown in Table 2. NR4A1 agonists C-DIM12, TMPA, cytosporone B and dihydroergotamine significantly increased CD169+ lineage generation from THP1D cells treated with the extended immodulin peptide immX3AVD, whereas C-DIM8, a known NR4A1 antagonist, significantly reduced it. Spironolactone (RXRγ agonist) increased, whereas HX531 (RXRγ inhibitor) decreased CD169+ macrophage generation. PKC inhibitor, bisindolylmaleimide IX, and cyclosporin A stimulated CD169+ macrophage generation, consistent with the known role of PKCbeta in nuclear retention of Nur77 and of cyclosporin A in Nur77 transcription.[23] C646 and IQ1 (EP300 inhibitors) and CPI-637 (an inhibitor of the EP300/CREB-binding protein complex) significantly reduced CD169+ generation, consistent with a central role for EP300 histone acetyl transferase. The above data, taken together, suggest that CD169+ differentiation depends on a signaling cascade involving EP300, Nur77 and RXR, and further suggests heterodimerization of Nur77 with the RXRγ isoform. Interestingly, toll-like receptor and STING signaling appear to modulate CD169 marker stimulation.

Generation of Tregs by CD169+ macrophages

As indicated below, CD169+, Clec12a+ and Clec9a+ monocyte-derived lineages are thought to be specialized antigen cross-presenting cell types that promote immune tolerance in cell death environments. Lack of pro-inflammatory consequences is thought to be a feature of CD169+ cross-presentation. GAD65-specific T-cells (i.e. CD4+ and CD8+ T-cells pre-selected for specific activation using GAD65 antigen) were used in a co-culture system to explore GAD65-specific presentation for a tolerization outcome, as opposed to activation.[13-15] THP1D cells were treated with PMA for 24 hours in 96-well plates and then exposed to 1 ug/ml GAD65 peptide immunogen in the presence of immX3AVD, immX3dSdVdD or immX5AVD peptide (0.66 uM) for an additional 24 hours. The peptides stimulated CCL22, IL10 and TGFbeta in supernatants (Fig.2 bottom panel). Cells were washed twice in PBS buffer and ~10e5 cells of pre-selected T-cells specific to the GAD65 antigen (Cellero LLC, Memphis, TN) were added to each well in RPMI-1640 medium. Incubation proceeded for an additional 96 hours, at which time cell extracts were assayed by ELISA for human FoxP3, a classical marker for Tregs. Both immX3AVD and immX3dSdVdD (but not immX5AVD) strongly stimulated Treg marker FoxP3.

Immodulin peptides stimulate myogenesis.

C2C12 myoblast cells (ATCC, Manassas, VA) in DMEM medium containing 2% horse serum were treated with immX3AVD peptide (0.66 uM) for four days, in the presence or absence of the indicated PPAR and LXR agonists (2 uM). immX3AVD peptide significantly stimulates myotube formation and creatine kinase activity in cell extracts, an activity further stimulated by agonists for PPAR-alpha, PPAR–delta, TR-beta, FXR and AMK, but diminished by LXR agonist GW3965 (Fig. 2, bottom panel).

Mechanistic insights

Fig. 3E confirms the importance of Nur77/NR4A1 and MEF2D function in the THP1D assay. The involvement of MEF2D in Nur77 transcription in muscle cells has been previously documented.[23] A trend to inhibition was also noted for anti-RXRγ siRNA, but it did not reach significance (p=0.07). Interestingly, anti-DMT1 siRNA did not inhibit at all, confirming mechanistic independence from the classical iron-uptake pathway [27]. At pH 7.4 (but not 5.2) PIP2 competes effectively against FITC-HMWHA for binding to holotransferrin-bound immX3AVD. The highly similar peptide immX5AVD exhibits different behavior (Fig. 3A). In both cases, excess cold HMWHA (but not LMWHA) successfully competes for binding to peptide. This is consistent with PIP2-modulated transfer of iron-bound immodulin from a transferrin carrier to a HMWHA (but not LMWHA) glycan carrier at the plasma membrane. Furthermore, Figure 3B shows that peptide::holotransferrin complex (but not peptide alone) binding to HMWHA::CD44 complex is preferentially competed with excess cold HMWHA, but not LMWHA. These results, taken together, are consistent with selective targeting of the holotransferrin carrier-bound immodulin peptide immX3AVD — and, by extension, bound iron — to HMWHA:CD44 complexes at PIP2-rich membrane domains, or in endosomes originating at such domains. Further support for this concept comes from the differential competitive effect of excess HMWHA (but not LMWHA) in cell culture assays of CD169+ macrophage differentiation and myogenesis (Fig. 3C). Moreover, Fig. 3D shows that both macrophage polarization and myogenesis are

significantly compromised by LY411575, an inhibitor of γ-secretase, and by CK-666, an inhibitor of Arp2/3 actin complexes.

Discussion

Mesenchymal cell-macrophage crosstalk is believed to modulate musculoskeletal homeostasis.[13] This study demonstrates that nanomolar concentrations of immodulin peptides are sufficient to stimulate myogenesis in C2C12 myoblasts and tolerogenic CD169+, Clec9a+ and Clec12a+ lineages in PMA-differentiated THP1D human macrophages. This is a rare demonstration of a small "natural" peptide domain driving differentiation processes in distinct cell types in concert, possibly revealing a natural link between musculoskeletal tissue regeneration and immune homeostasis. In addition, the relevance of 3/5/6 clade immodulins to immune self-tolerance in general, and Tregs in particular, is suggested by their ability to stimulate FoxP3 in T-cells in an immunogen-specific manner, and by the abundant production of CCL22, IL-10 and TGFbeta by immodulin-differentiated CD169+ macrophages, as shown in this study. CD169+ macrophages have a strong capacity to phagocytose apoptotic cells in the process of restoring immune homeostasis by secreting CCL22 whilst presenting antigens and recruiting Tregs through the CCL22 CCR4 axis, thereby inducing immune tolerance.[14,15] Similarly, Clec12a recognizes uric acid crystals formed during cell death and has been implicated in the downregulation of ROS, cytokines and NK cytotoxicity.[16] Another Dectin-class receptor, Clec9a, binds to F-actin exposed by dead cell debris and downregulates neutrophil recruitment.[17] Monocyte-derived efferocytotic phagocytes are endowed with high competence to cross-present external (engulfed) antigens from dead cells on MHC-I molecules to CD8+ T-cells. Such exceptional forms of antigen presentation are thought to lie at the crossroads of immune and tolerogenic outcomes.[18]

Macrophage functions (such as non-inflammatory efferocytosis) are associated with actin-branching Arp2/3 complex-dependent cytoarchitectures and, in polarized mesenchymal cells, PIP2 colocalizes spatially and temporally with membrane domains containing pericellular hyaluronan nets and hyaluronan receptor CD44-bound cytoplasmic actin adaptors such as ezrin [19]. In other contexts, γ-secretase-mediated cleavage of CD44 followed by release and nuclear transport of its intracellular domain (CD44-ICD) can trigger transcription of MMP-9, a modifier of extracellular matrix in inflammatory contexts.[20] Molecular size variants of hyaluronan, HMWHA and LMWHA, both of which bind CD44, have been associated with anti-inflammatory and pro-inflammatory contexts, respectively.[21,22] Taken together, these established observations help understand the highly specific binding patterns of immodulins to ECM ligands demonstrated in this study. The iron-binding immX3AVD immodulin peptide binds HMWHA preferentially over LMWHA in the presence of holotransferrin. The peptide is dislodged from holotransferrin by PIP2, which is abundant in the preferred membrane environments for internalization of HMWHA, a fact that may facilitate assembly of iron-glycan-CD44 complexes in mesenchymal stem cells,[7] while also possibly facilitating increased nuclear transport of immodulin-iron-glycan-CD44 complexes into the nucleus by virtue of the peptide's nuclear localization signal. Taken together, iron transport via this proposed model could simultaneously drive the differentiation of novel, "tolerizing" monocyte-derived subclasses via an EP300/RXRγ/Nur77-dependent transcriptional process, while also stimulating myogenesis in myoblast precursors using the aforementioned iron-glycan-driven mechanism.[7]

Through the use of siRNA and specific chemical inhibitors, the underlying mechanism for CD169+ macrophage differentiation has been confirmed to be EP300- and Nur77-dependent. As shown here, in vitro binding of immodulins 3/5/6 to RXRγ is strongest and the RXRbeta isoform does not bind at all. Moreover, the RXRγ agonist, spironolactone, sigificantly stimulates the CD169 marker in cultured macrophages, whereas a number of pan-RXR agonists fail to do so. The stimulating effect of the PKCbeta inhibitor, bisindolylmaleimide IX, is also corroborative for a central role of Nur77 in the underlying mechanism. PKC inhibition prevents nuclear export of Nur77 and pro-apoptotic engagement of Nur77 with mitochondria.[23] Nur77/NR4A1 agonists (C-DIM12, cytosporone B, TMPA and dehydroergotamine), but not agonists of either NR4A2 or NR4A3 (cilostazol, 6-mercaptopurine), enhance CD169 marker generation, whereas C-DIM8, a Nur77 inhibitor, reduces it. Nur77 has been suggested to play a role in musculoskeletal differentiation.[11] In the present study, anti-MEF2D and anti-NR4A1 siRNAs each inhibit immodulin-stimulated CD169+ macrophage differentiation. In an EP300-dependent process in muscle cell differentiation, Nur77/NR4A1 gene expression is driven by an EP300/MEF2 complex.[23] Direct inhibitors of EP300/CBP (C646, IQ-1, CPI-637) inhibit, whereas chemical inhibitors that restrict alternative EP300 binding partners such as NFAT (cyclosporin A) enhance differentiation.

D-amino acid XVD tripeptides added as C-terminal extensions to immodulins may aid the ability of these peptides to bind iron and withstand endoprotease digestion.[24] As shown here, extended immodulins stimulate CD169+ macrophage formation more strongly, albeit under the influence of ECM cues — as shown by the enhancing effects of hypoxia-mimic cobalt chloride and of lactate, a key metabolite of glycolysis. Remarkably, immX3AVD peptide binds hyaluronan more strongly at pH 5.2 than at pH 7.4, whereas heparin (an inhibitor of CD169 marker generation in the THP-1D assay) is bound less strongly

at low pH. Remarkably, these effects are all ferric iron-dependent.

CD169+ macrophages have previously been described. What is novel here is the ability to drive CD169+ differentiation in cell culture. Given current interest in the CD169+ macrophage lineage, the availability of this procedure may aid future mechanistic investigations. It is also quite surprising that immodulins should exhibit this particular pro-differentiation activity. Over seven thousand research papers have been published on the parental molecule, without ever raising suspicion about this particular IGF-independent activity.

It is a weakness of this study that only limited imaging data are presented. The conclusions reached in this study can be strengthened by imaging, and addressing this shortcoming will be an objective of future investigations.

In conclusion, immodulins drive the generation of novel tolerogenic CD169+, Clec9a+ and Clec12a+ macrophage lineages, accompanied by the generation of secreted chemokines such as CCL22, IL-10 and TGF-beta. Immodulins also stimulate myogenesis in myoblasts. Further study should address the question of whether the CD169+ macrophages described in this work can effectively present antigens to lymphocytes of the adaptive immune system in live animals, as they appear to do in the mixed culture experiments reported here; and whether the outcome of such presentation is tolerizing in vivo, as predicted by this study. A possible nexus of immodulins, CD44, glycoaminoglycans, transferrin and iron in a musculoskeletal environment affected by hypoxic insult, glycolytic acidification or inflammation raises intriguing questions, such as: how, in detail, are uptake and nuclear transport of immodulins managed in mesenchymal cells, given the diversity of endocytic pathways employed by iron-carrier systems in such contexts? And, what is the precise makeup of the chromatin complexes that activate transcriptional reprogramming in macrophages and mesenchymal cells in response to immodulins? These questions point to a vast mechanistic landscape for future studies to explore.

List of acronyms

BSA - bovine serum albumin
CCL22 – C-C motif chemokine 22
CD44 - CD44 antigen
CK - creatine kinase
DBD - DNA-binding domain
DMEM – Dulbecco's Modified Eagles Medium
ECM - extracellular matrix
ELISA – enzyme-linked immunosorbent assay
EM - extracellular matrix
FBS - fetal bovine serum
GAGs - glycosaminoglycans
HMWHA - high molecular weight hyaluronan
IGF - insulin-like growth factor
IGFBPs - insulin-like growth factor binding proteins
IL-10 – interleukin-10
IL-4 - interleukin-4
NR - nuclear receptor
NR4A - nuclear receptor 4A
Nur77 - nuclear receptor 4A1
PMA - phorbol 12-myristate 13-acetate
PPAR - peroxisome proliferator-activated receptor
RXR - retinoid X receptor
SD - standard deviation
TGFbeta – transforming growth factor beta
THP-1 – THP-1 cell line
Ybp – years before present

Contributions of Authors

The author confirms sole responsibility for study conception and design, project coordination, laboratory bench work, data collection, analysis and interpretation of results, and manuscript preparation. He has read and approved the final edited typescript.

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Conflict of Interest

The author declares that he has no conflicts of interest to report regarding the present study.

Ethical Publication Statement

I confirm that I have read the position of the Journal on issues related to ethical publication and I affirm that this report is consistent with those guidelines.

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