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# Selective Roles for Toll-Like Receptors 2, 4 and 9 in the Systemic Inflammation and Immune Dysfunction Following Peripheral Tissue Injury

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## Abstract

**Background—**Toll-like receptors (TLRs) detect endogenous ligands released after trauma and contribute to the proinflammatory response to injury. Post-traumatic mortality correlates with the extent of the immuno-inflammatory response to injury which is comprised of a complex regulation of innate and adaptive immune responses. Although TLRs are known to modulate innate immune

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responses, their role in the suppression of lymphocyte responses following traumatic tissue injury is unclear.

**Methods—**This study used a murine model of severe peripheral tissue injury, involving muscle crush injury and injection of fracture components, to evaluate the roles of TLR2, 4 and 9 in the early and delayed immuno-inflammatory phenotype. Post-traumatic immune dysfunction was measured in our trauma model using the following parameters: *ex-vivo* splenocyte proliferation, Th1 cytokine release and iNOS induction within splenic myeloid-derived suppressor cells (MDSC). Systemic inflammation and liver damage were determined by circulating interleukin-6 levels and hepatocellular injury.

**Results—**Suppression of splenocyte responses after injury was dependent on TLR4 and 9 signaling as was post-traumatic iNOS upregulation in splenic MDSC. TLR2 was found to have only a partial role through contribution to inhibition of splenocyte proliferation. This study also reveals the involvement of TLR2 and 4 in the initial systemic inflammatory response to traumatic tissue injury, however, this response was found to be TLR9-independent.

**Conclusions—**These findings demonstrate the previously unidentified role of TLR2, 4 and 9 in the T-cell associated immune dysfunction following traumatic tissue injury. Importantly, this study also illustrates that TLRs play differing and selective roles in both the initial proinflammatory response and adaptive immune response after trauma. Furthermore, the results in the TLR9-deficient mice establishes that the upregulation of early pro-inflammatory markers do not always correlate with the extent of sustained immune dysfunction. This suggests potential for targeted therapies that could limit the immune dysfunction through selective inhibition of receptor function following injury.

### Keywords

Injury; Toll-like receptor; lymphocyte; MDSC; immune dysfunction

## Background

Traumatic tissue injury releases endogenous ligands which serve as triggers for the immuno-inflammatory response following injury. Exaggerated and sustained levels of this post-traumatic response have been correlated with subsequent patient morbidity and mortality[1]. The early pro-inflammatory component of this response involves excessive activation of innate immune pathways whereas immune dysfunction is described as a depression in adaptive immunity and includes in part T-cell dysfunction[2].

Toll-like receptors (TLRs) are pattern-recognition receptors that were discovered based on their central role in the recognition of microbial molecules as part of host defense[3]. A subset of TLRs also recognize endogenous molecules and can trigger activation of inflammatory pathways in the setting of sterile inflammation[4,5,6]. In the setting of trauma TLR2[7], TLR4[8], and TLR9[9] have been shown to selectively participate in the pro-inflammatory response to hemorrhagic shock or tissue trauma. It is unknown if these same TLRs are involved in the regulation of pathways that lead to the sustained suppression of the immune responses, specifically lymphocyte responses, seen after trauma.

Here, we carried out experiments in a murine model of severe peripheral tissue injury to establish if TLR2, TLR4 and/or TLR9 contribute to either the early pro-inflammatory response or sustained immune dysfunction after injury. Our findings show that TLR4 is strongly involved in both components of the response. In contrast TLR2 was found to mediate systemic inflammation with little effect on immune dysfunction while TLR9 was shown to only contribute to the suppression of the adaptive T-cell function. Notably, our findings highlight a disconnect between the TLR-driven mechanisms and suggest a receptor-

specific separation of the components of the immuno-inflammatory response to sterile tissue injury.

## Methods

### Animal Care

This research protocol complied with the regulations regarding the care and use of experimental animals published by the National Institutes of Health, and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

All mice used in this study were male, aged 8–12 weeks. TLR4 knockout ($TLR4^{-/-}$), $TLR2^{-/-}$ and TLR9-mutant ($TLR9^{CpG1/CpG1}$) mice[23], all on a C57BL/6 background, were bred at the University of Pittsburgh and originally provided by Dr. R. Medzhitov (Howard Hughes Medical Institute, Yale University), Dr. J. Kolls (University of Pittsburgh) and Dr. B.Beutler (The Scripps Research Institute) respectively. Age-matched wild type (WT) C57BL/6 mice were obtained from Charles River Laboratories International (Wilmington, MA). TLR4-mutant mice (C3H/HeJ) and the corresponding WT strain (C3H/HeOuJ) were obtained from Jackson Laboratories (Bar Harbor,ME).

### Experimental Groups

The mice were randomly allocated to trauma or control groups. Control untraumatized mice were used to obtain physiologic baseline results. Prior to being euthanized under inhalational anesthesia, the control untraumatized mice receive no experimental manipulation and no other anesthesia. The traumatized mice underwent pseudofracture (PF), a peripheral tissue injury model under general anesthesia with pentobarbital sodium (70mg/kg, IP; Ovation Pharmaceuticals, Deerfield, IL) and with supplemental inhalational isoflurane (Abbott Laboratories, Chicago, IL) as needed. Buprenorphine (0.1mg/kg SC; Bedford Laboratories, Bedford, OH) was administered after recovery from anesthesia as an analgesic.

The pseudofracture model was performed as previously described[10,11]. In brief, soft tissue injury in the form of a muscle crush to both posterior thighs was followed by an injection into the crushed tissue of a suspension of pulverized bone. The bone suspension was prepared from both pulverized femurs and tibias of age- and weight-matched syngeneic donor mice resuspended in phosphate buffered saline (PBS). The animals were allowed full freedom after the procedure. Mortality of the procedure was 0%.

Mice that received control or inhibitory CpG oligodeoxynucleotides (100 μg) were injected subcutaneously both at time of and at 24hr after trauma.

### Reagents

Saponin was from Sigma-Aldrich Co. (St.Louis, MO); mouse Interleukin-6 (IL-6), IL-2, Interferon-γ (IFN-γ) enzyme-linked immunosorbent assay (ELISA) kits from R&D systems Inc. (Minneapolis, MN); RPMI 1640 and L-Glutamine from Lonza BioWhittaker (Walkersville, MD); heat-inactivated fetal bovine serum (FBS), non-essential amino acids (NEAA) and sodium pyruvate from Hyclone Lab., Fisher Scientific, (Logan, UT); penicillin-streptomycin (pen/strep) and 2-mercaptoethanol (2-ME) from Gibco, Life Technologies Corp., (Grand Island, NY); concanavalin A (Con A) from GE healthcare Corp. (Piscataway, NJ) and antiCD3ε mAb (clone 145-2C11), anti-CD16/CD32, BD cytofix/ cytoperm from BD Biosciences (San Jose, CA, USA). Fluorescently-labeled antibodies for flow cytometry were purchased from either eBiosciences (San Diego, CA) or BD Biosciences (San Jose, CA). Control CpG (ODN2088 Control; 5′-TCCTGAGCTTGAAGT –3prime;) and inhibitory CpG (ODN2088 miCpG; 5prime;-TCCTGGCGGGGAAGT

-3prime;) oligodeoxynucleotides were purchased from Fisher Scientific Eurofins MWG Operon (Huntsville, AL).

### Sample collection and preparation

Traumatized mice were euthanized 6, 12 or 48hr after trauma and compared with unmanipulated controls. At the conclusion of each experiment, animals were sacrificed with an overdose of isoflurane, then blood was collected by cardiac puncture and spleens were aseptically harvested.

Plasma was measured for levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with HESKA Dri-chem 4000 (Heska Loveland CO; slides from FUJIFILM Corp., Japan) analysis and IL-6 levels by ELISA, performed according to the manufacturer's instructions.

Single cell suspensions were prepared from spleens for both culture and flow cytometric analysis as previously described[12]. In brief, the separated splenocytes were resuspended in RPMI 1640 supplemented with 10%FBS, l-glutamine, pen/strep, NEAA, sodium pyruvate, 2-ME and cell viability of 90% was confirmed with trypan blue stain. Parallel cultures were carried out in 96-well round-bottom tissue-culture plates (BD Falcon, BD Biosciences (San Jose, CA) at 1 x $10^5$ viable cells/well and were stimulated with T-cell specific mitogens concanavlin A (2.5 μg/ml) or antiCD3ε mAb (1μg/ml) for 72hr at 37°C. Splenocyte culture supernatant was collected at 48hr for cytokine analysis. Tritiated thymidine (1μCi/well; Perkin Elmer Inc., Waltham, MA) was added for the last 18hr of culture. Proliferation was assessed through thymidine incorporation using a Topcount scintillation counter (Perkin Elmer Inc., Waltham, MA) and measured as counts per minute (cpm).

For flow cytometric analysis, splenocytes were incubated with anti-CD16/CD32 (Fc block) for 10min at 4°C. Cells were then stained for surface markers for 20min at 4°C with the following fluorescently labeled mAbs: anti-CD19, anti-CD90.2, anti-CD11b, anti-Gr-1. Cells were then incubated with BD cytofix/cytoperm for 20min at 4°C and washed with 0.1% saponin, stained with anti-iNOS (clone 6/iNOS/NOS Type II) for intracellular staining at room temperature for 1hr, washed with 0.1% saponin buffer and immediately analyzed. A Becton Dickinson LSR II flow cytometer was used for analysis of samples; FlowJo v.7.6.5 software (Tree Star Inc., Ashland, OR) was used for data analysis.

### Statistical analysis

Statistical significance ($p<0.05$) was assessed by Student's t-test and ANOVA analysis using SigmaPlot 11.0 software (Systat Software Inc., San Jose, California, USA). The results presented in the study are expressed as the mean ± standard error or mean (SEM).

## Results

### Peripheral tissue trauma elicits an early TLR4- and TLR2-dependent systemic inflammatory response with hepatocellular injury

The pseudofracture model (PF) was established and validated to simulate the severe peripheral tissue trauma seen with bilateral femur fracture[10]. However, because no bones are broken, the model permits the study of both early and delayed time points. We have previously shown that the early (6hr) systemic inflammatory response and remote organ injury in this model is TLR4-dependent[13]. Here, we tested whether TLR2 and TLR9, receptors known to be involved in the pro-inflammatory response to either hemorrhagic shock[9] or tissue trauma[7], are involved in the early responses in the PF model. Circulating IL-6 levels were used as a representative biomarker of the systemic inflammatory response

and hepatocellular injury as a marker of liver damage, as an example of one form of remote organ damage. We have previously shown that PF leads to a significant elevation of both IL-6 and ALT levels within the circulation at 6hr following injury in wild type C57Bl/6 mice[12,13], hence, this study uses the 6hr time point after PF to evaluate these markers. As shown in Figure 1, PF led to significant elevations in both IL-6 and ALT levels at 6hr, in comparison with uninjured controls. Also, as expected, this was dependent on TLR4 signaling as TLR4$^{-/-}$ mice exhibited minimal increases in IL-6 and ALT levels following PF. Injured TLR2$^{-/-}$ mice also exhibited minimal increases in IL-6 and ALT levels, while injured TLR9-mutant mice showed significantly elevated levels similar to those observed in WT mice after injury. Thus, the initial pro-inflammatory response following severe peripheral tissue injury is dependent on both TLR2 and TLR4, but independent of TLR9 signaling.

### Post trauma suppression in splenocyte function is TLR4 dependent

The capacity of splenocytes isolated from injured animals to respond to known mitogens is frequently used to assess immune dysfunction after trauma[2]. We have previously shown that PF leads to a profound suppression of splenocyte, specifically T-lymphocyte, proliferation and Th1 cytokine production in response to Con A and anti-CD3 antibody[12]. The nadir in the responses is reached at 48hr post injury[12], as such, this study uses this time point after PF to evaluate the splenocyte responses. To determine if TLR4 is also involved in the trauma-induced immune dysfunction, we examined splenocyte responses in global TLR4$^{-/-}$ mice, and mice with a mutant form of TLR4 that is defective in TLR4 signaling (TLR4-mutant (C3H/HeJ)). As shown in Figure 2, WT mouse strains, C57BL/6 (control for TLR4$^{-/-}$) and C3H/HeOuJ (control for TLR4-mutant) mice exhibited the expected suppression in splenocyte proliferation as well as IFN-γ and IL-2 release (in response to Con A) at 48hr after PF. In contrast, the TLR4$^{-/-}$ and TLR4-mutant strains exhibited no decrease in proliferation or cytokine production after PF. In fact, proliferation (TLR4-mutant) and IL-2 release (TLR4$^{-/-}$) was increased significantly after injury in the TLR4-deficient strains. These data provide evidence that TLR4 signaling is also involved in the altered splenocyte responses characteristic of trauma models.

### The role of TLR2 in depressed splenocyte proliferation after injury

To determine whether the role of TLR2 in the early post-traumatic response was associated with a role in late post-traumatic immune suppression we evaluated splenocyte responses from control and PF injured WT C57BL/6 and TLR2$^{-/-}$ mice. Unlike splenocytes from injured WT mice, splenocytes from injured TLR2$^{-/-}$ mice showed no decrease in proliferative capacity (Fig. 3A). However, cytokine release from stimulated splenocytes of injured TLR2$^{-/-}$mice was significantly depressed when compared to that of uninjured TLR2$^{-/-}$ mice, a finding similar to the WT C57BL/6 response (Fig. 3B,C). These findings identify a role for TLR2 in dysfunctional lymphocyte proliferation after peripheral tissue injury, but no role in lymphocyte Th1-cytokine release. Despite the involvement of TLR2 in the systemic inflammatory response to injury, a disconnect is identified here where only a partial role is observed in the late splenocyte dysfunction.

### TLR9-dependent immune dysfunction following peripheral tissue injury

Although TLR9 was not found to be involved in the early pro-inflammatory response in PF, we sought to determine if it had a role in the altered splenocyte responses following PF. Both injured and control WT (C57BL/6) and TLR9-mutant mice were sacrificed at 48hr after injury. Baseline values of splenocyte proliferation in TLR9-mutant mice were found to be similar to WT mice (Fig. 4). In contrast, the reduction in splenocyte proliferative activity seen in injured WT mice, was not found in TLR9-mutant mice at 48hr following PF.

Similarly, the post-traumatic suppression of splenocyte cytokine production seen in WT mice was not observed in injured TLR9-mutant mice.

To further confirm the role of TLR9 in depressed lymphocyte responses after trauma, we injected WT mice with either control or inhibitory CpG oligonucleotides to selectively block the activation of TLR9. Injured WT mice that received the control CpG showed the expected depression in splenocyte responses at 48hr following trauma. However, the WT mice that received inhibitory CpG-injections did not show the injury-induced depression of splenocyte responses. Collectively, these results show that TLR9 blockade attenuates depressed T lymphocyte responses induced by peripheral tissue trauma.

### A role for TLR4 and TLR9 signaling in the upregulation of iNOS within splenic Gr-1$^{hi}$-CD11b$^{hi}$ MDSC after trauma

An upregulation of iNOS following injury is well-described and we have previously shown the contribution of iNOS to the depressed lymphocyte responses after peripheral tissue injury[12]. To determine if TLRs contribute to iNOS expression after PF we evaluated the intracellular iNOS expression of the splenic Gr-1$^{hi}$-CD11b$^{hi}$ MDSC subset in TLR4$^{-/-}$, TLR2$^{-/-}$and TLR9-mutant mice. These MDSC are a cell type known to show iNOS upregulation at 12hr following trauma[12].

As expected the injured WT mice showed an upregulation of intracellular iNOS by flow cytometry at 12hr following trauma (Fig. 5). In parallel to these findings, splenic Gr-1$^{hi}$-CD11b$^{hi}$ MDSC from injured TLR2$^{-/-}$ mice showed a similar increase in iNOS expression. In contrast, TLR9-mutant mice did not show any change in iNOS expression in spleen cells at 12hr after injury in comparison with respective uninjured controls. However, splenic Gr-1$^{hi}$-CD11b$^{hi}$ MDSC subset from TLR4$^{-/-}$ mice actually showed reduced iNOS expression at the 12hr time point following PF in comparison with respective baseline controls. Overall, these data show an association between TLR4 and TLR9 signaling and the upregulation of iNOS in MDSC in the spleen after peripheral tissue trauma.

## Discussion

Traumatic injury induces an immuno-inflammatory response, the degree of which has been shown to correlate with subsequent morbidity and mortality[1]. Exaggerated responses are thought to contribute to multiple organ dysfunction and/or a state of immunosuppression[5]. The early pro-inflammatory component of this response involves excessive activation of innate immune pathways whereas immune dysfunction is described as a depression in adaptive immunity including T-cell dysfunction[2,14]. Here, we show that TLR2, 4 and 9 play selective roles in both the early pro-inflammatory and sustained immune dysfunction after extremity injury in mice.

Toll-like receptors (TLRs) are important pattern-recognition receptors that serve the function of detecting pathogens and initiating the immune response[3]. TLRs are expressed on many cell types and have been shown to both activate innate immunity and also modulate adaptive immunity[3]. In the setting of sterile tissue injury TLRs recognize endogenous ligands termed DAMPS (damage-associated molecular patterns) and initiate a proinflammatory response[2,4,5,6] as has been demonstrated after hemorrhagic shock, trauma and ischemia[15,16,17]. Nevertheless, the involvement of each of the TLRs in injury-induced immunosuppression remains unclear. Our study demonstrates that TLRs play differing and selective roles in both the systemic inflammatory response and in the adaptive immune response to sterile tissue trauma. For example, we have shown that TLR4 serves to mediate systemic inflammation, the suppression of T-cell proliferation, Th1-cytokine release and iNOS upregulation. In contrast, TLR2 has little effect on immune dysfunction despite its

role in mediating systemic inflammation. Quite the opposite, TLR9 has almost no effect on early systemic inflammatory responses to peripheral tissue trauma but can act to inhibit lymphocyte proliferation and Th1-cytokine release while having a minimal role in iNOS upregulation.

The role of TLRs in the early proinflammatory response to acute tissue injury has been well-recognized. Experimental models of hemorrhagic shock[8,15,18], femur fracture[16], tissue injury[13] and ischemia[17,19,20] have all shown evidence of TLR4 mediated danger signaling. Although the triggers of TLR4 signaling following trauma are continually under investigation, many different ligands including high mobility group box 1 (HMGB1)[4] have been identified. Studies using TLR2$^{-/-}$ mice indicate that TLR2 also contributes to trauma-induced responses as seen in pulmonary contusion[7] and burns[21]. Our findings also suggest a role for both TLR4 and TLR2 in the early proinflammatory response in our model of sterile tissue injury.

TLR9 is a receptor that has been shown to be solely responsible for the recognition of CpG DNA motifs, as TLR9-deficient and -mutant mice are unresponsive to CpG DNA[22,23]. Recent studies from our laboratory and others added TLR9 to the growing list of pattern recognition receptors involved in the hemorrhagic shock-induced pro-inflammatory response[9,24]. We therefore hypothesized that TLR9 blockade following PF would attenuate systemic hyperinflammation. Thus, unlike TLR4 which appears to contribute to the induction of inflammation both during ischemic insults and to tissue trauma, TLR9 appears to be only involved in the pro-inflammatory response to ischemia and shock. This may be the result of the release of DNA from mitochondria injured or rendered dysfunctional in the setting of ischemia[24]. It is interesting to find that TLR9 does contribute to the altered splenocyte responses after peripheral tissue injury. Our results do not identify the source of the TLR9 ligands, but the effect of the inhibitory oligonucleotides suggest that an endogenous source of DNA may be involved.

In this study we have identified a unique role for TLR4 and 9 and also a partial role for TLR2 signaling in suppressed splenocyte, specifically T-lymphocyte, responses following trauma. Multiple mechanisms have been implicated in TLR modulation of adaptive immune responses. It is well-established that TLR signaling indirectly modulates T-cell mediated adaptive immunity through antigen presenting cells (APC)[25,27,31] of the innate immune system. However TLRs have recently been shown to be functionally expressed on T-cells[28,29] resulting in modulation of T-cell activation directly by TLR ligands[26,30]. After trauma, a depression of the function of cells from both the innate and adaptive immune systems has been shown[2]. TLR involvement in innate cell dysfunction has been described[32,33, 34] and must also be considered due to significant contribution of these cells to the adaptive response. We expect that the mechanisms are specific to each receptor and may involve responses of several cell types.

Regulatory effects of TLRs on T-cell function have also been described through myeloid-derived suppressor cells (MDSC) in the context of murine tumor models[35,36,37]. We and others have shown MDSC to be implicated in depressed T-cell function after trauma[12,38]. We have previously established the contribution of iNOS-derived NO to T-cell dysfunction following peripheral tissue injury and identified iNOS-induction within a subpopulation of MDSCs. Interestingly, in this study, analysis of MDSC in this model has shown that TLR4 and TLR9 modulate the production of NO by upregulation of iNOS in MDSC suggesting that one mechanism for TLR4 and 9 mediated depression of splenocyte responses could be through iNOS upregulation in MDSC.

In summary, this study demonstrates the previously unidentified roles of TLR2, 4 and 9 in the T-cell mediated adaptive immune dysfunction following traumatic tissue injury. Taken in the context of previous studies demonstrating a role for TLRs in similar trauma-induced immunosuppression models, a paradigm emerges where several TLRs driven by diverse DAMPs are involved in the host immune imbalance to injury. Table 1 summarizes the relationships between the early pro-inflammatory response induced by peripheral tissue to the sustained depression in splenocyte responses. Whereas the two responses appear to be at least partially linked for TLR2 and 4, there is a clear disconnect for TLR9. Thus, it can be concluded that the early IL-6 elevations are not always proportional to the extent of sustained immune suppression. These results also suggest that targeted therapies could be developed to limit the immune dysfunction by selectively inhibiting receptor function at some interval after injury.

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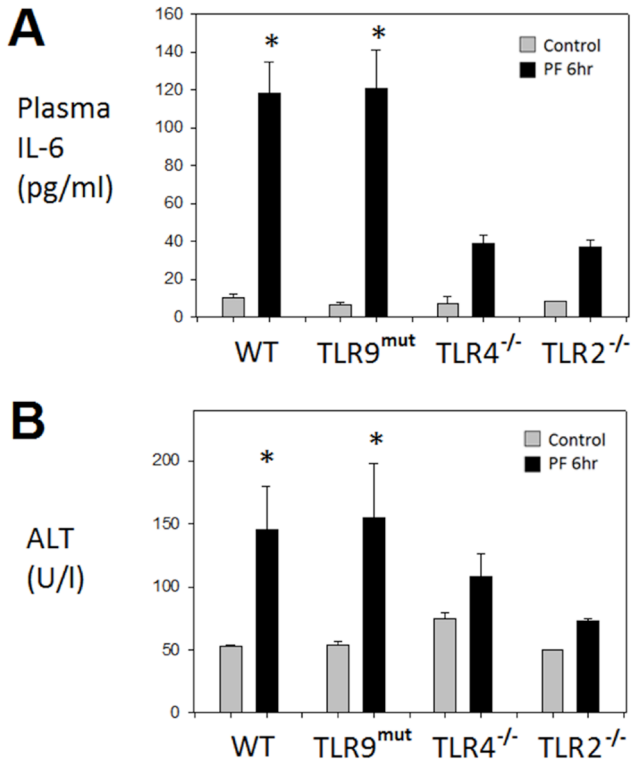


**Figure 1. Early systemic hyperinflammatory response to peripheral tissue trauma is TLR4-and TLR2-dependent, but TLR9-independent**

Plasma IL-6 (A) and plasma alanine aminotranferase (ALT) (B) in WT, TLR9-mutant, $TLR4^{-/-}$, $TLR2^{-/-}$ mice at 6hr following pseudofracture (PF) or in unmanipulated controls. Data shown as mean±SEM; * = $p < 0.05$ vs. respective control; n=4–9 mice/group at each time point; representative of 2 independent experiments.

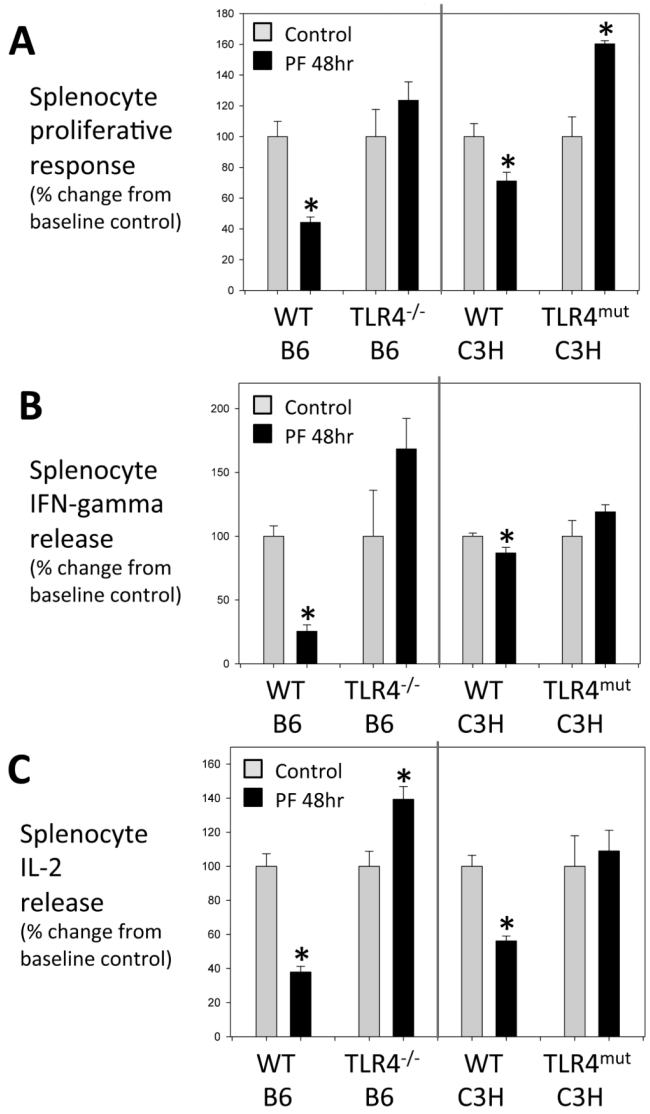


**Figure 2. TLR4-dependent post-traumatic lymphocyte dysfunction**

Splenocyte function at 48h after pseudofracture (PF) or in unmanipulated controls after concanavalin A (2.5μg/mL) stimulation: proliferation measured in counts per minute (cpm) of tritiated thymidine uptake (A), IFN- release (B) and IL-2 release (C). $TLR4^{-/-}$ mice compared to background C57BL/6 (WT B6) strain. TLR4-mutant mice (C3H/HeJ) compared to background strain C3H/HeOuJ (WT C3H). Data presented as percent change from baseline control. Data shown as mean±SEM; * = $p < 0.05$ vs. respective control; n=4–10 mice/group; representative of 2 independent experiments.

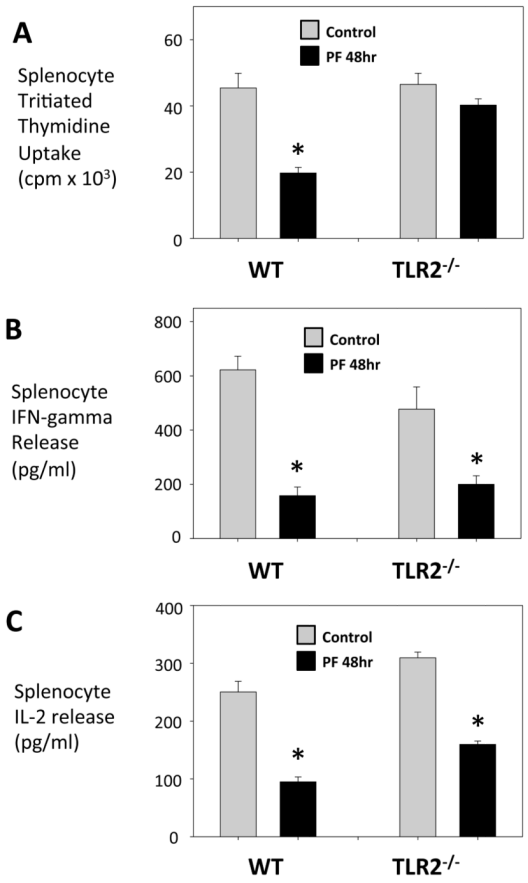


**Figure 3. A partial role for TLR2 in depressed lymphocyte responses after injury: splenocyte proliferation but not IFN-g, IL-2 release involves TLR2**

Splenocyte function in WT and TLR2$^{-/-}$ mice at 48h after pseudofracture (PF) or in unmanipulated controls after concanavalin A (2.5μg/ml) stimulation: proliferation measured in counts per minute (cpm) of tritiated thymidine uptake (A), IFN-g release (B) and IL-2 release (C). Data shown as mean±SEM; * = $p < 0.05$ vs. respective control; n=4–10 mice/group.

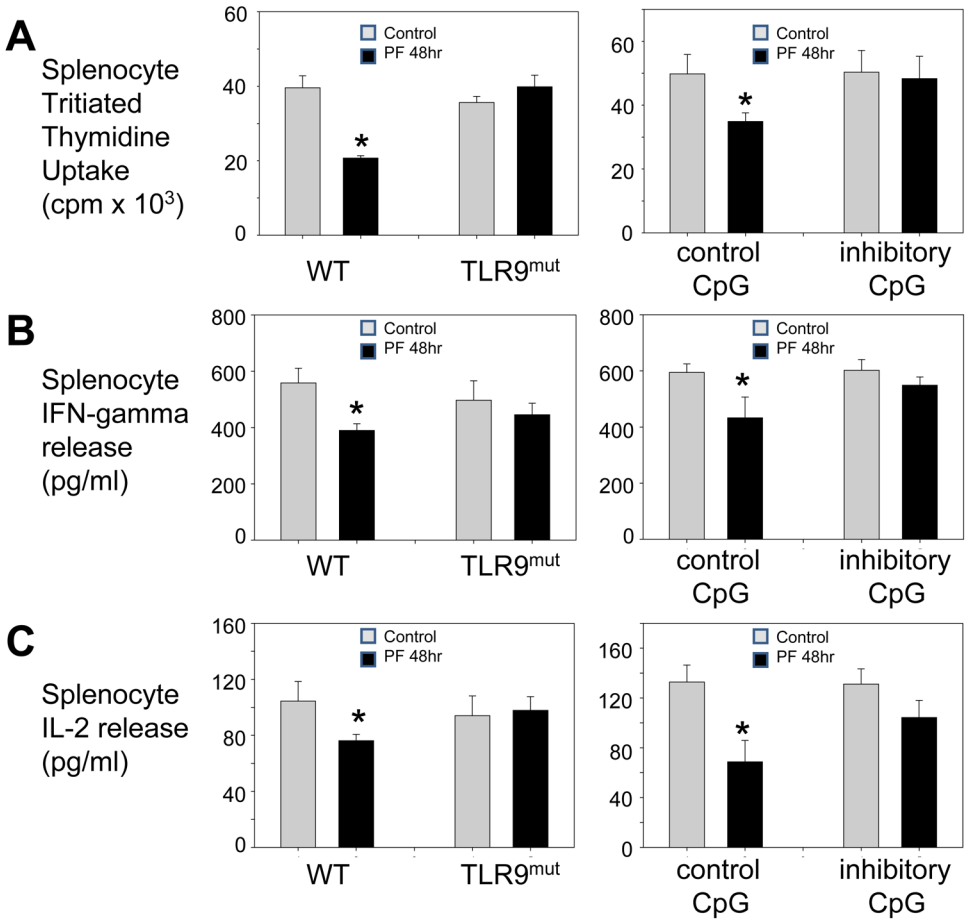


**Figure 4. Depressed lymphocyte responses following trauma are TLR9-dependent**

Splenocyte function of WT, TLR9-mutant and WT mice +CpG oligonucleotides at 48h after pseudofracture (PF) or in unmanipulated controls. Splenocyte responses after antiCD3e (1mg/mL) stimulation: proliferation measured in counts per minute (cpm) of tritiated thymidine uptake (A), IFN-g release (B), IL-2 release (C). WT mice that received control or inhibitory CpG oligodeoxynucleotides (100 μg) were injected subcutaneously both at time of and at 24h after trauma. Data shown as mean±SEM; * = $p < 0.05$ vs. respective control; n=6–8 mice/group; representative of 3 independent experiments.

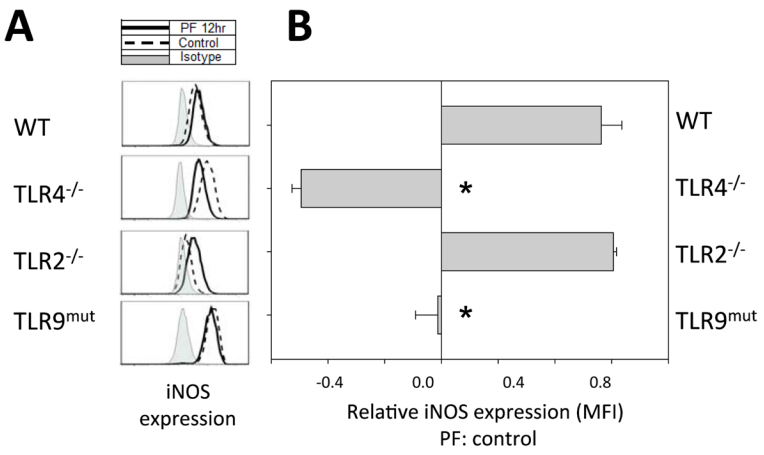


**Figure 5. iNOS induction in splenic Gr-1$^{hi}$-CD11b$^{hi}$ MDSC at 12h after trauma is TLR4-and TLR9-dependent**

(A) FACS analysis of intracellular iNOS expression in splenic Gr-1$^{hi}$-CD11b$^{hi}$ MDSC from WT, TLR4$^{-/-}$, TLR2$^{-/-}$ and TLR9-mutant mice at 12h following pseudofracture (PF) or in unmanipulated controls. Cells were gated through a CD19$^{+}$CD90.2$^{+}$ exclusion gate. (B) Relative change in mean fluorescence intensity (MFI) of iNOS expression at 12h after trauma in comparison to respective unmanipulated control. Data shown as mean±SEM; * = $p < 0.05$ vs. WT; n=2–3 mice/group; representative of 2 independent experiments.

**Table 1**

The comparative involvement of TLRs 4, 2 and 9 in the immuno-inflammatory response to peripheral tissue trauma.

| | TLR 4 | TLR 2 | TLR 9 |
|---|---|---|---|
| Early systemic inflammation | +++ | +++ | – |
| Late splenocyte dysfunction | +++ | + | +++ |