TOLL-LIKE AND ADENOSINE RECEPTOR EXPRESSION IN INJURED SKELETAL MUSCLE

GORDON L. WARREN, PhD,[1] TRACY HULDERMAN, BS,[2] ANGIE LISTON, BS,[2] and PETIA P. SIMEONOVA, MD, PhD[2]*

[1] Division of Physical Therapy, Georgia State University, P.O. Box 4019, Atlanta, Georgia 30302, USA
[2] Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

Accepted 7 December 2010

ABSTRACT: *Introduction:* Many aspects of skeletal muscle regeneration are now considered to be controlled by the innate immune system, specifically macrophages, but the mechanisms for activation and modulation of the innate immune system during injury are not well understood. *Methods:* We analyzed the expression of toll-like receptors (TLRs) and adenosine receptors during traumatic skeletal muscle injury. mRNA expression and immunostaining of these receptors were evaluated in mouse skeletal muscle injured by freezing. *Results:* Expression of nearly all mammalian TLRs was induced at 1 and/or 3 days postinjury with a common trend for higher expression at day 3. Injury also elicited a dramatic increase in the expression of adenosine receptors A_{2B} and A_3 but not A_1 and A_{2A}. *Conclusions:* Both receptor types may be potential targets for stimulation of skeletal muscle tissue regeneration and functional restoration after injury.

Muscle Nerve **44:** 85–92, 2011

Many aspects of skeletal muscle regeneration are now considered to be controlled by the innate immune system.[1] We and others have observed that reduction of monocyte/macrophage infiltration into injured muscle obstructs muscle regeneration as indicated by a delay in the appearance of regenerating myofibers and an increase in the occurrence of intramuscular adipocytes.[2–4] Macrophages are the phagocytic cells that clear tissue debris after tissue damage. Furthermore, increasing evidence has demonstrated that macrophages can enhance the replication and differentiation of muscle precursor cells.[5] Although macrophages are known as principal sources of mediators for tissue remodeling, including growth factors, the mechanisms for activating macrophages and the rest of the innate immune system during injury are not well understood.

An emerging theory is that the innate immune system during sterile inflammation, such as that occurring in most muscle injuries, may be activated through receptors similar to those that recognize components and products of microorganisms (i.e., pattern recognition receptors).[6] Toll-like receptors (TLRs), which function as pattern recognition receptors in mammals, are an evolutionarily conserved family of signal-transducing molecules that are thought to be critical for the induction of innate immunity. Some of these receptors (TLR1, 2, 4, 5, 6, and 11) are displayed on the surface of inflammatory cells, including macrophages, whereas others (TLR3, 7, 8, and 9) are associated with the membrane of intracellular organelles, such as endosomal vesicles.[7,8] The activation of TLRs induces a well-defined signal-transduction cascade through recruiting different adapter proteins. Subsequently, this can lead to activation of nuclear factor–κB (NF-κB), mitogen-activated protein kinase (MAPK), and other signaling pathways involved in control of inflammation.[9] In addition to TLR, monocyte/macrophage function can be shaped by signaling through adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3), which can sense a stressful environment and, accordingly, tune the function of the innate immune system.[10]

Minimal data exist regarding the involvement of TLRs or adenosine receptors in the tissue injury/repair process, especially for skeletal muscle. The purpose of our studies was to assess the expression of these receptors in skeletal muscle tissue after traumatic injury. Furthermore, we wished to test whether a specific agonist can be directly injected into the injured muscle for subsequent receptor activation. Our long-term goal is to determine whether TLRs and adenosine receptors can be therapeutic targets in the regeneration of skeletal muscle after traumatic injury.

METHODS

Animals. C57BL/6 mice (Jackson Laboratory), 8–10 weeks of age, were used in these studies. The animals were provided food and water ad libitum and maintained on a 12-h light/dark cycle. In preparation for muscle injury induction, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine administered intraperitoneally. Animal care and use procedures, including death by CO_2 asphyxiation, were conducted in

Abbreviations: A_1, adenosine A_1 receptor; A_{2A}, adenosine A_{2A} receptor; A_{2B}, adenosine A_{2B} receptor; A_3, adenosine A_3 receptor; cDNA, complementary deoxyribonucleic acid; C_T, threshold cycle, FITC, fluorescein isothiocyanate; HMGB1, high-mobility group B1; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor–kappaB; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT-PCR, reverse transcriptase–polymerase chain reaction; TA, tibialis anterior; Th, T-helper; TLR, toll-like receptor; VEGF, vascular endothelial growth factor

Key words: damage, inflammation, innate immune system, macrophages, regeneration

*Deceased December 16, 2009

Correspondence to: G. L. Warren; e-mail: gwarren@gsu.edu

Published online 12 April 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.22001

accordance with criteria outlined in the "PHS Policy on Humane Care and Use of Laboratory Animals" and *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, 1996); these procedures were approved by the National Institute for Safety and Occupational Health and the Georgia State University institutional animal care and use committees.

Induction of Freeze-Induced Muscle Injury. The procedure employed was identical to that described elsewhere.[11] In brief, a 1.5-cm-long incision was made through aseptically prepared skin overlying the left tibialis anterior (TA) muscle belly. Injury was induced by applying a steel probe cooled to the temperature of dry ice to the TA muscle belly for 10 s. The skin incision was then closed using silk suture followed by application of a liquid bandage (New-Skin; Medtech Products, Irvington, New York). In some histological analyses, the injured muscle was injected with 5 μl of 1.15 μg/μl fluorescein isothiocyanate (FITC)-labeled CpG (FITC ODN1668; InvivoGen, San Diego, California) prior to closure of the skin.

RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction. Upon dissection, TA muscles were immediately frozen in liquid nitrogen and stored at −80°C. The muscles were homogenized, and total RNA was extracted using a commercial kit (RNeasy; Qiagen, Valencia, California), following the manufacturer's protocol. cDNA was synthesized from 3 μg of RNA using Superscript II (Life Technologies, Gaithersburg, Maryland). Gene expression for TLRs was determined using 0.5 μg cDNA on a SYBR Green–based real-time polymerase chain reaction (PCR) array (PAMM-018; SABiosciences, Frederick, Maryland), as per the manufacturer's instructions. The array also includes members of the TLR signaling family as well as key mediators of the TLR signaling pathway, including genes that code main downstream proteins. The gene list is included in Table S1 (see Supplementary Material). Follow-up, confirmatory real-time PCR was performed on TLR2, TLR4, TLR6, TLR8, and TLR9 using TaqMan gene expression assays (Applied Biosystems, Carlsbad, California) [assay IDs of Mm00442346_m1 (*Tlr2*), Mm00445274_m1 (*Tlr4*), Mm02529782_s1 (*Tlr6*), Mm01157262_m1 (*Tlr8*), and Mm00446193_m1 (*Tlr9*)]. Adenosine receptor (A_1, A_{2A}, A_{2B}, and A_3) expression was also determined using TaqMan gene expression assays [assay IDs of Mm01308023_m1 (*Adora1*), Mm00802075_m1 (*Adora2a*), Mm00839292_m1 (*Adora2b*), and Mm00802076_m1 (*Adora3*), respectively]. All real-time PCR was run on a quantitative PCR system (Mx3000P; Stratagene, La Jolla, California). The comparative threshold cycle (C_T) method was used to calculate the relative concentrations. This method involved obtaining C_T values for the transcript of interest, normalizing to average of the two housekeeping genes, *Gapdh* and *Hsp90ab1*, and comparing the relative increases between control and experimental samples. Analyses were run on five muscle samples per combination of muscle type (injured vs. uninjured) and time postinjury (1 vs. 3 days).

Immunostaining. For histology and immunohistology, muscles were embedded in Tissue Tek OCT (Andwin Scientific; Addison, IL). The so-called optimal cutting temperature is approximately −20°C when in the cryostat but the tissue is actually embedded and frozen at −160°C (i.e., the temperature of melting isopentane), frozen in melting isopentane, and stored at −80°C. Using a microtome cryostat at −20°C, 10 cross-sections (10 μm thick) were cut at each of six levels equally spaced along the length of the TA muscle. Sections at each level were stained for routine hematoxylin–eosin or used for immunohistology. Rat monoclonal antibodies for Mac-1 (BD PharMingen, San Diego, California) and F4/80 (Abcam, Cambridge, UK), as well as rabbit polyclonal antibodies for TLR9 (Imgenex, San Diego, California), TLR2 (Imgenex), and A_{2B} (Millipore, Billerica, Massachusetts), were applied in an immunostaining protocol conducted on acetone-fixed cross-cryosections as described elsewhere.[12] The antibodies were diluted in 5% blocking serum to a 10-μg/ml final concentration and applied to the sections for 1 h at room temperature. Specific secondary antibodies conjugated to Cy3 or FITC (Jackson ImmunoResearch Labs, Weston Grove, Pennsylvania) were used in concentrations suggested by the manufacturer. Mounting medium containing DAPI was used for nuclear localization. A laser confocal microscope (LSM 510; Carl Zeiss, Inc., Thornwood, New York) was used to obtain images. Laser lines at 364, 488, and/or 543 nm were used depending on the fluorophore(s) in use. Control experiments without primary antibody demonstrated that the immunostaining signals observed were specific. All immunohistological evaluations were conducted on at least 4 mice per group.

Statistics. Differences in mRNA transcript levels between control and injured muscles at a given time after injury were analyzed using unpaired *t*-tests or Mann–Whitney *U*-tests when assumptions of normality or equal variance were violated. False discovery rate adjustments[13] were applied to the α level to control for type I error inflation due to the multiple comparisons. All statistical testing was conducted using SigmaStat, version 3.5 (Systat Software, San Jose, California), with an α level of 0.05, except as noted previously.

RESULTS

Using a real-time PCR array, it was determined that the expression of nearly all mammalian TLRs was induced at 1 and/or 3 days postinjury with a common trend for higher expression at day 3 (Table 1). This timing effect is consistent with the peak level of macrophage influx being observed to occur in TA muscle at 3 days postinjury.[14] The genes of the universal TLR adapter protein MyD88 and the related transcription factors of the NF-κB family were also upregulated in the injured TA muscle, demonstrating potential functional activities of the TLRs [Table 1, and Table S1 (Supplementary Material)]. The expression of select TLRs (2, 4, 6, 8, and 9) was analyzed using TaqMan RT-PCR (Fig. 1). These TLRs were found to be markedly upregulated in injured muscle compared with control muscle at 3 days postinjury; the fold increase was higher compared with the one assessed by the PCR array. The intracellular TLRs (8 and 9) were expressed at higher levels compared with the cell-surface TLRs (2, 4, and 6) in injured muscle.

The expression of adenosine receptors was characterized by TaqMan RT-PCR (Fig. 2). The injury elicited a dramatic increase in the expression of *Adora2b* and *Adora3* but not in *Adora1* and *Adora2a*. Similarly to the TLRs, adenosine receptor expression trended higher at 3 days compared with 1 day postinjury.

The protein levels of select TLRs and adenosine receptors were evaluated by immunostaining (Fig. 3). TLR9 protein was found at high levels in injured muscle at 3 days postinjury. Simultaneous immunostaining for both TLR9 and Mac-1 or F4/80, leukocyte or specific macrophage surface marker, respectively, indicated localization of TLR9 on macrophages surrounding the damaged myofibers (Fig. 3A and B). The uninjured part of the muscle did not exhibit TLR9 immunostaining (not shown). Immunostaining for adenosine receptor A_{2B} demonstrated a prominent positive response throughout the injured muscle but not in the uninjured area. A_{2B} protein was associated with damaged fibers and partially with leukocyte (macrophage) surface markers (Fig. 4).

To test the feasibility that specific TLR and/or adenosine receptor agonists/inhibitors could be used to modulate the regeneration processes in injured muscle through shaping macrophage activities, we injected 6 μg of FITC-labeled CpG, a TLR9 agonist, into injured TA muscle. As illustrated in Figure 5a, the CpG was still present in the muscle 1 day after injection, spread mainly throughout the injured area where it was injected. CpG fluorescence at this time-point could also be observed diffusely throughout the uninjured part of the muscle (Fig. 5b) and was also concentrated within leukocytes as indicated by its colocalization with Mac-1 (Fig. 5c–e).

DISCUSSION

To gain an understanding of the potential role of TLRs in skeletal muscle injury/repair mechanisms, we conducted transcription profiling of these receptors as well as related adapter and signaling molecules. The findings demonstrate that injured skeletal muscle expresses most members of the TLR family and many related molecules, such as Myd88, NF-κB, and interleukin-1β. The trend for this expression was to increase from 1 to 3 days postinjury, demonstrating a possible association with the regulation of macrophage function and tissue regeneration. We speculate that the general upregulation of the TLR family is beneficial in nature and not maladaptive. This upregulation potentially provides a primary or secondary mechanism for the necessary activation of macrophages after muscle injury. We have demonstrated that macrophages are essential for muscle regeneration and that macrophage depletion after injury results in delayed and impaired regeneration.[3]

Interestingly, the three most highly expressed TLRs after injury (*Tlr7*, *Tlr8*, and *Tlr9*) were ones that are intracellular in nature and whose traditionally described ligands are viral RNA or bacterial DNA. It is not clear how these TLRs may play a role in the activation of the innate immune system after muscle injury in which the skin remains intact. Although TLRs were discovered as molecules that sense microbial invasion of tissue and trigger the ensuing inflammatory response, accumulating evidence indicates that they also play an important role in sterile inflammation such as that occurring in damaged tissue when the overlying skin remains intact. Multiple molecules released from injured tissue, for example, hyaluronic acid, heparin sulfate, and high-mobility group B1 (HMGB1), can activate these receptors.[15,16] Our study has also demonstrated that specific small molecules, modulators of these receptors, such as CpG, a TLR9 agonist, can be injected directly into the injured muscle and remain localized there for at least 1 day after injury.

The regulation of immune responses, achieved by targeting TLRs, is one of the main goals in the development of new therapeutics for human immune or inflammatory diseases. For example, it has been demonstrated that synthetic TLR7 agonists (imiquimod and resiquimod), TLR9 agonists (CpG oligonucleotides), and TLR4 agonists (lipid A analogs) are efficacious as vaccine adjuvants.[6] It has been demonstrated paradoxically that asthma, an allergic inflammatory disease with a T-helper

Table 1. Gene expression of toll-like receptors and related proteins.

Gene name	Symbol	Fold change	
		1 day	3 days
Lymphocyte antigen 86	*Ly86*	7.62†	111†
Toll-like receptor 9	*Tlr9*	17.4†	75.7†
Toll-like receptor 7	*Tlr7*	3.75*	48.5†
Toll-like receptor 8	*Tlr8*	3.40†	40.2*
Chemokine (C–X–C motif) ligand 10	*Cxcl10*	13.3†	39.4†
Myeloid differentiation primary response gene 88	*Myd88*	7.81†	26.6†
Interleukin-6 receptor alpha	*Il6ra*	5.57†	22.3†
Bruton agammaglobulinemia tyrosine kinase	*Btk*	4.10*	16.1†
Interleukin-1 receptor, type I	*Il1r1*	3.97†	15.3†
Toll-like receptor 1	*Tlr1*	2.94*	13.4†
Interleukin-1 beta	*Il1β*	13.3†	8.03*
Peptidoglycan recognition protein 1	*Pglyrp1*	10.8†	10.7†
Chemokine (C–C motif) ligand 2	*Ccl2*	8.18*	10.2*
CD86 antigen	*Cd86*	3.21*	8.61†
Tumor necrosis factor receptor superfamily, member 1a	*Tnfrsf1α*	3.49*	7.07†
Eukaryotic translation initiation factor 2 alpha kinase 2	*Eif2ak2*	1.97*	6.86†
Nuclear factor of kappa light-chain gene enhancer in B-cells 1, p105	*Nfκb1*	2.02*	6.59†
Toll-like receptor adaptor molecule 1	*Ticam1*	1.68*	6.59†
Mitogen-activated protein kinase kinase kinase 1	*Map3k1*	1.33	6.39*
Toll-like receptor adaptor molecule 2	*Ticam2*	1.39	6.22†
Lymphocyte antigen 96	*Ly96*	1.51*	6.04†
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	*Nfkb2*	2.91*	6.03†
Interferon regulatory factor 1	*Irf1*	2.02*	5.80†
V-rel reticuloendotheliosis viral oncogene homolog A (avian)	*Rela*	3.23†	5.73†
Toll-like receptor 6	*Tlr6*	1.58	5.29†
Tumor necrosis factor, alpha-induced protein 3	*Tnfaip3*	2.21*	5.26*
TANK-binding kinase 1	*Tbk1*	1.77†	5.16†
CD14 antigen	*Cd14*	3.80*	4.86†
TNFRSF1A-associated via death domain	*Tradd*	2.05*	4.64†
Inhibitor of kappaB kinase beta	*Iκbkβ*	1.69*	4.53†
Reticuloendotheliosis oncogene	*Rel*	1.91*	4.18†
Toll-like receptor 5	*Tlr5*	2.27†	4.10†
CD80 antigen	*Cd80*	3.09*	4.06*
Toll-like receptor 4	*Tlr4*	2.38	3.95
Human immunodeficiency virus 1 Rev binding protein	*Hrb*	1.43*	3.85†
Mitogen-activated protein kinase 8 interacting protein 3	*Mapk8ip3*	1.42*	3.84†
Interleukin-10	*Il10*	1.43	3.71†
Tumor necrosis factor	*Tnf*	1.53	3.41*
Toll-like receptor 2	*Tlr2*	1.64	3.17†
Nuclear factor of kappa light-chain gene enhancer in B-cells inhibitor, alpha	*Nfκbiα*	1.90*	3.15†
Prostaglandin-endoperoxide synthase 2	*Ptgs2*	2.98*	1.52
Toll-like receptor 3	*Tlr3*	1.32	2.87*
Pellino 1	*Peli1*	1.41*	2.78†
Interleukin-6	*Il6*	2.69*	1.55
Interleukin-1 receptor–associated kinase 2	*Irak2*	1.88*	2.61*
Jun oncogene	*Jun*	1.24*	2.58†
CCAAT/enhancer binding protein (C/EBP), beta	*Cebpβ*	2.56*	2.14*
C-type lectin domain family 4, member e	*Clec4e*	2.24*	2.43*
Interferon regulatory factor 3	*Irf3*	1.73*	2.42*
Nuclear factor related to kappa B binding protein	*Nfrκb*	1.51†	2.41†
Heat shock protein 1A	*Hspa1a*	2.21*	0.89
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	*Nfκbil1*	1.23	2.21*
Interleukin-1 receptor–associated kinase 1	*Irak1*	0.93	2.10*
Toll-interleukin 1 receptor (TIR) domain–containing adapter protein	*Tirap*	1.37*	2.08†
ELK1, member of ETS oncogene family	*Elk1*	1.08	2.02*
Toll interacting protein	*Tollip*	1.71*	2.01†
Interleukin-12A	*Il12a*	0.49†	0.75
Peroxisome proliferator-activated receptor alpha	*Ppara*	0.34†	0.23†

TA muscles, obtained from mice at 1 and 3 days postinjury, were analyzed for mRNA transcripts using a SYBR green–based real-time PCR array. The expression was normalized to the average of Gapdh and Hsp90ab1 expression from the same samples and is presented as the fold change relative to uninjured muscle. Genes up- or downregulated by twofold at any time-point are included (the complete gene list is given in Table S1 in Supplemental Material).

**$P < 0.05$, but $P \geq$ false discovery rate–adjusted alpha-level.*

†$P <$ false discovery rate–adjusted alpha-level.

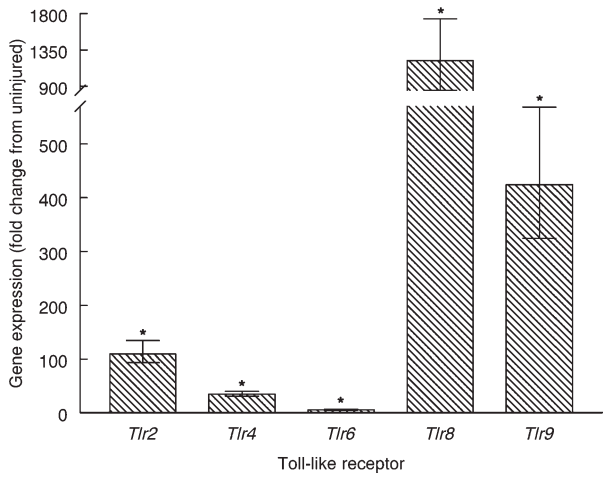

FIGURE 1. Gene expression of toll-like receptors 2, 4, 6, 8, and 9 in injured skeletal muscle. TA muscles, obtained from mice at 3 days postinjury, were analyzed for mRNA transcripts using real-time PCR. The expression was normalized to the average of *Gapdh* and *Hsp90ab1* expression from the same samples and presented as a fold increase above control (uninjured muscle). Values represent mean ± SE ($n = 5$). *Significantly different expression in injured muscle compared with uninjured muscle ($P <$ false discovery rate–adjusted α-level).

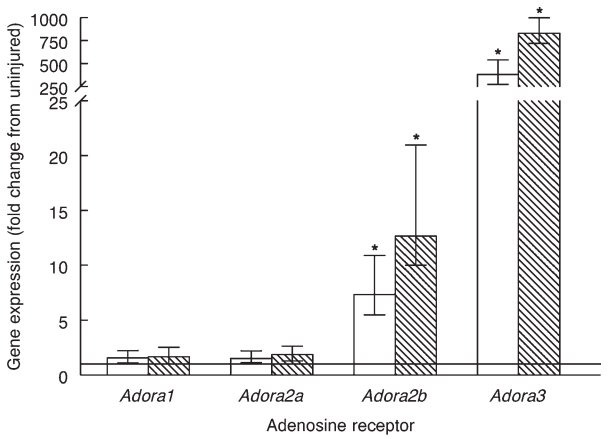

FIGURE 2. Gene expression of adenosine receptors in injured skeletal muscle. TA muscles, obtained from mice at 1 day (open) and 3 days (pattern) postinjury, were analyzed for mRNA transcripts using real-time PCR. The expression was normalized to the average of *Gapdh* and *Hsp90ab1* expression from the same samples and presented as a fold increase above control (uninjured muscle). Values represent mean ± SE ($n = 5$). *Significantly different expression in injured muscle compared with uninjured muscle ($P <$ false discovery rate–adjusted α-level).

type 2 (Th2-type) immune response, can be treated by TLR stimulation of the innate immune system.[6] Specifically, CpG, a TLR9 agonist, induces production of Th1-type cytokines, which normalizes Th-cell activity and prevents the symptoms of asthma. Minimal data exist regarding the role of these receptors in the tissue injury/repair process, especially for skeletal muscle. However, it has been reported recently that modulation of TLR by (1-3)-

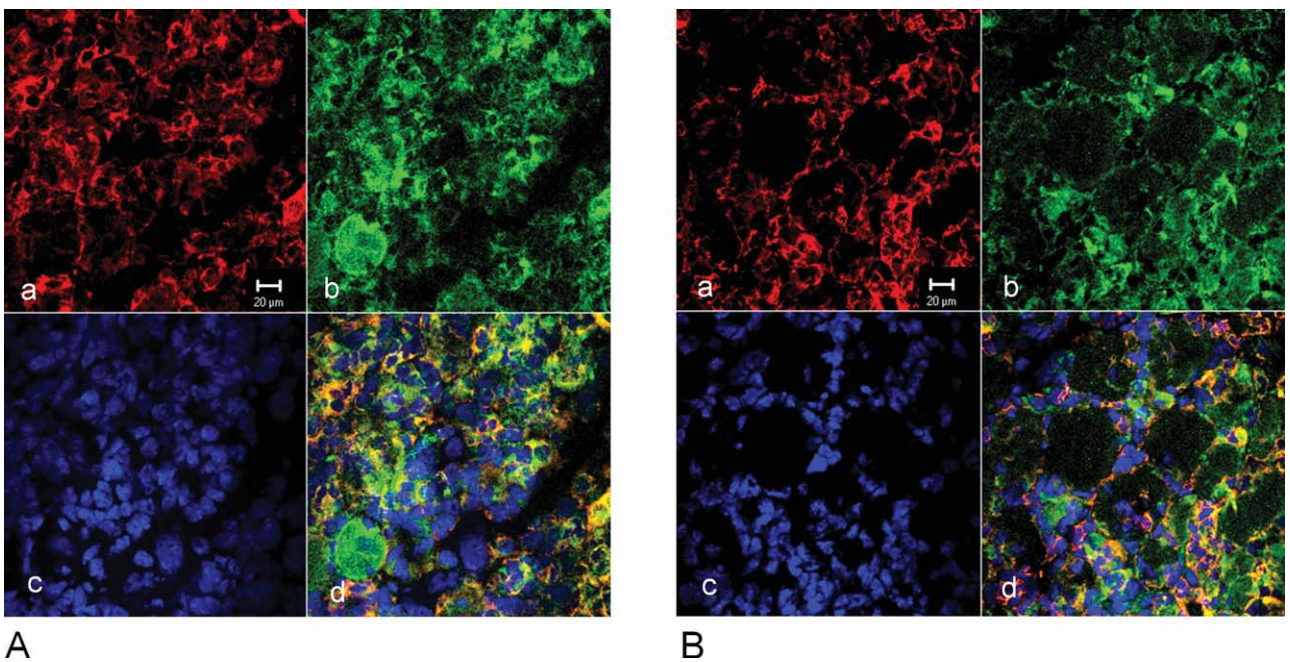

FIGURE 3. Immunofluorescence analysis of toll-like receptor 9, Mac-1, and F4/80 in TA muscle 3 days postinjury. Images are representative of those obtained in muscles from 4 C57BL/6 mice. Nuclei were stained with DAPI and appear blue. Original magnification: ×40; bars = 20 μm. Control experiments without primary antibody demonstrated that immunostaining signals observed were specific. (**A**) Toll-like receptor 9–F4/80 (a macrophage marker) localization: (**a**) image of F4/80 staining in red; (**b**) image of TLR9 staining in green; (**c**) image of DAPI staining; and (**d**) a merged image showing colocalization of TLR9 and F4/80, which produced a yellow–orange color. (**B**) Toll-like receptor 9–Mac-1 (a leukocyte marker) localization: (**a**) image of Mac-1 staining in red; (**b**) image of TLR9 staining in green; (**c**) image of DAPI staining; and (**d**) a merged image showing colocalization of TLR9 and Mac-1, which produced a yellow–orange color. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

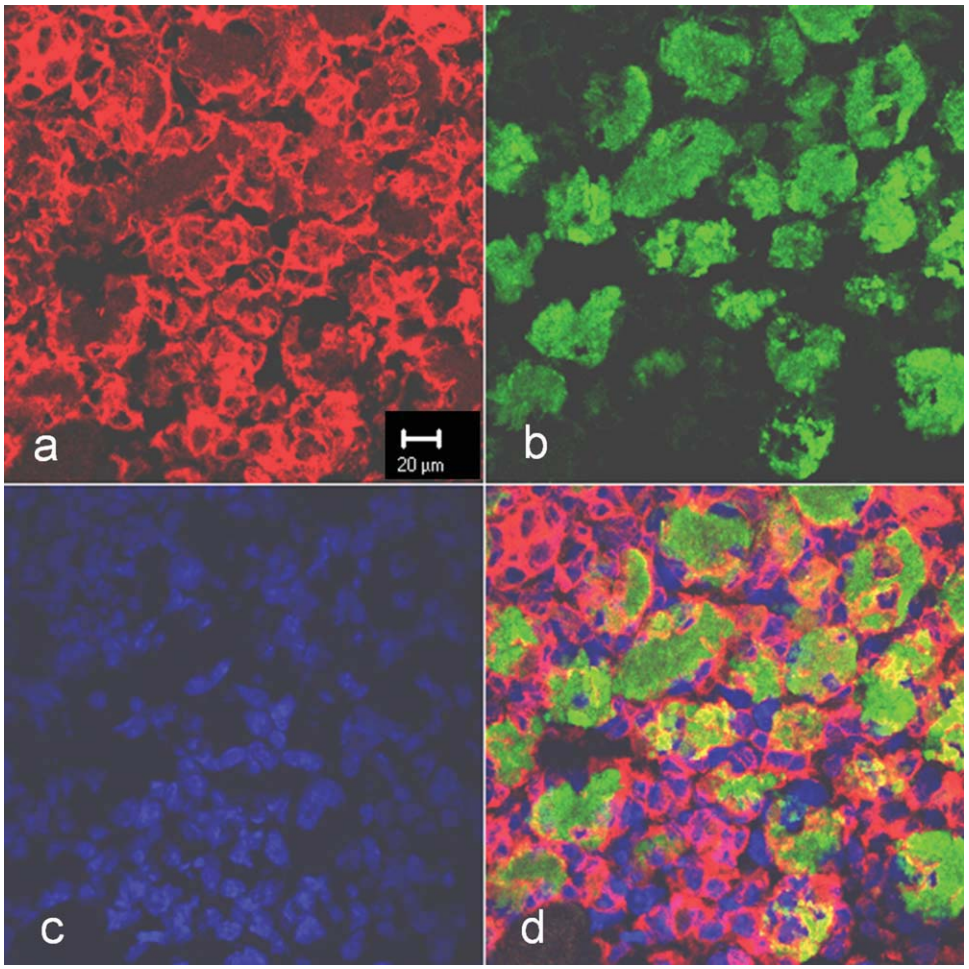

FIGURE 4. Immunofluorescence analysis of adenosine receptor 2b and Mac-1 in TA muscle 3 days postinjury. Images are representative of those obtained in muscles from 4 C57BL/6 mice. Nuclei were stained with DAPI and appear blue. Original magnification: ×40; bars = 20 μm. Control experiments without primary antibody demonstrated that immunostaining signals observed were specific. (**a**) Image of Mac-1 staining in red; (**b**) image of adenosine receptor 2b staining in green; (**c**) image of DAPI staining; and (**d**) a merged image showing colocalization of A_{2B} and Mac-1, which produced a yellow–orange color. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

β-D-glucan, a polymer of glucose, protects against myocardial ischemia–perfusion injury through shifting the TLR4-mediated MyD88 activation to PI3K/Akt pathway activation.[17]

In addition to TLRs, adenosine receptors are involved in regulation of the macrophage function and inflammation, mainly as anti-inflammatory molecules.[18] In our study, injured skeletal muscle was characterized by expression of *Adora2b* and *Adora3* at high levels. Adenosine is produced by many different tissues and cell types and is elevated in response to a variety of signals, including hypoxia and ischemia.[19] Agonists of A_{2A} can help balance the expression of inflammatory and anti-inflammatory cytokines in murine and human macrophages.[20] Lower concentrations of adenosine activate the high-affinity A_1, A_{2A}, and A_3 receptors, whereas high adenosine concentrations stimulate the low-affinity A_{2B} receptor.[10] Thus, the more pronounced cell distress is, the more adenosine should be released and the more A_{2B} receptors should be activated.[10] Muscle injury in this study induced significant expression of *Adora2b*, consistent with a massive destruction of the tissue. The role that A_{2B} plays in skeletal muscle injury and disease is not clear. Our finding of *Adora3* expression also at high levels in injured muscle is perplexing. This is one of the most enigmatic adenosine receptors.[21] As with cytoprotection of the heart, A_1, A_{2A}, and A_3 receptors have been implicated in protecting skeletal muscle from ischemia and reperfusion injury.[22] In contrast to A_1 and A_{2A} receptors, activation of A_3 is not associated with cardiac or hemodynamic depression, and thus the A_3 receptor has been suggested as a potential therapeutic target in ameliorating ischemia–reperfusion injury of skeletal muscle. Recently, administration of an A_3 agonist in mice has been found to reduce the blood level of creatine kinase, a marker of muscle injury, after an injurious bout of

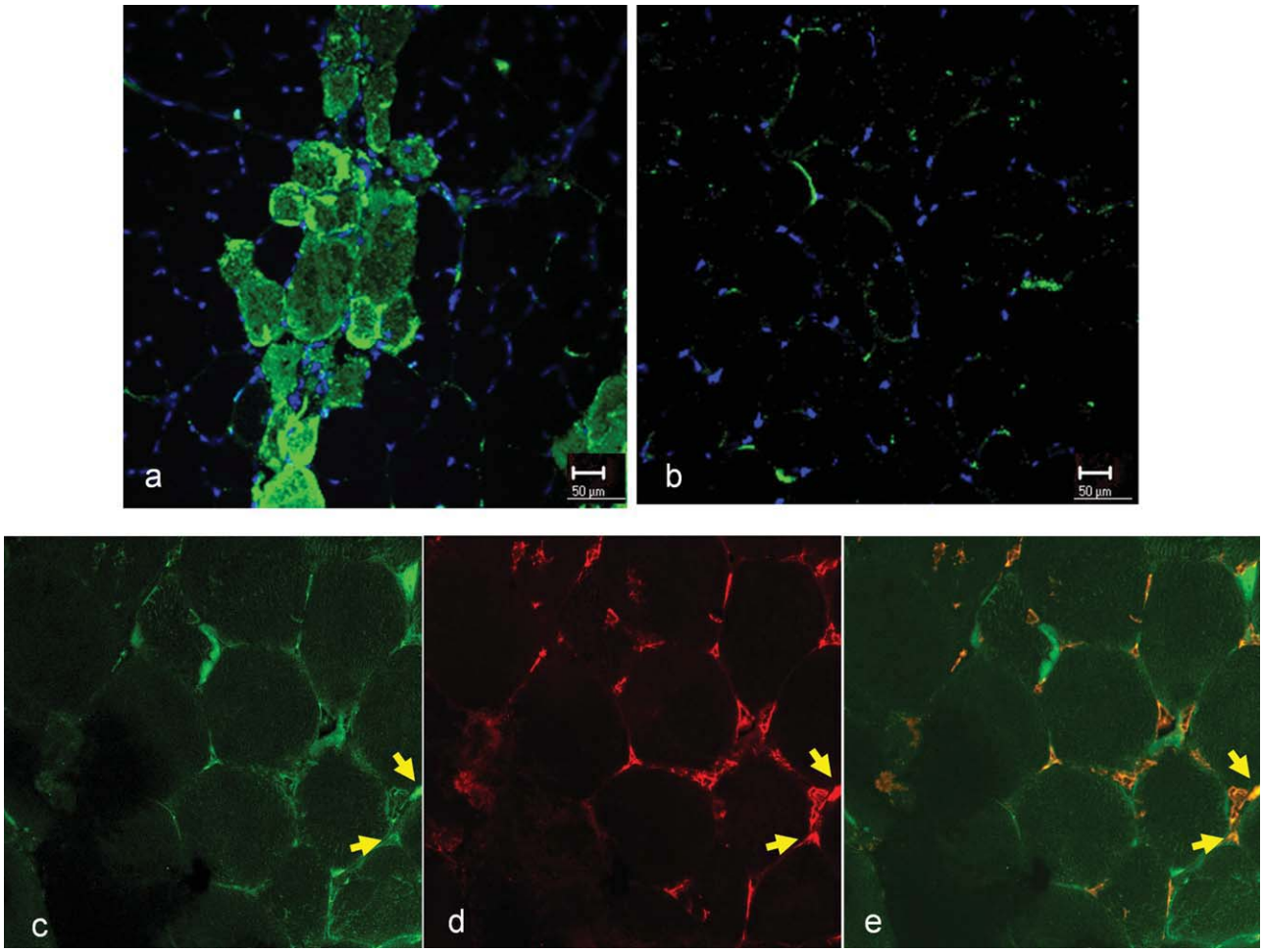

FIGURE 5. Distribution of CpG, a toll-like receptor 9 agonist, in TA muscle 1 day postinjury. CpG, labeled with FITC, was injected in the injured TA muscle (see text). Images are representative of those obtained in muscles from 4 C57BL/6 mice. Nuclei were stained with DAPI and appear blue. Original magnification: ×20; bars = 50 μm. (**a**) CpG in an injured portion of TA muscle; (**b**) CpG in an uninjured portion of TA muscle; (**c, d**) images of CpG (green) and Mac-1 (red) in a double-stained section; and (**e**) a merged image showing colocalization of CpG and Mac-1, which produced a yellow–orange color. Arrows indicate CpG localization and Mac-1–positive cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

downhill running.[23] The A_3 agonist's mechanism of action was presumed to be by an effect on the inflammatory response.

The administration of TLR and adenosine receptor agonists to injured skeletal muscle may be useful in modulating skeletal muscle regeneration, especially in conditions of delayed or incomplete recovery after injury. Interestingly, an interaction between TLRs and adenosine receptors in macrophage activation, resulting in reduced inflammation and stimulation of the angiogenic program, has been reported.[24,25] In these studies, it was demonstrated that vascular endothelial growth factor (VEGF) expression by murine macrophages is synergistically upregulated by agonists of TLRs 2, 4, 7, or 9 and an agonist acting through adenosine receptor A_{2A}. It has been suggested that the angiogenic phenotype of macrophages is important in wound healing and, indeed, activation of the A_{2A} receptor has been associated with enhanced skin wound healing.[26] Although different tissues and injury models may exhibit distinct expression patterns of TLRs and adenosine receptors as well as interactions between them, the expression of these receptors after skeletal muscle injury suggests that they may be potential targets for stimulation of skeletal muscle tissue regeneration and functional restoration after injury.

This research was supported in part by a grant from the Department of Defense/U.S. Army Institute of Surgical Research (W81XWH-07-1-0515).

REFERENCES

1. Tidball JG. Inflammatory processes in muscle injury and repair. Am J Physiol Regul Integr Comp Physiol 2005;288:R345–R353.
2. Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J Exp Med 2007;204:1057–1069.
3. Summan M, Warren GL, Mercer RR, Chapman R, Hulderman T, van Rooijen N, et al. Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. Am J Physiol Regul Integr Comp Physiol 2006;290:R1488–R1495.

4. Tidball JG, Wehling-Henricks M. Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. J Physiol 2007;578:327–336.
5. Chazaud B, Brigitte M, Yacoub-Youssef H, Arnold L, Gherardi R, Sonnet C, et al. Dual and beneficial roles of macrophages during skeletal muscle regeneration. Exerc Sport Sci Rev 2009;37:18–22.
6. Ulevitch RJ. Therapeutics targeting the innate immune system. Nat Rev Immunol 2004;4:512–520.
7. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–995.
8. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001;1:135–145.
9. Kawai T, Akira S. TLR signaling. Semin Immunol 2007;19:24–32.
10. Hasko G, Pacher P, Deitch EA, Vizi ES. Shaping of monocyte and macrophage function by adenosine receptors. Pharmacol Ther 2007; 113:264–275.
11. Warren GL, O'Farrell L, Summan M, Hulderman T, Mishra D, Luster MI, et al. Role of CC chemokines in skeletal muscle functional restoration after injury. Am J Physiol Cell Physiol 2004;286: C1031–C1036.
12. Warren GL, Hulderman T, Mishra D, Gao X, Millecchia L, O'Farrell L, et al. Chemokine receptor CCR2 involvement in skeletal muscle regeneration. FASEB J 2005;19:413–415.
13. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 1995;57:289–300.
14. Warren GL, Summan M, Gao X, Chapman R, Hulderman T, Simeonova PP. Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. J Physiol 2007;582: 825–841.
15. Akashi-Takamura S, Miyake K. TLR accessory molecules. Curr Opin Immunol 2008;20:420–425.
16. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. FASEB J 2006;20:9–22.
17. Li C, Ha T, Kelley J, Gao X, Qiu Y, Kao RL, et al. Modulating Toll-like receptor mediated signaling by (1→3)-beta-D-glucan rapidly induces cardioprotection. Cardiovasc Res 2004;61:538–547.
18. Gao ZG, Jacobson KA. Emerging adenosine receptor agonists. Expert Opin Emerg Drugs 2007;12:479–492.
19. Pang CY, Neligan P, Zhong A, He W, Xu H, Forrest CR. Effector mechanism of adenosine in acute ischemic preconditioning of skeletal muscle against infarction. Am J Physiol 1997;273:R887–R895.
20. Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM, Vizi ES. Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. J Immunol 1996;157:4634–4640.
21. Gessi S, Merighi S, Varani K, Leung E, MacLennan S, Borea PA. The A3 adenosine receptor: an enigmatic player in cell biology. Pharmacol Ther 2008;117:123–140.
22. Zheng J, Wang R, Zambraski E, Wu D, Jacobson KA, Liang BT. Protective roles of adenosine A1, A2A, and A3 receptors in skeletal muscle ischemia and reperfusion injury. Am J Physiol Heart Circ Physiol 2007;293:H3685–H3691.
23. Wang R, Urso ML, Zambraski EJ, Rader EP, Campbell KP, Liang BT. Adenosine A3 receptor stimulation induces protection of skeletal muscle from eccentric exercise-mediated injury. Am J Physiol Regul Comp Physiol 2010;299:R259–R267.
24. Leibovich SJ, Chen JF, Pinhal-Enfield G, Belem PC, Elson G, Rosania A, et al. Synergistic up-regulation of vascular endothelial growth factor expression in murine macrophages by adenosine A(2A) receptor agonists and endotoxin. Am J Pathol 2002;160:2231–2244.
25. Pinhal-Enfield G, Ramanathan M, Hasko G, Vogel SN, Salzman AL, Boons GJ, et al. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. Am J Pathol 2003;163:711–721.
26. Montesinos MC, Desai A, Chen JF, Yee H, Schwarzschild MA, Fink JS, et al. Adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A(2A) receptors. Am J Pathol 2002;160:2009–2018.