Myoinjury transiently activates muscle antigen-specific CD8+ T cells in lymph nodes in a mouse model.

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Title

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Running head: Myoinjury and CD8$^+$ T-cell activation

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Objective. To investigate the influence of myoinjury on antigen presentation to T-cells in the draining lymph nodes (dLNs).
Methods. Muscle crush was performed in mice injected with exogenous OVA, and in the Tg SM-OVA mouse expressing OVA as a muscle specific self-antigen. Antigen exposure and the resulting stimulation of T-cell proliferation was assessed in dLNs by transferring CFSE-labelled OVA-specific $CD8^+$ and $CD4^+$T-cells from OT-I and OT-II mice and by measuring the dilution of CFSE which directly reflects their proliferation. The role of moDCs in T-cell priming was assessed using pharmacologic blockade of DC migration. Immunofluorescence was used to detect $CD8^+$ T-cells, inflammatory monocyte-derived DCs (moDCs) and MHC-I-expressing myofibers in crushed muscle, and to assess expression of perforin, interferon (IFN)γ, IL-2, IL-10 and TGFβ1.
Results. OVA injection into intact muscle induced strong proliferation of $CD4^+$ and $CD8^+$T-cells indicating efficient exposure of soluble antigens in dLNs. OVA-specific $CD8^+$ T-cell proliferation in dLNs required myoinjury in Tg SM-OVA mice and was unaffected by pharmacologic inhibition of moDC migration. At d7 post-injury activated $CD8^+$T cells expressing perforin, IFNγ and IL-2 were transiently detected in crushed muscle, and these cells closely contacted MHC-class I^+ regenerating myofibers. From the d7 time point, immunosuppressive cytokines IL-10 and TGFβ1 were conspicuously expressed by $CD11b^+$cells, and $CD8^+$T cells rapidly disappeared from the healing muscle.
Conclusion. Myofiber damage induces a bout of muscle antigen-specific $CD8^+$T-cell proliferation in dLNs. Activated $CD8^+$T-cells transiently infiltrate the injured muscle with prompt control by immunossupressive cues. Inadequate control might favour sustained autoimmune myositis.

Idiopathic inflammatory myopathies (IIMs) are autoimmune diseases with distinct histopathological features suggesting either (i) humorally mediated processes, primarily targeting the microcirculation in dermatomyositis (DM) and myofibers in autoimmune necrotizing myopathies; or (ii) $CD8^+$ T cell mediated and MHC-I restricted autoimmune attack of myofibers in polymyositis (PM) and inclusion body myositis (IBM) (1).

Pathophysiological studies have mainly explored how muscle cells can participate in immune cell interactions in polymyositis (2). In this setting, myofibers strongly express MHC-class I molecules at their surface (1) and become invaded by auto-invasive T cells (3) expressing perforin (4). Clonal expansions of T cells are found in muscle and blood (5-8), and autoinvasive T cells exhibit selective gene rearrangement of their T-cell receptor (TCR) with restricted aminoacid sequences in the complementary-determining region 3, suggesting muscle antigen-driven T-cell expansion (9).

Adult myofibers are one of the few cell types in the body that do not express MHC-class I molecules (10). Therefore, they cannot serve as antigen presenting cells (APCs) at steady state (2). In contrast, cultured myoblasts constitutively express MHC-class I molecules, and upregulate these molecules upon exposure to interferon (IFN-γ), lipopolysacharide (LPS) and other cytokines (11). It remains to be elucidated whether *in vivo* myofiber expression of MHC molecules is induced by pro-inflammatory cytokines or by infectious agents or by a nonspecific response to tissue injury and regeneration or by a combination of these factors (12).

Attention has recently focussed on the specific role of regenerating myofibers in IIM pathophysiology. These cells preferentially express (i) myositis autoantigens (13), (ii) proinflammatory Toll-like receptors 3 and 7 which bind and respond to nucleic acids and endogenous ligands (e.g. necrotic debris) (14), and (iii) MHC-class I molecules allowing autoantigen presentation and eventually inducing endoplasmic reticulum stress (15). Taken together, these findings suggest immature fiber-driven amplification of the immune response.

We previously studied the coordinate immune cell reaction induced by myoinjury and found that resident muscle macrophages govern the inflammatory response to mouse myoinjury by specifically attracting neutrophils and circulating monocytes (16). Normal muscle hosts very little, if any, conventional dendritic cells (DCs), but, about 7 days post-injury, a conspicuous subset of $CCR2^+$ monocyte-derived $CD11c^{int}$cells with APC function can be detected in the regenerating muscle (16). These monocyte-derived DCs (moDCs), also called "inflammatory" DCs (17), are distinct from preexisting migratory or lymphoid organ-resident myeloid (mDCs), and from plasmacytoid (pDC) (18). Such moDCs have been shown to induce memory $CD8^+$T cell expansion on secondary antigen encounter in peripheral tissues (19).

Whether myoinjury could influence T-cell activation *in vivo* remains unknown. The primary activation of naïve T cells after local antigen challenge takes place within the draining lymph nodes (dLNs) and spleen, not in the peripheral tissue. In these lymphoid organs, "professional" APCs have the unique capacity to strongly amplify a minute subset of cognate naïve T-cells patrolling the lymphoid tissues (18). The respective contributions of the different DC subsets to these primary immune responses are still incompletely delineated.

Using ovalbumin (OVA) as a model antigen, we investigated for the first time the influence of crush myoinjury on OVA-specific $CD4^+$ and $CD8^+$T-cells in dLNs. This was achieved by adoptive transfer of TCR transgenic OVA-specific $CD8^+$ and $CD4^+$T cells labelled with CFSE, allowing assessment of proliferation-associated fluorescence dilution in dLNs. Using

Tg SM-OVA mice expressing OVA as a muscle autoantigen (20), we observed that myocrush induces OVA-specific $CD8^+$T-cell proliferation in dLNs, not requiring moDC migration from the injured muscle to dLN. Activated $CD8^+$T-cells transiently infiltrated the injured muscle but the T-cell reaction to myoinjury was rapidly controlled, likely through immunosuppressive cues physiologically associated with muscle healing.

MATERIALS AND METHODS

Mouse strains. C57BL/6 mice were purchased from Janvier (Paris, France). Transgenic mice harboring OVA-specific T cell receptor (TCR) were purchased from Jackson (Maine, USA). They included OT-I (C57BL/6) mice, for $CD8^+$ T cells, and OT-II (C57BL/6) for $CD4^+$ T cells. Briefly, OT-I mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes. The transgenic TCR was designed to recognize OVA residues 257-264 in the context of MHC-I ($H2K^b$) presentation. OT-II mice express the mouse alpha-chain and beta-chain TCR that pairs with the CD4 co-receptor and is specific for chicken OVA323-339 in the context of MHC-II (I-Ab).

For obtaining OT-I and OT-II mice carrying allotypic CD45.1 phenotype, we bred OT mice ($CD45.2^+$) with Ly-5 mice (B6.SJL-$Ptprc^aPep3^b$/BoyJ) carrying the CD45.1 antigen purchased from Charles River (Wilmington, MA). Donor mice used for adoptive transfer were F1 mice, double positive for CD45.1 and CD45.2 (21,22).

TgSM-OVA mice express a membrane-bound form of OVA under the control of MCK-3E, a mutated form of muscle creatine kinase. Skeletal muscle-restricted OVA expression has been documented by extensive RT-PCR-based tissue screening (20).

Mice were 6-12wk of age and handled according to European community guidelines.

Mouse model of myoinjury. In a preliminary study, we first induced myoinjury by direct injection of notexin, a snake venom with phospholipase A2 activity, as previously reported (16). Notexin inhibited both $CD8^+$ and $CD4^+$T-cell proliferation. Consistently, notexin exerted dose-dependent cytotoxicity on T lymphocytes *in vitro* (data not shown). Therefore, we used mechanical crush to avoid interference of toxic effects on immune cell responses. After cutting the skin, the tibialis anterior (TA) muscle was exposed, dissociated, and entirely crushed from the distal tendon to the proximal extremity by two series of repeated short (5sec) forceps applications (23). The contralateral TA muscle was used as an uninjured control. In sham experiments, crush was replaced by simple skin incision and suturation.

Adoptive transfer and analysis of T cell priming. TCR transgenic $CD8^+$ or $CD4^+$T-cells were isolated from spleens and LNs of F1 [OT-I or OT-II×Ly-5] mice, purified by negative selection using magnetic beads (Cat.no. 114.17D and 15D, Dynal Biotech, Invitrogen, Carlsbad, CA), and labeled with the intracellular CFSE dye by 10min incubation at 37°C (C34554, Invitrogen). One day after crush, CFSE-labeled OT-I and OT-II-derived T cells were co-transferred at 1:1 ratio, by tail vein injection (5×10^6cells per mouse), to wild type or Tg SM-OVA mice. Soluble OVA (Sigma-Aldrich, St. Louis, MO) was injected, or not, into the crushed TA muscle (10ug per mouse) the day after T cell transfer. Proliferation of $CD4^+$ and $CD8^+$T cells was assessed at d4 and d7 post-injury, or to the corresponding d3 and d6 post-transfer in uninjured animals: $CD45.1^+$ $CD8^+$ and $CD45.1^+$ $CD4^+$ cells were collected from dLNs of the $CD45.2^+$ recipient mice and their *in vivo* activation was measured by flow cytometry on the basis of proliferation-dependent CFSE fluorescence loss (24). Uncrushed mice with T cell transfer served as controls.

To inhibit DC migration to dLNs we used the BW245C prostaglandin analog, an agonist of PGD2 receptor (25) (100nM, Cat.no.12050, Cayman Chemical, MI), injected into crushed muscle at d2 and d4 post-injury, compared to DMSO (vehicle control).

Cell sorting and flow cytometry analysis. Popliteal and inguinal dLNs were removed under magnifying glasses, minced and gently digested using 0.1% collagenase B (Roche Laboratories, Basel, Switzerland). Cell viability was assessed by Trypan blue and propidium iodide. For isolating inflammatory cells from mouse muscle, fascia of the crushed TA was removed first. Muscle was dissociated in DMEM containing collagenase B 0.1% and pronase 1% at 37°C for 60min and 45min respectively. $CD45^+$ cells were isolated using mouse CD45 MicroBeads (Miltenyi Biotec, CA).

We used the CyAnADP High-Performance Flow Cytometer (Dako, Glostrup, Denmark). Flow cytometry antibodies (Abs) obtained from eBioscience (San Diego, CA) included: PE-coupled F4/80, CD8a, B220 and CD4; APC-coupled anti-CD8a; FITC-coupled anti-mPDCA-1, Ly-6C, MHC-I, CD5 and CD11b; PercP-Cy5.5-coupled anti-CD11c and CD4; PE-Cy5-coupled anti-CD11c; APC-Cy7-coupled anti-CD45.1. Results were analyzed by OFFLINE analysis of Summit software (Dako).

Immunohistochemistry. Snap-frozen whole TA muscle was transversally cryosectioned, and either stained with hematoxylin and eosin or prepared for immunofluorecence: fixation by cold acetone, incubation with rat anti-mouse biotinylated Abs against CD8a (clone 53-6.7, BD Pharmingen, NJ; or eBioscience, San Diego, CA), mouse monoclonal Ab against MHC class I $H2k^d$ cross reacted with the a3 domain of $H\text{-}2K^b$ (ab25334, clone 34-1-2S, Abcam, Cambridge, UK), rabbit anti-mouse Perforin Ab (#3693, Cell Signaling, Danvers, MA), chicken polyclonal anti-Laminin Ab (Abcam), rabbit anti-mouse CD3e Ab (clone 500A2, BD Pharmingen), rat anti-mouse CD11b Ab (MCA711, Sero Tec, Oxford, UK; or eBioscience), and biotin anti-mouse CD11c(HL3) Ab (BD Pharmingen), anti-B220 (eBioscience), anti-cytokines IFNγ (eBioscience), , IL2, IL10, and TGFβ1 (Santa Cruz, Heidelberg, Germany). AlexaFluor 488-conjugated goat anti-mouse IgG (564513, Invitrogen), Cy3-conjugated donkey anti-chicken and goat anti-rabbit IgG (703-166-155, Jackson, West Groove, PA), TRITC-conjugated mouse anti-rat IgG (40-212-025-104, Jackson ImmunoResearch) and PE-conjugated-Streptavidin (554061, BD Pharmingen) were used as secondary Abs. Nuclei were counterstained with DAPI. Slides were observed with Zeiss Axioplan 2 microscope (Iena, Germany), and data collected using the Zeiss Apotome and Axiovision 4.1 software.

Statistical analysis. Each experiment was performed in at least three different animals. Provided that results were consistent in triplicate experiments, flow cytometry results were illustrated by one representative experiment for each tested condition. Stastistical analyses were done using the Student's *t* test, a p value <0.05 being considered as significant.

RESULTS

Myoinjury has no influence on vigorous $CD8^+$ and $CD4^+$T-cell proliferation induced by exogenous OVA in WT mice. Crush of TA muscle in WT mice induced myofiber necrosis and degeneration accounting for about 40% of the muscle cross-sectional area at d4 post-injury (Fig.1A). Damage was gradually replaced by smaller regenerating myofibers, and centrally nucleated myofibers becoming prominent at d7. Accordingly, these time points were taken as the detection time points of necrosis and regeneration respectively. Conspicuous mononuclear cell

infiltrates were detected at both d4 and d7 (Fig.1A). At d10, myorepair was almost complete (Fig.1A).

T-cell priming in dLNs was assessed by flow cytometry measurement of proliferation-dependent fluorescence loss of CFSE-labelled OVA-specific CD45.1$^+$ CD8$^+$ and CD4$^+$ T-cells transferred to CD45.2$^+$ WT recipients.

In the absence of crush, i.m. injection of exogenous OVA challenge resulted in a vigorous expansion of both CD8$^+$ and CD4$^+$ OVA-specific T cells in dLNs, as expected from studies using systemic OVA challenge (21, 22). Proliferation rate of OVA-specific CD8$^+$ T cells was in the 85-87% range at d3 and 75-82% at d6 post-transfer. That of CD4$^+$ T cells was 80-83% at d3 and 52-55% at d6 (Fig.1B).

In crushed OVA-injected mice, T cell proliferation was similar to that of intact OVA-injected animals: proliferation of CD8$^+$ T cells was 86-91% at d4 and 84-87% at d7 post-crush, and that of CD4$^+$ T cells was 75-77% at d4 and 50-53% at d7 (Fig.1B).

Notably, crush alone elicited no OT-I and OT-II T-cell proliferation at d4 and d7 (all proliferation rates <10% for both CD8$^+$ and CD4$^+$ T-cells, Fig.1B), confirming specificity of the observed T-cell proliferations.

Myoinjury induces CD8$^+$, but not CD4$^+$, T cell proliferation in Tg SM-OVA mice. Because myofiber damage may unmask self-antigens and, in so-doing, elicit an adaptive immune response, we next used the TgSM-OVA mouse (20) that was constructed to selectively express OVA as a self-antigen in skeletal muscle (Fig. 1C).

In the absence of both crush and exogenous OVA injection, TgSM-OVA mice showed low proliferation of OVA-specific CD8$^+$ and CD4$^+$T-cells in dLNs (all rates <12% at d3 and d6 post transfer) (Fig.1B).

Crush alone in TgSM-OVA mice induced neither CD8$^+$ nor CD4$^+$ T-cell priming at d4 (proliferation rates <8%). In contrast, CD8$^+$, but not CD4$^+$, T-cell priming was observed at d7 post-injury. At this time point, proliferation rate of CD8$^+$ T cells was 28-39% (*vs* 10-12% in intact mouse), that of CD4$^+$T cells was 5-7% (identical to intact mouse) (Fig.1B). Notably, myoinjury-induced T-cell priming selectively affecting CD8$^+$T-cells was also found in spleen of TgSM-OVA mice at d7 (proliferation rate 24-32%) (data not shown).

We also injected exogenous OVA into muscle the day after T cell transfer. T-cell responses to OVA were similar in WT and TgSM-OVA mice, regardless of crush, for both CD8$^+$ and CD4$^+$ T-cells. This was assessed by no more than 5-10% variations between experiments comparing WT *vs* TgSM-OVA mice, and crushed *vs* intact mice (Fig.1B).

Since it was previously observed that OT-II cells are less sensitive to OVA antigen than OT-I cells (20), we further examined the effects of adjuvant stimuli on T-cell priming. LPS (100µg) injected into intact muscle of Tg SM-OVA mice had no effect on CD4$^+$T-cell proliferation (all proliferation rates<6%), whereas the proliferation rates of CD8$^+$ T cell slightly increased to 12-16% at d4 and 22-28% at d7. In crushed animals, LPS challenge induced earlier and strongly increased CD8$^+$ T-cell priming compared to crush alone. LPS injected into the crushed muscle at d2 post-injury was associated with specific CD8$^+$T-cell proliferation of 35-38% at d4, and 60-73% at d7. In contrast, CD4$^+$T-cell priming remained unaffected by LPS challenge (proliferation rates <10% at d4 and d7) (Fig.2A, B).

Both resident and inflammatory DCs increase in dLNs after myoinjury. Specific T cell activation is initiated by physical interactions between naive T cells and APCs in secondary

lymphoid structures (18). To determine changes in number and type of APCs in dLNs after myoinjury we performed muscle crush in WT mice and extracted dLN mononuclear cells at d0 (no crush), d4 and d7 post-injury for flow cytometry analysis. We defined resident mDCs as $CD11c^{hi}$Ly-$6C^{-}CD8a^{+}$/$CD8a^{-}$ cells; migratory "inflammatory" moDCs as $CD11c^{int}CD8a^{-}$ cells; pDCs as $CD11c^{int}$mPDCA-1^{+} cells; macrophages as $CD11c^{-}$Ly-$6C^{+}F4/80^{+}$; and B cells as $CD11c^{-}B220^{+}$ cells.

In normal dLNs (d0), mononuclear cells included about 0.35% macrophages, 1% of resident DCs ($CD8a^{+}$ 0.27 %, $CD8a^{-}$ 0.61%), 0.62% migratory DCs, 0.03% pDCs, and 41.5% B cells. At d4 after myoinjury, mDCs, moDCs, and pDCs remained stable, whereas macrophages slightly increased (0.78%), and B cells decreased (29.7 %), suggesting some maturation into plasma cells. In contrast, at d7 there was dramatic increase of DCs involving both mDCs (8.2%, including $CD8a^{+}$ 2.99%, and $CD8a^{-}$ 5.21%), and inflammatory moDCs (5.73%), but not pDCs (0.04%), whereas macrophages tended to return to normal value (0.42%) and B cells remained stable (29.8%) (Fig.3A,B).

These results support previous contention that inflammatory moDCs migrate into dLNs at the muscle regenerating stage (16), and, in addition, show strong coincident increase of resident mDCs.

DC migration blockade does not alter myoinjury-induced $CD8^{+}$T-cell priming in Tg SM-OVA mice. To examine if migratory "inflammatory" moDCs elicited by myoinjury may take part into T-cell priming in dLNs, we used a pharmacologic migration inhibitor called BW245C. This synthetic prostaglandin analog was previously shown to potently inhibit migration of both Langerhans cells and dermal DCs from skin to dLNs (25, 26). Three conditions were compared in WT mice myocrushed at d0 and intramuscularly injected with OVA at d1: (i) no BW245C injection; (ii) a single i.m. injection of 100nm BW245C at d2; and (iii) two i.m. injections of 100nm BW245C at d2 and d4. Mononuclear cells were extracted from dLN at d9 and immunophenotyped by flow cytometry using the hematopoietic marker CD45, the myeloid cell marker CD11b, and the DC marker CD11c. The number of DCs ($CD45^{+}CD11b^{+}CD11c^{+}$ cells) in dLNs decreased in a dose-dependent manner upon BW245C administration (Fig.4A). With two doses of inhibitor, about one third of DCs remained detectable in dLNs, presumably corresponding to resident mDCs (Fig.4A).

Next, we analyzed the influence of DC migration blockade on $CD8^{+}$ T-cell priming in myoinjured Tg SM-OVA mice. Mice underwent OT-I T-cell transfer (d1 post-crush) and then received 2 doses of BW245C (d2 and d4). As assessed by CFSE dilution (d9), these mice had T-cell proliferation rates similar to controls receiving no BW245C. Thus, crush-associated $CD8^{+}$ T-cell priming was unaffected by inflammatory DC migration blockade. This remained true when exogenous OVA (i.m. injection at d2) was added to crush (Fig.4B). In the setting of exogenous OVA administration, OT-II T-cell priming was similarly unaffected by DC migration blockade (data not shown).

Cytotoxic $CD8^{+}$T cells transiently infiltrate the regenerating muscle tissue. Muscle antigens have been shown to induce the migration of T lymphocytes and immature DCs to the endomysium or perimysium during myositis (27). Although T-cell infiltration has been consistently observed in experimental models of autoimmune myopathies (28, 29), this has not been well documented after myoinjury.

At d4 and d7, crushed and intact TA muscles of WT mice were studied by double

immunostainings to assess the presence of cytotoxic T-cells (CTLs), inflammatory DCs, B cells and MHC-class I-expressing myofibers. $CD8^+$T cells were defined as $CD3\varepsilon^+CD8a^+$cells, while, $CD8a^+perforin^+$cells were taken as the functional CTLs. $CD11b^+CD11c^+$cells were considered as inflammatory DCs, and $B220^+$cells as B cells. Sarcolemmal MHC-class I expression was assessed by comparison to the basement membrane staining by Laminin-1.

Notably, when notexin was used to injure muscle, no $CD3^+CD8^+$T-cell infiltration could be detected in muscle at d4 and d7 (data not shown). In contrast, as shown in Fig.5, $CD8^+$ T-cells were detected in crushed muscle at d7. B cells were not detected. $CD8^+$ T-cells were scattered in the endomysium among other mononuclear cells (Fig.5A-C). A number of them conspicuously expressed perforin as CTLs do, and some closely contacted regenerating myofibers showing internal nuclei (Fig.5B). $CD8^+$T cells had decreased at d10 and disappeared at d15. To further assess migration of T cells to injured muscle, OT-I $CD8^+$T-cells labeled with CFSE were injected into the tail vein of crushed mice (d1 post-injury). $CFSE^+$ cells were neither found in uninjured muscles nor in the injured muscle at d4. In contrast, at d7, $CFSE^+$ cells were detected in the regenerating muscle, and showed perforin expression (Fig.5D). Detection of abundant $CD45.1^+$ cells contrasting with CD45.2 expression of the proper leucocytes of the recipient Tg SM-OVA mouse confirmed the presence of transferred OT-I cells in crushed muscle (Fig.5D). Contralateral intact muscles showed no $CD45.1^+$ cell infiltrates. At d7, but not before, some myofibers strongly expressed MHC-class I antigen and were in contact with infiltrated $CD8^+$cells (Fig.5C). Sarcolemmal fluorescence was neither observed in uninjured muscle nor when staining was performed with an isotypic antibody used as control (data not shown). The regenerating muscle was hugely infiltrated by moDCs co-expressing CD11b and CD11c (Fig.5E). Expectedly, MHC-class I antigen was strongly expressed by these cells. A number of $CD8^+$T-cells were associated with moDC infiltrates (Fig.5F). We were unable to sort a sufficient number of viable OT-I cells to test antigen-specific cytokine secretion *ex vivo*. This poor viability of OT-I cells was presumably due to multistep handling of tissue (including injured muscle tissue digestion, extraction of mononucleated cells, and $CD45^+$ cell selection before culture) combined with strong local immunosuppressive cues associated with myorepair (30). Consistently, immunofluorescent cytokine screening showed $CD8^+$cells expressing IFNγ (Fig.6A) and IL-2 at d7, whereas $CD11b^+$cells already expressed the immunosuppressive cytokine IL-10 (Fig.6B) and TGFβ1. After complete disappearance of $CD8^+$cells (d15), immunosuppressive $CD11b^+$cells expressing IL10 and TGFβ1 (Fig.6C) were still conspicuously present. Although not evaluated on the long -term, kinetics of myorepair appeared similar in WT mice and in TgSM OVA mice injected with OT-I cells.

DISCUSSION

Several experimental models of chronic autoimmune myositis have been recently proposed (20,28). It was not our intention to propose a new model of myositis. Instead, we used the inflammatory reaction phase following myoinjury to assess the impact of myofiber breakdown on adaptive immune responses. The main objective of the present study was to examine for the first time what was happening in dLNs after myoinjury. We injured muscle by mechanic crush rather than the more commonly used notexin injection (16,30). This approach was chosen because an exploratory study showed dose-dependent toxicity of notexin for lymphocytes, possibly explaining why $CD3^+$T-cells are not found in notexin-injured muscle. Consistently, notexin

injection abolished the vigorous expansion of both $CD8^+$ and $CD4^+$ OVA-specific T cells elicited in dLNs by injection of exogenous OVA into muscle, whereas T-cell responses were remarkably strong after crush.

In contrast to exogenous OVA challenge, endogenous muscle-specific OVA unmasked by crush injury in TgSM-OVA mice elicited selective $CD8^+$ T-cell proliferation in dLNs at d7. In contrast, $CD4^+$ T-cell proliferation was not elicited by crush in TgSM-OVA mice. Concurrent i.m. LPS injection increased proliferation of $CD8^+$T-cells but was unable to induce $CD4^+$T-cell priming. This was quite unexpected since a variety of $CD4^+$T-cell reactions have been documented in dLNs after acute inflammatory injuries, such as burn injury, brain injury, and surgical trauma (31-33). Why muscle autoantigen-induced $CD4^+$T-cell priming does not occur after myoinjury is unclear. It is possible that the amount of endogenous OVA released by myoinjury was unsufficient to elicit $CD4^+$ T-cell response as OT-II T cells are of lower affinity than OT-I and may therefore require a higher quantitity of Ag to be activated. In contrast, selective proliferation of autoantigen-responsive $CD8^+$ T-cells was reminiscent of the pathophysiological role of CTLs in human PM and its murine models (28,29).

DCs in lymphoid and non-lymphoid tissues encompass different subsets with specific functions in the initiation of both immunity and tolerance induction (18). In IIMs, mDCs are present in PM and IBM, whereas a distinct IFNα/β-producing pDC subset is typically found in DM (34). Crush myoinjury was associated with increase of mDC and moDC, but not pDC, in dLNs at d7. Strikingly little is known about the exact DC functions in muscle (35-37). We previously showed that injury elicits formation of "emergency" moDCs in muscle (16), differing from mDCs by an intermediate rather than high level of CD11c expression (37,42). These moDCs presumably attempt to substitute for the functions of conventional migratory mDC at play in other tissues (18). DCs responding to crush in dLNs included moDCs coming from the injured muscle, and dLN-resident mDCs, mainly generated from blood-borne progenitors (18). Migratory and resident DCs appear to play complementary roles in $CD4^+$ T-cell priming, but their respective implication in $CD8^+$ T-cell proliferation is less clear. In dLNs, resident DCs can capture antigens, either drained from peripheral tissues via the lymphatics or transferred from migratory DCs (39,40), and then trap and activate cognate naïve $CD4^+$ T-cells, while migratory DCs specifically support proliferation of these $CD4^+$T-cells (18). In the present study, efficient pharmacologic inhibition of moDC migration from the injured Tg SM-OVA mouse muscle to dLNs had no effect on OVA-specific $CD8^+$ T-cell proliferation. Thus, in our experimental setting, dLN resident mDCs were likely to play the central role in autoreactive $CD8^+$T-cell activation. This finding is in agreement with previous report that monocyte-derived cells are several orders of magnitude less efficient in presenting antigens to T cells than conventional mDCs (41).

$CD8^+$ T-cells, corresponding to activated IFNγ-producing cells and/or $perforin^+$ CTLs, were transiently detected at d7 after myoinjury but could not be expanded *ex vivo* to assess their response to muscle antigens. It is not formally possible to exclude non-specific activation of CTLs in the milieu. However, CTLs were found in close association with regenerating myofibers which transiently expressed MHC-class I antigens at this time point. $CD8^+$ T-cells were also intermingled with moDCs. Such moDCs have been identified in several infectious models (42), where they exert local microbicidal activity through production of TNF-α and NO (43), and induce expansion of effector (44) and memory (20) $CD8^+$ T-cells within peripheral tissues. In the setting of myoinjury, we previously showed that inflammatory monocytes are initially attracted by muscle

resident macrophages and convert locally into moDCs (16). These monocyte-derived cells initially express proinflammatory cytokines, the expression of TNF-α and IL-1β transcripts peaking at days 2 – 3 after notexin injury, and then switch to an anti-inflammatory phenotype supporting myogenesis, characterized by upregulation of IL-10 and TGF-β1 mRNAs observed from day 2 after injury and maintained throughout regeneration (30). Although an initial inflammatory phenotype of moDCs could have participated to the transient homing or expansion $CD8^+$ T-cell observed after myocrush, immunohistochemistry was consistent with rapid and protracted upregulation of the immunosuppressive cytokines IL-10 and TGFβ1 in moDCs, as previously documented by RT-PCR in the notexin model of myoinjury (30).

Muscle crush mimicks exercise-induced injury and is associated with self-limiting inflammatory reaction related to efficient regulatory mechanisms, that both favour rapid decrease of the post-injury inflammatory reaction and promote myorepair (30). Physiologic protective mechanisms also prevent occurrence of autoimmune attack against regenerating myofibers. In normal individuals, tolerance mechanisms avoid sustained activation of autoreactive T cells by inducing apoptosis, skewing phenotype, anergy and/or regulatory T cells (45). Although incompletely understood, tolerization is particularly strong in muscle and efficiently protects the tissue from immune attacks targeting muscle antigens (20). Consistently, it proved very difficult to induce experimental autoimmune myositis (EAM) in the literature. The most recent attempt necessitated the combination of up to 4 immunizations with muscle myosin emulsified in complete Freund adjuvant plus pertussis injection plus depletion of regulatory T-cells (28). Therefore, in our experimental setting, conspicuous modulation of the myorepair process by one injection of autoeactive T-cells was neither expected nor detected. It seems very likely that strong local production of IL-10 and TGFβ1 observed at time of myorepair (30,46), participated to the rapid disappearance of $CD8^+$ T-cell infiltration elicited by crush myoinjury. Although not evaluated herein, it is possible that other mechanisms such as the Treg response could participate to the control of auto-reactive T-cells after myoinjury, since administration of *ex-vivo* expanded Treg efficiently controls inflammation and ameliorates disease activity in EAM (28).

In conclusion, myofiber damage induces a bout of muscle antigen-specific $CD8^+$T-cell proliferation in dLNs. Activated $CD8^+$ T-cells transiently infiltrate the injured muscle with prompt control by immunossupressive cues. It seems likely that inadequate control of the $CD8^+$T-cell response could represent a prerequisite for the emergence of autoimmune myositis and that, in this context, myonecrosis may amplify autotoxic $CD8^+$T-cell mechanisms and sustain muscle inflammation.

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FIGURE LEGENDS

Figure 1. Myopathologic alterations and T cell reaction in dLNs after TA muscle crush.

A, Representative muscle sections stained by HE at different time points. d0, normal muscle; d4, full-blown necrosis and myophagcytosis; d7, small regenerating myofibers with central nuclei neighboured by mononuclear inflammatory cells; d10, decreased inflammatory changes in healing muscle. Bar, 50µm. B, Flow cytometric analysis of co-transferred CFSE-labeled $CD45.1^+$ OT-I and OT-II T cells (5×10^6 cells transferred to each mouse) extracted from dLNs of $CD45.2^+$ intact and muscle-crushed WT or SM-OVA Tg mice at d4 and d7 post-myoinjury, with or without i.m. injection of soluble OVA. Cells were gated for $CD45.1^+CD8^+$ or $CD45.1^+CD4^+$ and further analyzed for CFSE fluorescence. Data shown here correspond to one representative experiment out of three. The proliferation of transferred OT-I and OT-II cells were compared using Student's *t* test (*n*=3). Data represent mean±SD. C, Immunoperoxidase staining in SM-OVA mouse showing an OVA-expressing myofiber surrounded by inflammatory cells 9 days after crush. Bar, 10µm.

Figure 2. Effects of LPS on T-cell reactions in dLNs of Tg SM-OVA mice.

A, Flow cytometry assessing CFSE dilution indicate that LPS increases proliferation of $CD8^+$ OT-I, but not $CD4^+$ OT-II, cells. The most pronounced effect is observed at d7 after crush. One representative profile is shown for each tested condition (out of three consistent experiments). B, The percentage of $CFSE^{low}$ OT-I and OT-II cells (i.e. cells that have proliferated) is represented as mean±SD for each tested condition.

Figure 3. Resident and migratory DCs increase in number in muscle dLNs after myoinjury.

A, Flow cytometric analysis of the relative numbers of macrophage($CD11c^-$ $Ly\text{-}6C^+F4/80^+$), resident conventional DCs （cDCs, ， $CD11c^{hi}$ $Ly\text{-}6C^-$ $CD8a^+/CD8a^-$ cell), migratory DCs ($CD11c^{int}$ $CD8a^-$ cells), plasmacytoid DCs (pDCs, $CD11c^{int}$ mPDCA-1^+ cells), and B cell ($CD11c^-$ $B220^+$ cells) found in the dLNs at the day 0, 4 and 7 after crush myoinjury on WT C57 BL/6 mice. Results are representative of three independent experiments. B, The frequency of macrophage, resident cDCs, migratory DCs, pDCs, and B cell are represented. Data represent mean±SD.

Figure 4. Blockade of moDC migration by BW245C i.m. administration.

A, BW245C exerts dose-dependent inhibition of DC migration from injured muscle to dLNs of WT mice (flow cytometry results on cells extracted from dlLNs at d9 after crush; BW245C inhibitor was either not injected, or injected at d2, or injected at d2 and d4). B, BW245C has no effect on $CD8^+$ OT-I cell proliferation in dLNs of crushed Tg SM-OVA mice with or without exogenous OVA.i.m. administration (triplicate experiments, mean±SD).

Figure 5. $CD8^+$ T-cells migrate to the regenerating muscle tissue.

Immunofluorescence staining of the regenerating muscle at d7 after crush injury in WT or SM-OVA mice. A, $CD3\varepsilon^+CD8\alpha^+$ T cells scattered in the endomysium. B, $CD8\alpha^+Perforin^+$ CTLs surrounding regenerating myofibers with internal nuclei. C, Sarcolemmal MHC-I expression. The serial section showed that $MHC\text{-}I^+$ myofiber are closely contacted by $CD8\alpha^+$ cells. D, $CFSE^+$ cells appeared in regenerating muscle after i.v. adoptive transfer of $CD8\alpha^+CFSE^+$ T cells, and some of $CFSE^+$ cell co-expressed Perforin. Detection of $CD45.1^+$ cells confirmed infiltration of OT-I cells in the TgSM-OVA mouse muscle. E, $CD11b^+CD11c^+$ DCs appeared in regenerating muscle. F, $CD11C^+$ DCs closely associated with $CD8a^+$ cells. Bar, 50µm.

Figure 6. Cytokine expression in crushed muscle.

A, $CD8a^+$ cells expressing IFN-γ are transiently detected in the infiltrate at d7. B, most $CD11b^+$ cells already express the immunosuppressive cytokine IL-10 at d7. C, all $CD11b^+$ cells still strongly express the immunosuppressive cytokine TGFβ1 at d15, at time point associated with almost complete healing. Bar, 50µm.