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RESEARCH ARTICLE

Adaptive Immunity

TLR4 Transactivates CD8$^+$ T Lymphocytes upon Acute Sterile Tissue Injury

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ABSTRACT

Acute major tissue injury induces immune dysregulation that is characterized by the development of systemic sterile inflammation and an increased risk for opportunistic infections. Although the contribution of the innate immune system has been examined in detail, research on the impact of acute sterile tissue damage on the T cell compartment remains limited. In the current study, we used a clinically relevant mouse model for traumatic skeletal muscle injury to investigate the impact of sterile tissue damage on diverse subpopulations of CD4$^+$ Th and CD8$^+$ cytotoxic T cells in systemic and local lymphoid organs. For the first time, to our knowledge, we provide evidence that injury selectively induced the expression of the activation marker CD69 on naive and central/virtual memory CD8$^+$ T cells in the lymph nodes but not in the spleen of male mice. CD4$^+$ Th cells remained unaffected in both organs. The activation of CD8$^+$ T cells was dependent on signaling through TLR4. Within a few hours, injury triggered the expression of IL-12 in the lymph nodes in a TLR4-dependent manner. Blocking of IL-12 prevented the activation of naive and central memory CD8$^+$ T cells after injury. Thus, early after traumatic tissue damage, TLR4 transactivates naive and central/virtual memory CD8$^+$ T cells through innate cytokines in local lymph nodes, where they might modulate forthcoming local immune responses. *ImmunoHorizons*, 2021, 5: 298–306.

INTRODUCTION

Upon tissue damage, damage-associated molecular patterns (DAMPs) are released and cause sterile inflammation via TLR signaling. Acute major tissue injury induced by traumatic or surgical stress causes a broad dysregulation of the immune system (1). Severely injured patients suffer not only from severe systemic inflammation that is associated with remote organ damage but also display an enhanced susceptibility to nosocomial infections.

The systemic activation of the innate immune system as the origin of hyperinflammation after major injury is well known. In contrast, less information exists on the impact of tissue injury on the T lymphocyte compartment. Because foreign Ags that usually drive T cell responses during infectious diseases lack in sterile inflammation, a classical T cell response is not expected after acute tissue injury. However, recent studies have shown that increased numbers of naive and memory CD4 and CD8 T cells circulate in patients' blood within the first hour after major trauma (2). During this hyperacute phase, the transcription of genes involved in T cell signaling pathways is reduced in circulating mononuclear cells (3). In mice, the phosphorylation of signaling molecules involved in TCR engagement increases within minutes in CD4$^+$ regulatory T cells (Treg) of skin-draining lymph nodes after burn injury (4). These findings suggest that T lymphocytes

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Abbreviations used in this article: DAMP, damage-associated molecular pattern; qPCR, quantitative PCR; T_{CM}, central memory T cell; $T_{Eff/EM}$, effector/effector memory T cell; T_n, naive T cell; Treg, regulatory T cell; T_{VM}, virtual memory T cell.

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receive so far unknown signals very early after tissue injury. We have previously shown that T cells in the lymph node that drain the site of traumatic skeletal muscle injury impair the adaptive immune response to foreign Ags, indicating that T cells undergo functional changes after injury. This suppressive activity of the T cells establishes within 24 h after injury and is independent of an enhanced frequency of $Foxp3^+$ Treg (5).

Activation of $CD4^+$ Th and $CD8^+$ cytotoxic T cells occurs when their TCR recognizes a specific Ag peptide presented via MHC class II or class I, respectively. The TCR-induced signaling cascade leads to the expression of the activation marker CD69 (6), initiates cell proliferation, and ultimately promotes the polarization of the T cells toward a distinct cytokine secretion pattern. Diverse T cell subpopulations may be distinguished according to their expression of CD62L and CD44. Naive T cells (T_n) that have not yet recognized their cognate Ag express CD62L but are weakly positive for CD44. The recently activated $CD62L^-CD44^{hi}$ effector/effector memory T cells ($T_{Eff/EM}$) express cytotoxic molecules and cytokines and are predominantly located in peripheral tissues. Central memory T cells (T_{CM}) are Ag-experienced T cells that mainly reside in lymph nodes and may be rapidly activated upon re-exposure to their cognate Ag (7). T_{CM} are characterized as $CD62L^+CD44^{hi}$. The complete process of Ag uptake/presentation by APCs and stimulation/polarization of specific T cells in the draining lymphoid organ usually requires up to seven days.

In addition, T cell activation may be induced by innate cytokines, such as IL-12, type I IFNs, and IL-18, and plays a role in immune defense early during bacterial infections (8). This alternative pathway of T cell activation is independent of the recognition of cognate Ag and is therefore much faster than the Ag-dependent counterpart (8). The most potent T cell subsets in Ag-independent activation are T_{CM} and virtual memory T cells (T_{VM}), a subset of T_{CM} that additionally express high levels of CD122, the common β-chain of the IL-2 and IL-15 receptors (9), and lack the expression of CD49d (10).

Considering the above-mentioned evidence of early T cell modulation after major injury, we hypothesize that any injury-induced signal would trigger the expression of the early activation marker CD69 on T cells. In this study, we used a clinically relevant murine model of traumatic skeletal muscle injury to analyze the expression of CD69 on diverse $CD4^+$ and $CD8^+$ T cell subpopulations as well as the cytokine expression pattern in the draining lymph node. We provide evidence that, within a few hours after skeletal muscle injury, signaling through TLR4 induces the expression of IL-12 in the draining lymph nodes and causes the activation of $CD8^+$ T_n, T_{CM}, and T_{VM} but not of $CD4^+$ T lymphocytes.

MATERIALS AND METHODS

Mice

Male 10- to 12-wk-old wild-type BALB/c mice were obtained from Janvier Labs (Saint Berthevin Cedex, France). Male VertX (IL-10 reporter mice) (11) breeding pairs were kindly provided by K. Couper, London School of Hygiene and Tropical Medicine, London, U.K., and $TLR4^{-/-}$ mice (12) breeding pairs were kindly provided by B. Ryffel, University of Orleans. On BALB/c background, they were bred in the local animal facility of the University Hospital Essen, Germany, and were used at the same age. All mice were kept under special pathogen–free conditions with standard rodent food and water ad libitum. Before onset of the experiments, all mice spent at least 1 wk in the local animal facility for acclimatization. The study implemented the ethical principles and guidelines for scientific experiments on animals of the Swiss Academy of Medical Sciences and were approved by the local Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia, Germany.

Induction of sterile skeletal muscle injury

Traumatic injury of both gastrocnemius muscles was induced as described previously (5). Briefly, after isoflurane inhalation (Forene; Abbott, Wiesbaden, Germany), ketamine (100 mg/kg; MEDISTAR, Ascheberg, Germany) and xylazine (10 mg/kg, Medistar, Ascheberg, Germany) were injected i.m. into the right fore limb. A 20-g weight was dropped from a height of 120 cm onto a cuboid (1 cm long × 2 cm wide × 1 cm high) that was placed on the gastrocnemius muscle. Animals with signs of unintended femur fracture according to clinical examination were excluded. Sham mice were anesthetized only. In some experiments, mice were treated with neutralizing Abs against IL-12p40 (500 μg/mouse; InVivoPlus anti-mouse IL12 p40, Bio X Cell, Lebanon, NH) or with the appropriate isotype control Ab 24 h before trauma or sham treatment. At different time points after trauma or sham treatment, mice were euthanized.

Preparation of lymph node and spleen cells

Popliteal lymph nodes were exposed and removed from surrounding tissue using anatomical tweezers. For quantitative PCR (qPCR) analyses, whole lymph nodes were immediately snap-frozen in liquid nitrogen. For flow cytometry, lymph nodes were carefully teased apart using two 23-gauge needles until all cells were released. Spleen cells were isolated using collagenase digestion followed by RBC lysis, as described before (13).

Cell culture

RPMI 1640 with stabilized glutamine (Biochrom, Berlin, Germany) supplemented with 10 mM HEPES (Biochrom, Berlin, Germany), 0.02 mg/ml gentamicin (Sigma-Aldrich, Taufkirchen, Germany), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany), and 10% FCS (Biochrom, Berlin, Germany) was used as cell culture medium.

$CD8^+$ T lymphocytes were purified from total spleen cells by negative selection using the CD8 T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), as recommended by the manufacturer. The purity of $CD8^+$ T cells was >95%, as

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determined by flow cytometry. $CD8^+$ T cells were seeded in 96-well plates (4×10^5 cells per well in 200 µl of cell culture medium) and were stimulated in the absence or presence of rIL-12 (20 ng/ml; R&D Systems, Wiesbaden, Germany) and IL-18 (20 ng/ml; MEDICAL & BIOLOGICAL LABORATORIES, Nagoya, Japan). After 18 h, the cells were harvested for flow cytometry.

Real-time qPCR

mRNA from frozen lymph nodes was isolated using the RNeasy Lipid Tissue Kit (QIAGEN, Hilden, Germany) in combination with the RNeasy Lipid Tissue Mini protocol. mRNA was reverse transcribed into cDNA using the Revert Aid H Minus First Strand cDNA Synthesis Kit from Thermo Fisher Scientific (Waltham, MA), according to the manufacturer's instructions. Real-time qPCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN, Hilden, Germany) and QuantiTect Primer Assay (QIAGEN) for IL-2, IL-6, IL-12p40, IL-15, IL-18, and GAPDH. Primers for IFN-α were 5′-ATGGCTAGGCTCTGTGCTTTCC-3′ (forward) and 5′-AGGGCTCTCCAGACTTCTGCTCTG-3′ (reverse). The $2^{-\Delta Ct}$ method (with ΔCt = Ct target − Ct housekeeping) was used to calculate the normalized expression of the target genes. GAPDH served as housekeeping gene.

Flow cytometry

Flow cytometry was performed as described previously (14). The following fluorochrome-labeled Abs were used for cell surface staining: anti-CD3ε (APC/Fire; clone 145-211), anti-CD4 (BV510; clone RM4-5), anti-CD8α (PerCp-Cy5.5; clone 53-6.7), anti-CD62L (PE-Cy7; clone MEL-14), anti-CD44 (AF488; clone IM7), anti-CD122 (PE; clone TM-β1), anti-CD69 (BV421; clone H1.2F3), anti–PD-1 (allophycocyanin; clone 129F.1A12), anti-CD49d (Alexa Fluor 647; clone R1-2). For intracellular staining of IFN-γ, GolgiPlug (BD Biosciences, Heidelberg, Germany) was added during the last 5 h of culture. After staining of surface molecules, cells were fixed and permeabilized and incubated with Abs against IFN-γ (PE; clone XMG1.2; BD Biosciences). Staining with appropriate isotype control Abs was used to define the threshold of specific binding. All Abs were purchased from BioLegend (San Diego, CA). Data were acquired using a FACSCanto II (BD Biosciences) and were analyzed using NovoExpress (ACEA Biosciences, San Diego, CA). $CD8^+/CD4^+$ T cell subsets were defined as follows: $CD3^+CD8^+/CD4^+CD62L^+CD44^{lo}$ (T_n), $CD3^+CD8^+/CD4^+CD62L^+CD44^{hi}$ (T_{CM}), $CD3^+CD8^+CD62L^+CD44^{hi}CD122^{hi}$ (T_{VM}), and $CD3^+CD8^+/CD4^+CD62L^-CD44^{hi}$ ($T_{Eff/EM}$).

Statistics

Data are presented as Tukey box plots or scatter plots with the median and interquartile range of individual mice. Statistical differences between two groups were analyzed by Mann–Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) using Graph Pad Prism 8.0 software.

RESULTS

Sterile traumatic tissue injury induces early activation of $CD8^+$ T cell subsets

To examine the impact of acute sterile tissue damage on $CD4^+$ and $CD8^+$ T lymphocytes in vivo, traumatic skeletal muscle injury was induced on both hind limbs (5). As expected, the concentration of creatinine kinase in the serum, a marker for skeletal muscle damage, increased within 3 h after injury (Fig. 1A). Enhanced levels of circulating IL-6 were observed 6 h after the insult (Fig. 1A), indicating systemic inflammation. The expression of the activation marker CD69 on diverse $CD4^+$ and $CD8^+$ T cell subsets in the draining lymph node and in the spleen as local and systemic lymphoid organ, respectively, was evaluated at different time points after skeletal muscle injury or sham treatment. $CD8^+$ T_n ($CD62L^+CD44^{lo}$) and T_{CM} (total $CD62L^+CD44^{hi}$) cells (Fig. 1B), transiently expressed higher levels of CD69 in the popliteal lymph node 24 h after injury (Fig. 1E) but not in the spleen (Supplemental Fig. 1). Likewise, $CD8^+$ T_{VM} cells that express high levels of CD122, the β-chain of the IL-2R and IL-15R (Fig. 1B) and lack the expression of CD49d (Fig. 1C), showed a clear increase in CD69 expression after injury in the lymph nodes that was maintained for at least 48 h (Fig. 1D, 1E). $CD8^+$ T_{VM} cells in the spleen remained unaffected (Supplemental Fig. 1). In contrast to $CD8^+$ T cells, none of the $CD4^+$ T cell subsets significantly changed the expression of CD69 after injury (Supplemental Fig. 2A). No alteration of the absolute number of each $CD8^+$ T cell subset in the lymph nodes was observed after injury (Supplemental Fig. 2B).

Several DAMPs are released upon tissue damage and may contribute to the development of sterile inflammation in a TLR-dependent manner (15). To address a potential involvement of TLR4 and TLR2 in $CD8^+$ T cell activation, trauma and sham treatment was performed with TLR4- and TLR2-deficient mice. In contrast to wild-type mice, no significant increase in CD69 expression on $CD8^+$ T cell subsets was observed in the lymph nodes of $TLR4^{-/-}$ mice after injury (Fig. 1F). $CD8^+$ T cell subsets in the lymph nodes of $TLR2^{-/-}$ mice also increased the expression of CD69 (Supplemental Fig. 2C). Thus, skeletal muscle injury induces sterile inflammation and the activation of $CD8^+$ T_n, T_{CM}, and T_{VM} cells in a TLR4- but not TLR2-dependent manner.

Sterile injury triggers proinflammatory cytokine expression in draining lymph nodes

The sterile nature of the traumatic insult argues against an Ag-specific T cell activation as origin of the rapid increase of CD69 expression on $CD8^+$ T cells. We considered that sterile tissue injury induced Ag-independent activation and examined the expression of cytokines that are known to induce the activation of T cells in the absence of their cognate Ag. The limited material that could be obtained from the popliteal lymph nodes was insufficient to perform protein analyses. Therefore, real-time qPCR was used to detect cytokine gene transcription from 3 h

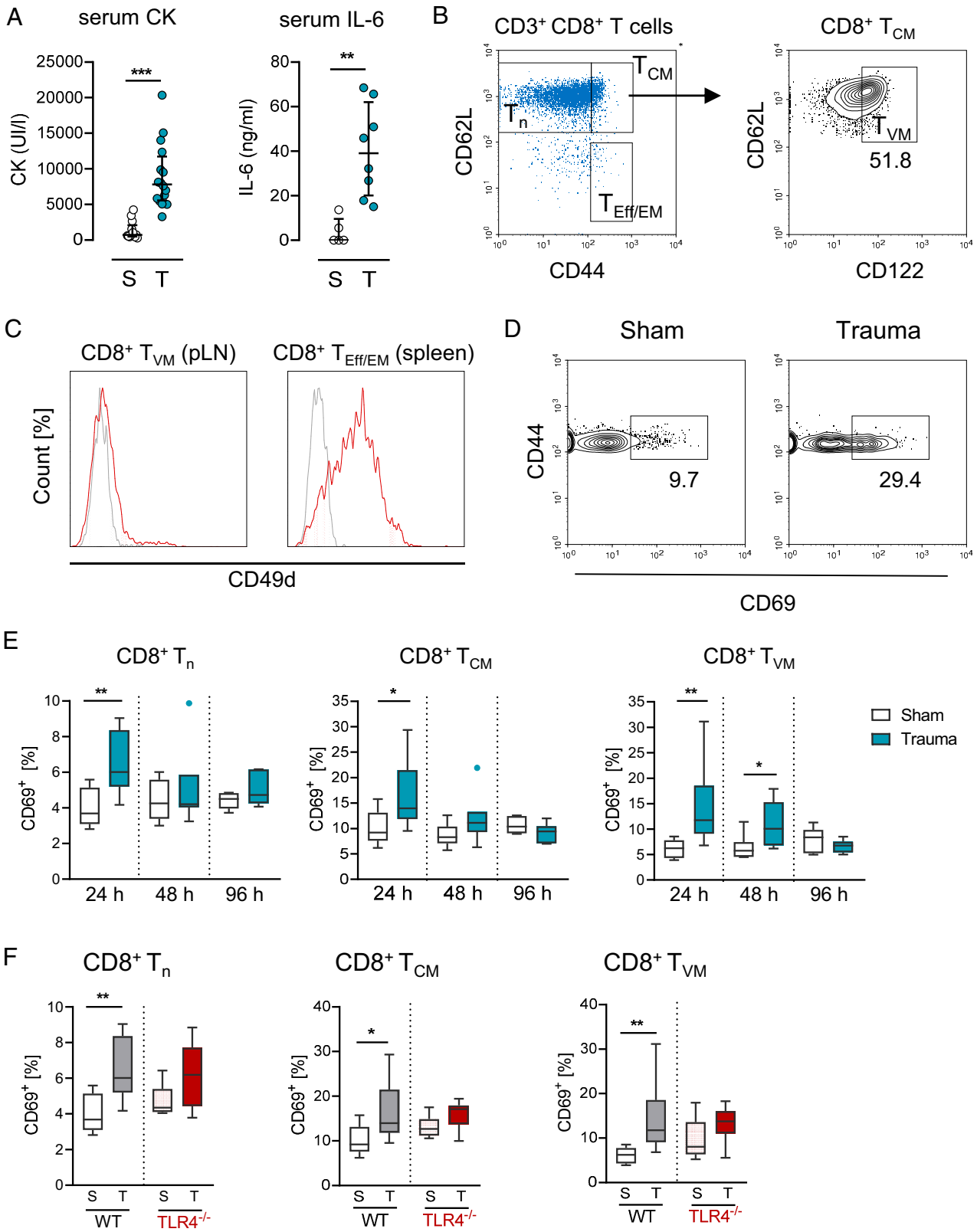

FIGURE 1. Traumatic skeletal muscle injury induces the activation of CD8+ T cells in the draining lymph node.

Sham treatment (S) or traumatic injury (T) was induced in wild-type (WT) or TLR4$^{-/-}$ mice. (**A**) Concentration of creatinine kinase (CK) and IL-6 in the serum of individual WT mice 3 and 6 h, respectively, after the treatment. (**B–E**) Popliteal lymph node cells were isolated at different time points after the treatment (n = 6–8 per group) and were analyzed for CD8+ T cell subsets and for the expression of CD69 by means **(Continued)**

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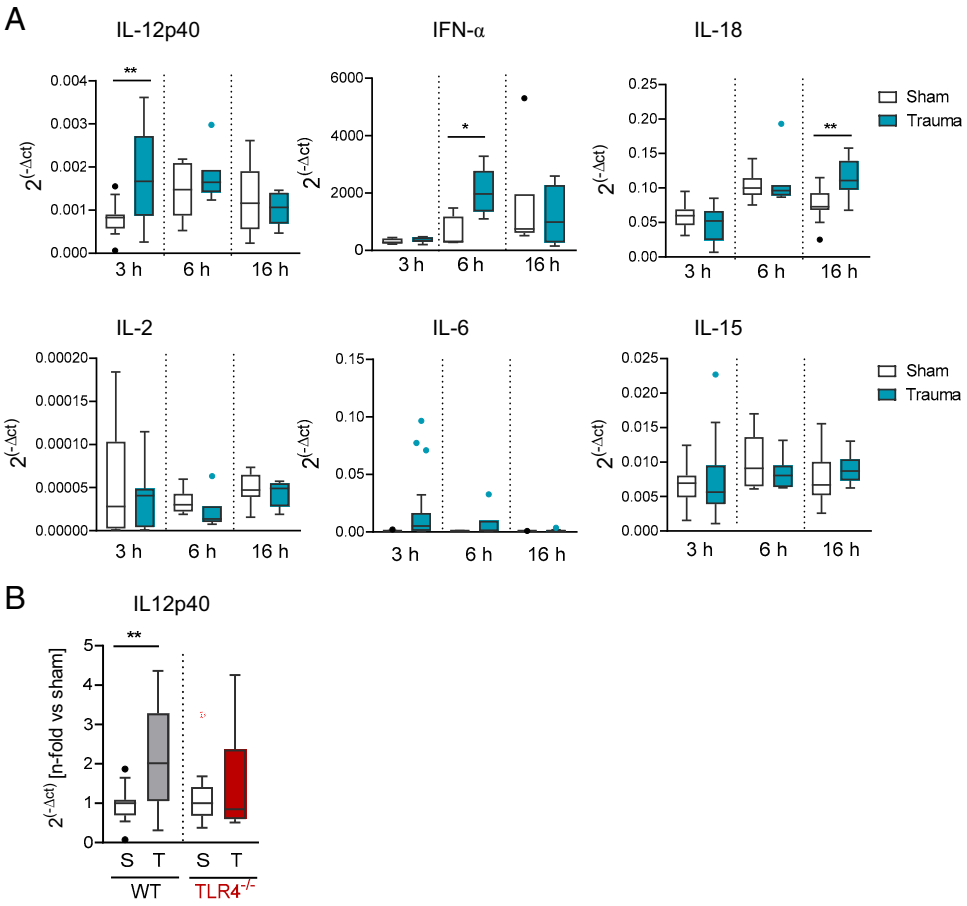

FIGURE 2. Cytokine expression in the popliteal lymph node after injury.

Wild-type (WT) or TLR4$^{-/-}$ mice underwent sham treatment or traumatic injury. At different time points thereafter, mRNA was isolated from popliteal lymph nodes and reverse transcribed into cDNA. Real-time PCR was performed for cytokines, as indicated. (**A**) Kinetics of gene transcription in WT mice. (**B**) Comparison of *Il12p40* gene transcription between WT and TLR4$^{-/-}$ mice 3 h after the treatment. To correct for interassay variations, data were normalized to the median value from sham mice (set as 1). The underlying raw data for WT mice are the same as in (A), for 3h. Data are pooled values from two to four experiments (each with three to four mice per group) and are presented as Tukey box plots with median. Statistical differences between sham und injured mice were analyzed by Mann–Whitney *U* test. *$p < 0.05$, **$p < 0.01$. S, sham treatment; T, traumatic injury.

to 16 h after injury or sham treatment. Increased levels of cytokine mRNA were detected for *Il12p40* (at 3 h), *Ifna* (at 6 h), and *Il18* (at 16 h) after injury (Fig. 2A). In contrast, *Il2*, *Il6*, and *Il15* mRNA expression did not change significantly after injury (Fig. 2A). Importantly, injury did not induce increased *Il12p40* mRNA expression in TLR4$^{-/-}$ mice (Fig. 2B). Thus, skeletal muscle injury rapidly triggers the expression of innate cytokines in the draining lymph node that involves signaling through TLR4.

IL-12p40 contributes to Ag-independent CD8$^+$ T cell activation in vivo

To examine whether IL-12 was involved in the activation of CD8$^+$ T cells after injury in vivo, mice were treated with neutralizing Abs against IL-12p40 or with isotype control Abs before trauma or sham treatment. Blocking of IL-12p40 prevented the injury-induced increase in CD69 expression on both CD8$^+$ T_n and T_{CM} cells (Fig. 3A). Additional in vitro analyses confirmed the stimulatory activity of IL-12 on CD8$^+$ T_n

of flow cytometry. (B) Gating strategy of CD8$^+$ T cell subsets in popliteal lymph node cells: T_n, CD62L$^+$CD44^{lo}; $T_{Eff/EM}$, CD62L$^-$CD44^{hi}; T_{CM}, CD62L$^+$CD44^{hi}; T_{VM}, CD62L$^+$CD44^{hi}CD122^{hi}. (C) T_{VM} in the lymph node did not express CD49d, in contrast to $T_{Eff/EM}$ in the spleen that served as positive control for CD49d expression (isotype control in grey; CD49d in red). (D) Representative contour plot of CD69 expression on T_{VM} from WT mice. Numbers indicate the frequency of positive cells. (E) Frequency of CD69$^+$ cells among CD8$^+$ T cell subsets 24, 48, or 96 h after the treatment. (**F**) Comparison of WT ($n = 8$) and TLR4$^{-/-}$ ($n = 6$) mice in terms of CD69 expression 24 h after the treatment. Data for WT mice are the same as in (D). Data are shown in scatter dot plots with median and interquartile range or Tukey box plots with median. The absolute number of the CD8$^+$ T cell subsets did not differ between WT and TLR4$^{-/-}$ mice (not shown). Statistical differences between sham and injured mice were analyzed by Mann–Whitney *U* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells; IL-12 was inefficient alone (data not shown) but triggered an increased expression of CD69 on $CD8^+$ T_n cells when combined with IL-18, which was also expressed after injury (Fig. 2A). Likewise, T_{CM} cells responded to IL-12 and IL-18 with increased CD69 expression and, in contrast to T_n cells, secreted IFN-γ (Fig. 3B). Thus, IL-12 triggers the activation of $CD8^+$ T_n and T_{CM} cells in vitro and after tissue damage in vivo.

Activated T_{VM} do not show a regulatory phenotype

Given the regulatory activity of T cells after injury (5) we asked whether $CD8^+$ T_{VM} cells expressed the regulatory molecules IL-10 and PD-1. To test this, injury was induced in VertX mice that express GFP under the control of the IL-10 promoter and allow the visualization of *Il10* gene transcription in vivo. *Il10* gene transcription was clearly visible in $CD69^+$ $CD4^+$ $T_{Eff/EM}$ cells (although without significant difference between sham and injury; Supplemental Fig. 4). $IL\text{-}10^+$ $CD8^+$ T_{VM} (Fig. 4A, 4B) and $PD\text{-}1^+$ $CD8^+$ T_{VM} (Fig. 4C, 4D) were present in the lymph nodes at low frequency and did not significantly change after injury. Thus, activated $CD8^+$ T_{VM} after injury do not show the phenotype of regulatory T_{VM}.

DISCUSSION

In the current study, we provide the first evidence, to our knowledge, that a TLR4/IL-12p40 axis rapidly triggers the activation of distinct subsets of cytotoxic $CD8^+$ T lymphocytes in draining lymph nodes after tissue damage.

In line with previous reports on other injury models, such as trauma-hemorrhagic shock (16), burns (17), and bone fracture (18), we observed increased levels of IL-6 in the blood, indicating a systemic inflammatory response to the traumatic skeletal muscle injury.

Within 24 h after injury, $CD8^+$ T cells but not $CD4^+$ T cells displayed an increased expression of the activation marker CD69 in the draining lymph node. Injury selectively activated $CD8^+$ T_n and T_{CM} (including T_{VM}) cells. Given that the size of the population of these $CD8^+$ T cell subpopulations did not increase after injury, we assume that the enhanced frequency of $CD69^+$ T cells reflects the activation of already-existing cells in the lymph nodes rather than the recruitment of $CD69^+$ cells from the circulation.

As previously shown (19), the mechanical disruption of skeletal muscle tissue caused the release of myocyte-specific intracellular molecules, such as myoglobin and creatinine kinase, into the circulation. Skeletal muscle-derived molecules additionally access the proximal lymph node through lymphatic vessels (20). Accordingly, muscle-derived molecules that were discharged from the damaged tissue most likely reached the lymph node and seemed to be sufficient to trigger the stimulation of $CD8^+$ T cells. In contrast, molecules that get access to the circulation were diluted, a putative explanation for the lack of T cell activation in the spleen after injury. A similar spatial distribution of T cell activation has been described for burn injury, in which early T cell activation is found in the skin-draining lymph nodes but not in the spleen (21). Yet, burn injury triggers $CD4^+$ Treg (4), not $CD8^+$ T cells, as we observed after skeletal muscle injury.

Trauma hemorrhage that indirectly induces tissue damage through ischemia/reperfusion injury causes the activation of T cells in the spleen but only when combined with musculoskeletal tissue damage (22, 23). This observation supports the above proposed assumption that the activation of $CD8^+$ T cells in the spleen requires a larger extent of tissue damage to exceed the

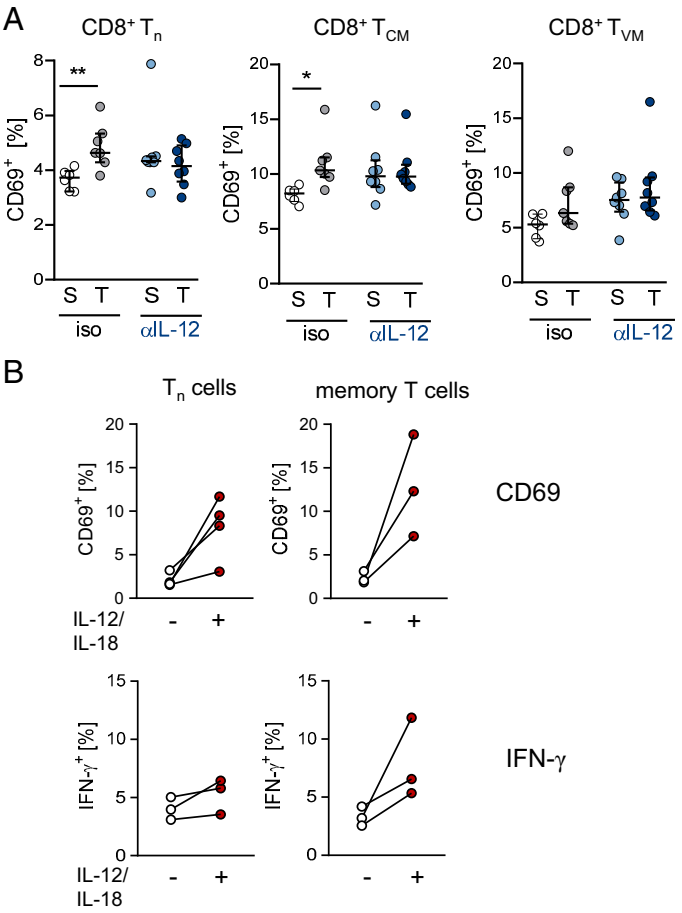

FIGURE 3. IL-12 is responsible for the activation of $CD8^+$ T cell subsets after traumatic injury.

(**A**) Mice received neutralizing Abs against IL-12p40 (αIL-12) or isotype control Abs (iso) before sham treatment (S) or traumatic injury (T). 24 h later, the expression of CD69 on diverse $CD8^+$ T cell subsets was determined. Gating of T cells was performed as described in Fig. 1B. Data from individual mice (n = 6–8 mice per group) are shown. Horizontal lines indicate the median and interquartile range. Statistically significant differences were tested using the Mann–Whitney U test. (**B**) Purified splenic $CD8^+$ T cells from naive mice were cultured in the absence (–) or presence (+) of rIL-12 and IL-18, and the expression of CD69 and intracellular IFN-γ was determined in naive and total $CD44^{hi}$ memory T cells. Representative dot plots for the gating strategy and expression of CD69 and IFN-γ are shown in Supplemental Fig. 3. Graphs show pooled data from three independent experiments. *$p < 0.05$, **$p < 0.01$.

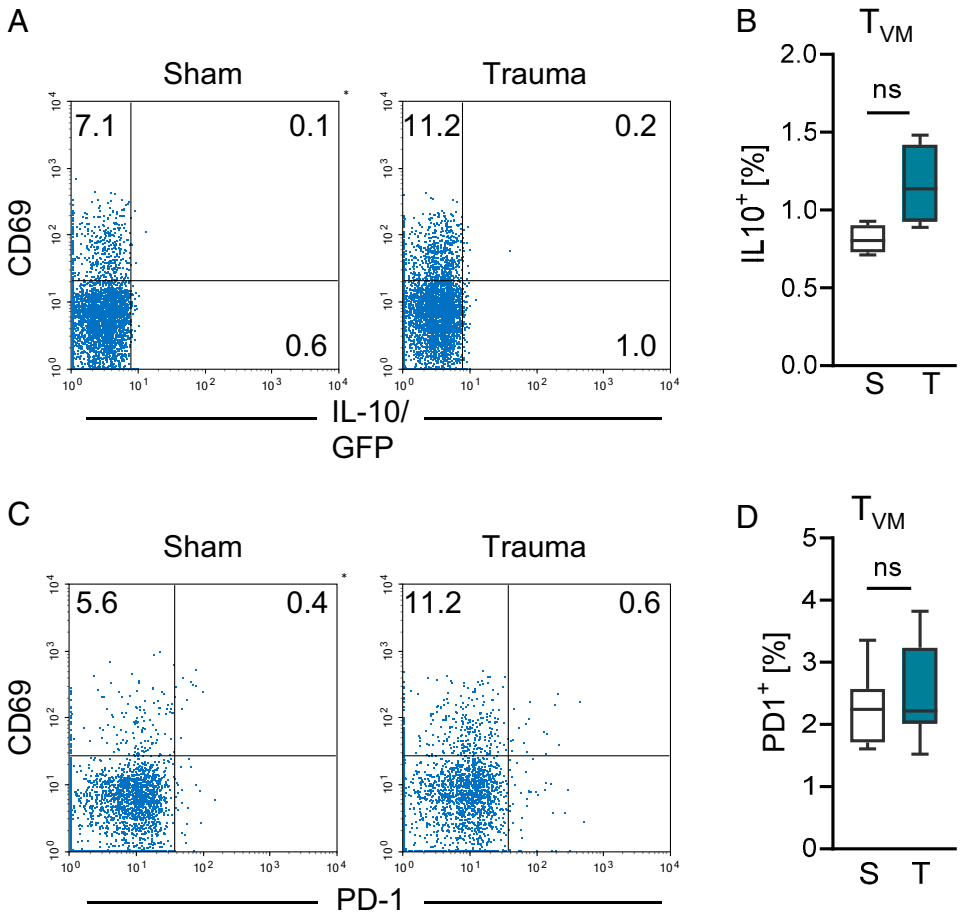

FIGURE 4. T_{VM} do not express increased levels of IL-10 or PD-1 after traumatic injury.

VertX mice (**A** and **B**) or wild-type mice (**C** and **D**) underwent sham treatment (S) or traumatic injury (T). Forty-eight hours later, the expression of IL-10/GFP (A and B) and PD-1 (C and D) was determined for gated T_{VM} from popliteal lymph nodes. (A) Representative dot plots of IL-10/GFP expression on T_{VM}. The threshold for GFP expression was set using cells from naive nontransgenic mice. (B) Pooled data for GFP/IL-10^+ T_{VM} from n = 4 mice per group. (C) Representative dot plots of PD-1 expression on T_{VM}. (D) Pooled data for PD-1^+ T_{VM} from n = 4 mice per group. Data are expressed as Tukey box plots. Statistical significance was tested using Mann–Whitney U test. ns, not significant.

threshold of stimulatory molecules in the circulation. Alternatively, previous studies did not discriminate between T cell subpopulations and therefore might have missed the increased expression of CD69 on the minor subsets T_{CM} and T_{VM}.

Mechanistically, we discovered that the activation of $CD8^+$ T cells in the lymph node after injury was dependent on TLR4 but not on TLR2. There is evidence that TLRs are not only expressed on innate immune cells but also on T lymphocytes. Whereas TLR2 is known to serve as a costimulatory molecule in T cell activation (24–26) the relevance of TLR4 in the functionality of murine T cells is debated. A recent study shows that the activation of $CD4^+$ Treg after burn injury is at least partially dependent on TLR4, but it is unknown whether intrinsic TLR4 signaling in Treg is involved (27). In this study, we demonstrate that TLR4-dependent CD8 T cell activation after skeletal muscle injury occurred in *trans* through IL-12p40, the common subunit of bioactive IL-12 and IL-23. TLR4 signaling in as yet unidentified cells led to the expression of *Il12p40* transcripts in the lymph nodes as early as 3 h after injury. Because of the limited size of popliteal lymph nodes, we could not isolate sufficient material to prove the presence of IL-12 and/or IL-23 protein. Innate immune cells, such as dendritic cells, but also B lymphocytes may release IL-12 and IL-23 and thus are a potential cellular source of these cytokines after skeletal muscle injury. Numerous DAMPs, such as heat shock proteins, S100 proteins, and high-mobility group B (HMGB) 1, are known to be released upon tissue damage and may act as TLR4 ligands (15). The identity of the DAMP(s) that trigger TLR4 signaling in case of skeletal muscle injury, as well as their target cell and the cellular source of IL-12p40 in the lymph nodes, are currently under investigation.

We observed that T_n and T_{CM} cells in the draining lymph node increased the expression of CD69 after skeletal muscle damage. Upon uptake of foreign Ags in the tissue, dendritic

cells migrate into the draining lymph node, where they stimulate Ag-specific T cells. This process requires several days and is in contrast to the rapid activation of $CD8^+$ T cells that we observed after injury. Alternatively, T cells may be activated by multiple innate cytokines in the absence of its cognate Ag (8). T_{CM} cells, especially, respond rapidly to the combination of IL-12 with IL-18, type I IFNs, and other cytokines with an increased expression of CD69 and the release of IFN-γ. Thereby, IL-12 triggers a cross-talk between the β1-chain of the IL-12R and the TCR signaling pathway (28). This polyclonal activation of T_{CM} cells is known to contribute to the early immune defense during infectious diseases (29). Given that neutralization of IL-12p40 prevented the increase in CD69 expression in vivo, we assume that cytokine-induced rather than Ag-dependent $CD8^+$ T cell activation occurred after injury.

IFN-α and IL-18 were expressed in the draining lymph node after skeletal muscle injury, although at later time points than IL-12p40. Because IFN-α and IL-18 synergize with IL-12, these two cytokines might favor the Ag-independent activation of $CD8^+$ T cells after injury. This assumption is supported by the finding that T_n as well as memory $CD8^+$ T cells increased the expression of CD69 after stimulation with IL-12 together with IL-18 in vitro. γc cytokines, such as IL-15, IL-2, and IL-7, induce Ag-independent activation of $CD8^+$ T_n cells (30). To our knowledge, we provide the first evidence that IL-12 may also activate $CD8^+$ T_n cells in vitro and in vivo. Importantly, although $CD8^+$ T_n cells must have received a stimulatory signal, as indicated by the expression of CD69, they maintained their characteristic expression of the adhesion molecule CD62L. Upon recognition of the specific Ag by the TCR or upon stimulation with γc cytokines, T_n cells lose the expression of CD62L (30, 31) and may enter the circulation. Whether the maintenance of CD62L expression on activated $CD8^+$ T_n cells after injury affects their migratory behavior remains to be investigated in future work.

The question arises. What consequence does the cytokine-induced activation of $CD8^+$ T cells after injury have? Ag-independent activation of $CD8^+$ T cells is crucial for the immune defense against invading pathogens (32). In contrast, the activation of T cells specific for endogenous Ags that are released after injury-induced tissue damage would cause autoimmunity. Indeed, the development of self-reactive T cell responses has been shown after skeletal muscle injury in mice but only when the frequency of autoreactive T cells had been artificially enhanced in advance through adoptive transfer of such T cells (33). Because an increased frequency of autoimmune diseases after injury has not been reported so far, neither for patients nor for naive mice, it is unlikely that the activated $CD8^+$ T cells that we found after skeletal muscle injury promoted the development of autoimmunity. We also did not find an increased expression of IL-10 and PD-1 that would point to a regulatory capacity of T_{VM} as an alternative consequence of the cytokine-induced activation. Therefore, the question whether the activation of CD8 T_{VM} after injury is beneficial or detrimental for the host remains unanswered to date and warrants further examination.

In summary, sterile inflammation induced by acute tissue injury triggers the activation of $CD8^+$ T_n, T_{CM}, and T_{VM} cells through integration of TLR4 signaling and cytokine production in the local lymph node. These findings might point to an as yet unrecognized activity of distinct $CD8^+$ T cell subpopulations in local lymph nodes after acute sterile tissue injury that might modulate forthcoming local immune responses.

DISCLOSURES

The authors have no financial conflicts of interest.

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