T\textsubscript{reg} cells limit IFN-\(\gamma\) production to control macrophage accrual and phenotype during skeletal muscle regeneration

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Skeletal muscle regeneration is a highly orchestrated process that depends on multiple immune-system cell types, notably macrophages (MFs) and Foxp3\textsuperscript{+}CD4\textsuperscript{+} regulatory T (T\textsubscript{reg}) cells. This study addressed how T\textsubscript{reg} cells rein in MFs during regeneration of murine muscle after acute injury with cardiotoxin. We first delineated and characterized two subsets of MFs according to their expression of major histocompatibility complex II (MHCIIm) molecules, i.e., their ability to present antigens. Then, we assessed the impact of T\textsubscript{reg} cells on these MF subsets by punctually depleting Foxp3\textsuperscript{+} cells during the regenerative process. T\textsubscript{reg} cells controlled both the accumulation and phenotype of the two types of MFs. Their absence after injury promoted IFN-\(\gamma\)-production, primarily by NK and effector T cells, which ultimately resulted in MF dysregulation and increased inflammation and fibrosis, pointing to compromised muscle repair. Thus, we uncovered an IFN-\(\gamma\)-centered regulatory layer by which T\textsubscript{reg} cells keep MFs in check and dampen inflammation during regeneration of skeletal muscle.

Macrophages | T\textsubscript{reg} cells | Muscle repair | Interferon-\(\gamma\) | Muscle regeneration

Skeletal muscle undergoes regeneration at steady state, in muscular dystrophies, and subsequent to acute injury, such as after cardiotoxic (CTX) treatment (1). Muscle damage initiates a highly orchestrated process, in which a pool of generally quiescent muscle progenitor cells, termed satellite cells, are activated (2, 3). Satellite cells undergo asymmetric division, proliferation, and differentiation into postmitotic precursors, which subsequently fuse into multinucleated myotubes. Myotubes then form new, or fuse to existing, myofibers and proceed through a stage of terminal differentiation and growth. While myogenic precursor cells are essential for muscle regeneration, they are not sufficient on their own. Several types of immunocytes also play key roles (4).

Macrophages (MFs) are important players in skeletal muscle repair. Ablation of the myeloid compartment, in particular MFs, by multiple approaches results in defective repair, prolonged necrosis, and increased adipogenesis and fibrosis in the injured muscle (5–7). MFs can sense injury, phagocytose debris, mount an inflammatory response, and promote muscle regeneration via secretion of insulin-like growth factor 1 (IGF1), growth differentiation factor 3 (GDF3) and, likely, other factors (8, 9). The function of muscle MFs changes with their phenotype over the course of regeneration. Proinflammatory MFs accumulate early upon injury and express abundant tumor necrosis factor (TNF)-\(\alpha\) (6), which induces expression of myogenic transcription factors (10) and promotes apoptosis of fibroblastic fibro/adipogenic progenitors (FAPs) (11). Switching of muscle MFs to a proregenerative phenotype results in higher production of transforming growth factor (TGF)-\(\beta\) (6), which stimulates survival of FAPs, with concomitant deposition of cell matrix in the regenerating muscle (11). While the phenotypic switch is necessary for efficient muscle repair (12–14), the mechanisms governing it are largely unknown.

T\textsubscript{reg} cells accumulate in murine skeletal muscle after acute injury, within the dystrophic muscles of mdx mutant mice, and in the muscle of patients with dystrophinopathies (15–17). Punctual depletion of muscle T\textsubscript{reg} cells results in impaired regeneration, prolonged inflammation, and fibrosis (15, 16). Inefficient accumulation of T\textsubscript{reg} cells upon injury also correlates with sarcopenia in aged mice, which can be improved by boosting muscle T\textsubscript{reg} cells via injection of the cytokine, interleukin (IL)-33 (18). Loss of T\textsubscript{reg} cells reduces the effectiveness of muscle progenitor cells, likely reflecting an impact of the growth factor, amphiregulin (15, 17). T\textsubscript{reg} cells also regulate the inflammatory milieu surrounding the regenerating myofibers, notably the phenotype of muscle MFs (15, 16).

Here, we address the impact of muscle T\textsubscript{reg} cells on MF accumulation and phenotype during murine skeletal muscle repair after acute injury. Our findings highlight a critical role for T\textsubscript{reg} cells in reigning in a local IFN-\(\gamma\) response and, thereby, dampening proinflammatory MFs.

Results

Delineation of Distinct Subsets of Skeletal Muscle MFs According to MHCIIm Molecule Expression. Muscle MFs have often been parsed on the basis of Ly6c and/or CX3CR1 expression; however, neither of these markers has shown a strong association with phenotype after acute injury nor relevant in vivo functionality (6, 19). On the basis of potential functional divergence and our preliminary findings (i.e., differential sensitivity to T\textsubscript{reg} loss; see

Significance

Skeletal muscle relies on its regenerative capacity to recover after acute injury. Immune-system cells, notably macrophages and regulatory T cells, play critical roles during muscle regeneration. This study addressed the impact of regulatory T cells on macrophages during muscle repair. In a mouse model of acute injury, regulatory T cells controlled the composition and phenotype of muscle macrophages during muscle repair by limiting production of the inflammatory cytokine, interferon-\(\gamma\), produced by natural killer and effector T cells. Thus, we uncovered an interferon-\(\gamma\)-centered regulatory loop that can be further explored as a gateway to improved muscle therapies.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE110549). Raw data pertinent to interferon-\(\gamma\) response signature can be retrieved from the GEO repository (accession no. GSE110549).

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A Role for Treg Cells in Regulating the Accumulation and Phenotype of Muscle MFs, we chose the gating strategy depicted in Fig. 1A, which culminates in a distinction based on MHCII expression by F4/80hi cells. At steady state, the MHCII+ subset was more abundant than its MHCII− counterpart (Fig. 1B). The MHCII− subset showed more frequent and/or higher expression of CD64, Tim4, CX3CR1, CD206, and MerTK (Fig. S1A), all markers of tissue-resident MFs (20). However, the MHCII+ subset expressed more of the markers associated with migratory MFs: CD11b, CCR2. Lack of substantial Ly6c expression by either subset (Fig. S1C) argues that our gating strategy identified bona fide MFs and excluded monocytes. MHCII− MFs expressed higher levels of Ki67, a marker of cells in cycle (Fig. S1D), suggesting that this subset might have greater self-replenishing capacity, a property of tissue-resident MFs (21).

For a broader view, we compared the transcriptomes of MHCII+ and MHCII− MFs via microarray (Fig. 1C and D). According to pathway enrichment analysis of genes differentially expressed between the two subsets, the MHCII+ MFs were enriched for pathways involved in antigen processing and presentation, cell adhesion molecules (CAMs), and cytokine-cytokine receptor interactions, while the MHCII− MFs were enriched for pathways involved in metabolism, lysosome, and complement and coagulation cascades.

Fig. 1. Two subsets of MFs in skeletal muscle. (A) Gating strategy for cytofluorimetric delineation of skeletal muscle MF subsets at steady state. Numbers refer to fraction of cells within the designated gate. (B) Summaries of cell numbers (Left) and fractions (Right) of the MHCII+ and MHCII− subsets from 6- to 10-wk-old male C57BL/6J mice. Three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 by the unpaired t test. (C) Volcano plot of transcriptomic differences between the MHCII+ and MHCII− MF subsets isolated from skeletal muscle at steady state. Averages of duplicate samples. (D) Pathway enrichment analysis (KEGG) of transcripts from ≥ twofold differentially expressed genes. P values represent maximum EASE score determined according a modified Fisher Exact test (DAVID). Representative genes in these pathways are labeled in C. Cytofluorimetric dot plots (E) and quantification of cell number (F, Left) and frequency (Right) for MHCII+ vs. MHCII− MF subsets during regeneration after acute injury with CTX. Results are from three individual experiments.
Fig. 2. Changes in MF representation and phenotype in the absence of Treg cells. (A, Left) Immunofluorescence imaging of MFs and Foxp3+ Treg cells in a cross-section of TA muscle at day 7 after injury with CTX. Original magnification 400x and 4x zoom. White arrows: Treg cells and MFs within touching proximity. (A, Right) Quantification of the distances between Treg cells and the nearest MF on day 7 after injury. Measured manually from 20 separate field views from three mice. (B) Continuous Treg cell depletion regimen. (C-F) Analysis of diverse parameters following the regimen depicted in B: fraction of Foxp3+ Treg cells (C), number of CD45+ cells per gram tissue (D); representative dot plots (E, Left) and quantification of MHCII+ to MHCII− MF subset ratio (Right); representative dot plots and summary quantification of fraction of EdU+ MFs after a 4-h pulse just before tissue collection (F). DTR+; Foxp3+DTR−; DTR−; Foxp3+DTR+ littersmates. Numbers on dot plots refer to fraction of cells within the designated gates. Results are from three independent experiments. Statistics as per Fig. 1B. (G) Volcano plot comparing transcriptomes from MHCII+ MFs of Treg-depleted (as per B) vs. nondepleted mice. (H) List of top pathways (KEGG) enriched in the sets of genes ≥twofold differentially expressed (P < 0.05). Representative genes in these pathways are indicated in G. Statistics are as per Fig. 1D. ***P < 0.001.

expressed ≥twofold, the MHCII+ subset up-regulated transcripts encoding molecules involved in antigen processing and presentation (H2-Abl, Ciita), costimulation (Icosl), phagosomes (CD209a), and cytokine–cytokine receptor interactions (Il6, Il1r1, Ilr1l1) (Fig. 1 C and D). However, the MHCII− subset up-regulated transcripts associated with lysosomes (Cd63, Hexb, Cisl), glycosaminoglycan degradation (Hyal, Hgcd), metabolic pathways (Hyal, Hexb, Arg2, Aldh2), phagosomes (Ctsl, Msr1), and complement and coagulation cascades (Vsig4) (Fig. 1 C and D). While both MF types showed enrichment in transcripts involved in the phagosome pathway, different genes were involved in the two subsets. The MHCII+ subset also turned up transcripts encoding the growth factor, GDF3 (Fig. 1C), a PPARY-dependent factor that potentiates muscle regeneration in vivo and promotes myofiber fusion in vitro (further discussed in Discussion) (9).

There was little partitioning of the classical M1- and M2-type gene signatures (derived from Jablonski et al.; ref. 22) between the two subsets (Fig. S1F). There was more evident partitioning of certain gene signatures distilled by Xue et al. (23) for a set of polarization states of in vitro differentiated MFs: The MHCII− subset showed greater expression of the signature in response to ultrapure LPS + immune complexes and to glucocorticoids (Fig. S1F).

Both the MHCII+ and MHCII− MF subsets could be identified in normal skeletal muscle (Fig. S2A). They often accumulated
A Role for T<sub>reg</sub> Cells in Regulating the Accumulation and Phenotype of Muscle MFs. MFs and T<sub>reg</sub> cells colocalized in the regenerating areas of injured muscle, as illustrated for the tibialis anterior (TA) on day 7 after CTX treatment (Fig. 24). Most T<sub>reg</sub> cells occurred within 10 µm of a MF; in many cases, the two cell types were within touching distance.

To evaluate their impact on muscle MFs during the repair process, we punctually ablated T<sub>reg</sub> cells in mice expressing the diphtheria toxin receptor (DTR) under the dictates of Foxp3 regulatory elements [Foxp3-IRES-GFP-hDTR (Foxp3<sup>DTR</sup>)] (25). Every-other-day i.p. administration of diphtheria toxin (DT) during the week after CTX-induced injury (schematized in Fig. 2B) effectively reduced T<sub>reg</sub> cells (Fig. 2C), resulting in a concomitant increase in CD45<sup>−</sup> cells (Fig. 2D). The MHCII<sup>+</sup> MF subset was strongly diminished during this same time period (Fig. 2E). An increased MHCII<sup>+</sup> to MHCII<sup>+</sup> MF ratio was not evident until day 7 (Fig. S44). To assess how proliferation might contribute to this effect, we administered a 4-h pulse of 5-ethyl-2′ deoxyuridine (EdU) on day 7 after injury to Foxp3<sup>DTR</sup>- (T<sub>reg</sub>-depleted) vs. Foxp3<sup>DTR+</sup>- (T<sub>reg</sub>-replete) mice. MHCII<sup>+</sup> MFs proliferated more actively in the absence of T<sub>reg</sub> cells (Fig. 2F). There were too few MHCII<sup>+</sup> MFs in the DTR<sup>+</sup> mice to permit a parallel analysis of this subset.

Cytofluorometric analysis of muscle MFs using canonical markers of tissue-resident MFs (20) revealed a decrease in expression of CD206, CX3CR1, and Tim4 in the MHCII<sup>+</sup> subset in Foxp3<sup>DTR+</sup>- compared near autofluorescent myofibers, previously reported to be atrophic (24) (Fig. S2B).

We then asked how the two MF subsets evolved after acute injury of skeletal muscle with CTX. There was a rapid accumulation of MHCII<sup>−</sup> MFs, peaking in number at day 2 and declining to preinjury levels by day 7, while MHCII<sup>+</sup> MFs peaked in number at day 4 (Fig. 1E). Proportions of the two subsets reversed in dominance on days 1 and 2 but were restored to steady-state levels by day 7 (Fig. 1F). Transcriptome analyses revealed that the two MF subsets maintained their phenotypic distinction throughout the regeneration process, as signaled by enrichment of their respective steady-state signatures at all time-points (Fig. S34).

Thus, we have delineated two phenotypically distinct subsets of MFs within steady-state skeletal muscle. While the MHCII<sup>−</sup> subset seemed to be involved in homeostatic functions such as metabolism and degradation, the MHCII<sup>+</sup> subset had the potential to be more engaged with other immunocytes via its capacity to present antigens and express chemokines. The latter MF type predominated in the later phases of acute muscle injury.

**Fig. 3.** An enhanced IFN-γ response in muscle MHCII<sup>−</sup> MFs in the absence of T<sub>reg</sub> cells. (A) Volcano plot as in Fig. 2H, except the IFN-γ response up-signature, detailed description in Materials and Methods, is highlighted in red. (B) DT injection regimens for short-term (24-h) depletion of T<sub>reg</sub> cells at early or late phases of regeneration after injury with CTX, followed by analysis at the indicated time-points. Fraction of Foxp3<sup>+</sup>CD4<sup>+</sup> T<sub>reg</sub> cells (C); number of CD45<sup>−</sup> cells per gram of muscle (D); representative dot plots and summary quantification of MF subset ratios (E). Numbers on dot plots refer to fraction of cells within the designated gates. Data are from at least two independent experiments. Statistics are as in Fig. 1E. (F) Volcano plots of transcriptomes from MHCII<sup>−</sup> MFs of DTR<sup>+</sup> vs. DTR<sup>−</sup> mice upon short-term depletion of T<sub>reg</sub> cells beginning at day 1 or 7. Highlighted in red is the IFN-γ response up-signature, as in A. Averages of two to three replicates per group. P values according to the χ² test. ***P < 0.001.
DTR cells were required to rein in IFN-γ. We then turned to identifying the mechanisms by which Treg cells regulated MF subset proportions and properties during muscle regeneration. Pathway analysis of transcripts overrepresented in MHCI⁺ MFs in the absence of Treg cells pointed to an elevated response to IFN-γ (Fig. 2F). This notion was confirmed by overlaying an IFN-γ response up-signature onto a volcano plot of MHCI⁺ MF transcripts from DT-treated Foxp3<sup>DTR⁺</sup> vs. Foxp3<sup>DTR⁻</sup> mice (Fig. 3A). Analysis of the trajectory of the IFN-γ response signature revealed clusters of genes up-regulated in both MHCI⁺ and MHCI⁻ MFs at days 0, 1, 4, and 7 after injury (Fig. S5A). However, a large portion of the IFN-γ-induced signature genes was coregulated and highly overrepresented essentially only in MFs from Foxp3<sup>DTR⁺</sup> mice.

Next, we asked whether the IFN-γ response of MHCI⁺ MFs in Treg-depleted mice reflected an accumulated effect throughout the 7 d of DT treatment, or whether shorter windows of Treg insufficiency had a similar effect, as schematized in Fig. 3B. Short-term (24-h) DT treatment on either day 1 or 7 after injury depleted Treg cells, although seemingly more effectively at the earlier time-point (Fig. 3C). However, neither the accumulation of CD4<sup>+</sup> cells (Fig. 3D) nor the proportions of the two MF subsets (Fig. 3E) were significantly affected by these shorter Treg depletion regimens. Nonetheless, there was substantial up-regulation of the IFN-γ response up-signature by the MHCI⁺ MFs upon short-term Treg cell depletion on day 7, i.e., the late phase of repair. Notably, Cd274 transcripts, encoding PD-L1, a diagnostic IFN-γ-inducible gene, were clearly enriched. These data indicated that Treg cells were required throughout the process of regeneration to limit an overexuberant MHCI⁺ MF response to IFN-γ produced in the context of regeneration.

Sources of Muscle IFN-γ and Their Regulation by Treg Cells. To investigate which muscle lymphocytes produced IFN-γ during regeneration, we analyzed the dynamics of NK and effector T cell generation, we analyzed the dynamics of NK and effector T cell production, we depleted them during either an early or late window of regeneration, compared with a continuous 1-wk depletion (regimens schematized in Fig. 5A). Despite a partial recovery of Treg cells by day 7 (Fig. 5B), early ablation resulted in immunological effects similar to those of weeklong ablation: an increase in numbers of CD4<sup>+</sup> cells (Fig. 5C), and an augmentation in IFN-γ-producing NK, CD4<sup>+</sup> conventional (Conv), and CD8<sup>+</sup> T cells (Fig. 5D). In contrast, late ablation of Treg cells had no significant impact on these parameters (Fig. 5C and D) although the Treg cell compartment rebounded less than after early depletion (Fig. 5B).

In brief, muscle Treg cells were important in restraining NK and T cells and their potential to produce IFN-γ during regeneration. The Treg cell effect on IFN-γ production required their presence early but not late after injury.

MHCI⁺ MFs Contributed to Type 1 Inflammation During Muscle Repair. Since Treg cells controlled the proportion and phenotype of MFs during muscle regeneration, we asked whether antigen-presenting MFs played a role in the type 1 inflammation also stabilized the MF phenotype during the successive inflammatory and reparative phases of muscle regeneration.

During Muscle Regeneration, Treg Cells Limited the Response of Local MFs to IFN-γ. We then turned to identifying the mechanisms by which Treg cells regulated MF subset proportions and properties during muscle regeneration. Pathway analysis of transcripts overrepresented in MHCI⁺ MFs in the absence of Treg cells pointed to an elevated response to IFN-γ (Fig. 2F). This notion was confirmed by overlaying an IFN-γ response up-signature onto a volcano plot of MHCI⁺ MF transcripts from DT-treated Foxp3<sup>DTR⁺</sup> vs. Foxp3<sup>DTR⁻</sup> mice (Fig. 3A). Analysis of the trajectory of the IFN-γ response signature revealed clusters of genes up-regulated in both MHCI⁺ and MHCI⁻ MFs at days 0, 1, 4, and 7 after injury (Fig. S5A). However, a large portion of the IFN-γ-induced signature genes was coregulated and highly overrepresented essentially only in MFs from Foxp3<sup>DTR⁺</sup> mice.

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induced by acute injury. To address this question, we used mice with Lyz2-promoter/ enhancer-driven ablation of H2-Ab1 gene expression (MF-MHCII<sup>−/−</sup>) (Fig. 6A). These mice and their wild- type littermates (MF-MHCII<sup>+/+</sup>) showed comparable accumulation of CD4<sup>+</sup> Foxp3<sup>+</sup> <sub>T<sub>reg</sub></sub> and Foxp3<sup>+</sup> <sub>T<sub>conv</sub></sub> cells (Fig. 6B). However, there was a detectable decrease in the fraction of IFN-γ<sup>+</sup>CD4<sup>+</sup> <sub>T<sub>conv</sub></sub> cells and in total numbers of IFN-γ<sup>+</sup> NK and CD4<sup>+</sup> <sub>T<sub>conv</sub></sub> cells in the mutant mice (Fig. 6C). Since IFN-γ is known to induce MHCII expression in diverse cell types, these results suggest the existence of a positive feedback loop between accumulation of MHCII<sup>+</sup> MFs and IFN-γ<sup>+</sup> leukocytes.

**Effects of IFN-γ on Repair of Skeletal Muscle.** Increased production of and response to IFN-γ in the absence of <sub>T<sub>reg</sub></sub> cells led us to directly investigate the role of IFN-γ during muscle regeneration. We i.v.-injected recombinant (r)IFN-γ into mice on days 4 and 6 after CTX-induced injury, and asked to what extent IFN-γ, alone, could mimic the effects of <sub>T<sub>reg</sub></sub>-cell ablation (Fig. 7A and B). Expression of PD-L1, an IFN-γ-inducible gene, was augmented in muscle MFs of IFN-γ<sup>+</sup>-treated mice (Fig. 7C). There was also an increase in the CD45<sup>+</sup> population (Fig. 7D) and elevated numbers of IFN-γ<sup>−</sup>/NK and CD4<sup>+</sup> <sub>T<sub>conv</sub></sub> cells (Fig. 7E). Histological analysis of inflammation and fibrosis revealed that rIFN-γ-treated (at 10 μg per injection) mice had a significantly worse inflammation score (Fig. 7F) and a trend toward a higher fibrosis score (Fig. 7G) on day 7 of regeneration, which are indicators of ineffective muscle repair (26).

These data showed that injection of IFN-γ alone could at least partially mimic the proinflammatory, antiregenerative effects of <sub>T<sub>reg</sub></sub> cell ablation. Moreover, IFN-γ injection increased local production of IFN-γ.

**Discussion**

Acute injury of skeletal muscle provokes rapid accumulation of <sub>T<sub>reg</sub></sub> cells, which prevent excessive buildup of inflammatory myeloid cells and fibrosis in the regenerating muscle (15). This study has refined our view of skeletal muscle MF subsets based on expression of F4/80 and MHCII. Most importantly, it established that <sub>T<sub>reg</sub></sub> cells keep inflammation in check by limiting IFN-γ production in regenerating muscle. This finding raises a number of issues worthy of discussion.

First, skeletal muscle MFs have thus far been characterized as a bulk CD11b<sup>+</sup> population that expresses either the Ly6c or CX3CR1 surface markers (6, 19). This delineation not only failed to identify bona fide MFs but did not uncover functionality associated with expression of either marker through muscle regeneration, for example (like us) no clear partitioning into M1 and M2 populations (19). By gating on F4/80<sup>+</sup>/MHCII<sup>+</sup> MFs and parsing them according to MHCII expression, an indicator of antigen presentation, we identified two subsets of MFs that exhibited different transcriptional programs and, therefore, potential functions at steady state. Non-uniformity in expression of CD64, Tim4, CX3CR1, and CD11c within the MHCII<sup>+</sup> subset did suggest additional distinctions, but profiling at the single-cell level would be needed to fully resolve this heterogeneity. Cytokine/secretome and transcriptional analyses of the two MF subsets indicated that MHCII<sup>+</sup> MFs were primarily proinflammatory, in particular, by promoting inflammation during muscle repair. In contrast, MHCII<sup>−</sup> MFs showed enrichment in expression programs associated with tissue maintenance and proradiprogenitive factors, such as GDF3. Thus, parsing MF subsets according to markers with a direct functional readout (i.e., antigen presentation) was an effective initial step toward understanding their heterogeneity and function.

Second, <sub>T<sub>reg</sub></sub> cells were found in close proximity to MFs and were required to preserve MF subset proportions and properties. The disproportion in MF subsets in the absence of <sub>T<sub>reg</sub></sub> cells could potentially reflect increased proliferation of the MHCII<sup>+</sup> subset, greater conversion of the MHCII<sup>−</sup> cells, and/or death of the MHCII<sup>−</sup> cells. Indeed, the MHCII<sup>−</sup> subset had an elevated proliferation rate in the absence of <sub>T<sub>reg</sub></sub> cells. In addition, <sub>T<sub>reg</sub></sub> cells were essential for reining in NK and effector

![Fig. 5.](www.pnas.org/cgi/doi/10.1073/pnas.1800618115)
T cells poised to produce IFN-γ, which is known to activate the transcription factor, CIITA, resulting in up-regulation of MHCII expression in MFs (27). Thus, by limiting local IFN-γ production, T<sub>reg</sub> cells could prevent conversion of MHCII<sup>−</sup> MFs to other MF subsets. Gain-of-function experiments showed that systemic injection of rIFN-γ partially recapitulated the effects on MF subset accumulation and phenotype observed in T<sub>reg</sub>-depleted mice. Third, while the presence of NK cells in regenerating skeletal muscle has been noted by a few groups (17, 28), their functional importance remains a question. We discovered that muscle NK cells had a critical time window of efficacy. Punctual T<sub>reg</sub>-ablation protocols demonstrated that accumulation of T<sub>reg</sub> cells was required early (initial few days after injury) to keep late-stage inflammation in check, in particular excessive local IFN-γ production by NK and effector T cells. It has been reported that IFN-γ is required for muscle regeneration after acute injury: it accumulates in the late phase of muscle repair, and its blockade or genetic deficiency inhibits regeneration (28). Furthermore, supplementation of IFN-γ in a laceration model reduced fibrosis and improved muscle function (33). However, it has also been demonstrated that IFN-γ can be detrimental in models of chronic muscle injury, wherein inflammation and fibrosis persist (34). It directly inhibited expression of the Myog gene, which encodes a crucial muscle differentiation factor up-regulated in the late stages of muscle regeneration, thereby dampening myogenesis (35, 36). We hypothesize that IFN-γ, while required for effective regeneration, must be tightly controlled temporally and quantitatively to prevent overt inflammation and persistent damage.

Acute skeletal muscle injury is common in sports and other traumatic accidents. A role for the immune system in potentiation of muscle repair is by now quite apparent (7, 15, 16). Immunotherapies are increasingly espoused in a broad range of clinical contexts. The T<sub>reg</sub>-specific cell loop orchestrated through IFN-γ might provide a path to muscle immunotherapy.

**Materials and Methods**

**Mice.** C57BL/6J mice were purchased from Jackson Laboratory. B6.129P2-Lyz2<sup>tm5挑v/j</sup> (Ly5<sup>+</sup>) and B6.129 x 1-H2-Ab1<sup>tm1Koni</sup>/J (MHCII<sup>lox/lox</sup>) mice were purchased from Jackson Laboratory and bred in our colony to generate MF-MHCII<sup>++</sup> and MF-MHCII<sup>−</sup> littermates. Foxp3<sup>−</sup>-IRE6-GFP (Foxp3<sup><sup>cre</sup></sup>) mice were obtained from V. Kuchroo, Brigham and Women’s Hospital, Boston. Foxp3<sup>−</sup>-IRE6-GFP/DT (Foxp3<sup><sup>cre</sup></sup>) mice were obtained from A. Rudensky, Memorial Sloan-Kettering Cancer Center, New York. Six to 10-week-old male and female mice were used for all experiments, unless otherwise indicated. All mice were housed in our specific pathogen-free facility at Harvard Medical School. All experiments were conducted under protocols approved by Harvard Medical School’s Institutional Animal Care and Use Committee.

Foxp3<sup>−</sup>- and Foxp3<sup><sup>cre</sup></sup>- litters were i.p.-injected with DT (Sigma-Aldrich, D5654) in phosphate-buffered solution (PBS) at 25 ng/ml or 50 ng/ml of body weight for continuous or short-term T<sub>reg</sub> cell-depletion regiments, respectively. Protocols of DT administration for the different experiments are schematized in the corresponding figures. Male littersmates were used in these experiments unless otherwise indicated. For in vivo rIFN-γ administration, 5–10 μg of IFN-γ (Biolegend) were i.v.-injected into mice in sterile PBS, according to the protocols detailed in the corresponding figures.

For acute injury, mice were anesthetized with avertin (0.4 mg/g body weight) and injected with 0.03 ml per muscle of Naja mossambica CTX (0.03 mg/ml; Sigma-Aldrich) in one or more hindlimb muscles (TA, gastrocnemius, quadriceps), as previously described (15).

**Isolation and Analysis of Muscle Leukocytes.** Hindlimb muscles were excised, minced, and digested at 37 °C for 30 min in a solution containing collagenase II (2 mg/ml; Thermo Fisher Scientific), DNase I (150 μg/ml; Sigma-Aldrich), and Dulbecco’s modified Eagle media (DMEM) without phenol red (Thermo Fisher Scientific). Digested muscle was passed through a 70 μm filter and washed in DMEM without phenol red/2% FBS/2 mM EDTA. To separate the leukocyte fraction, we resuspended the digested muscle in 40% Percoll (Sigma-Aldrich), underlain with 80% Percoll, and spun for 25 min at 1,126 × g (without breaks). The interphase containing leukocytes was recovered, washed, and stained for analysis or sorting by flow cytometry.

Samples were stained with the following antibodies: anti-CD3 (145-2C11), antibodies (2C1; Thermofisher Scientific), CD4 <br>(GK1.5), CD8 (53-5-8), CD11b (M1/70), CD11c (N418), CD45 (30-F11), CD206 (SN52), CCR5 (4A9), CD3e (145-2C11), CD40 (2E6), CD206 (2C9), CCR7 (SDC1), CD45 (30-F11), and CD8<sup>-</sup> (53-5-8). Fluorescent data were acquired with a 4-laser LSR II or 4-laser Fortessa (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

For in vivo proliferation experiments, 1 mg of EdU (Thermo Fisher Scientific) was administered i.p. 4 h before analysis. After the tissue was collected, EdU detection was done after fixation and permeabilization using the...
Twenty thousand cells were double-sorted into staining, muscle infiltrates were collected at the penicillin/Biolegend, 10,000 U IFN-μ response up-regulated signature was derived from transcriptomes GolgiPlug (BD Biosciences) in complete βL**DTR** cell loss (DTR**www.pnas.org/cgi/doi/10.1073/pnas.1800618115 except fibrosis was assessed. All statistics as per Fig. 1 T cells per gram of muscle. (Injection. (P**to compensate for T**7**histology. As per PD-L1 to sites of inflammation. (Original magnification: 50

Fig. 7. Partial complementation of T**reg cell loss by IFN-γ injection. (A) Strategy for evaluating the ability of IFN-γ to compensate for T**reg cell loss (DTR**+). Numbers indicate fraction of cells in the designated gate. (B) Representative dot plots (Left) and summary quantification of MF subset ratios (Right). Results are from three independent experiments. (C) H&E histology: representative images (Left) and inflammation score (Right). Black arrows point to sites of inflammation. (Original magnification: 50x.) (Scale bars: 200 μm.) Scoring was assessed by two-independent, blinded scorers. (G) Gomori Trichrome histology. As per F except fibrosis was assessed. All statistics as per Fig. 18. *P < 0.05; **P < 0.01.

Foxt3 Fix/Perm buffer set (ebioscience). The Click-iT EdU (Thermo Fisher Scientific) reaction was performed according to the manufacturer’s instructions. Ki67 was stained intracellularly using the Foxt3 Fix/Perm buffer set.

For ex vivo intracellular IFN-γ staining, muscle infiltrates were collected at the indicated time-points after injury, and single-cell suspensions were stimulated for 3.5 h at 37 °C with 50 ng/ml phorbol 12-myristate 13-acetate, 1 μM ionomycin (both Sigma-Aldrich), and 1x GolgiPlug (BD Biosciences) in complete RPMI-1640 media, containing 10% FBS (Thermo Fisher Scientific), 1x penicillin/ streptomycin (Gemini Bio Products), 10 mM sodium pyruvate (Thermo Fisher Scientific), 55 μM β-mercaptoethanol (Sigma-Aldrich), 2 mM L-glutamine (Thermo Fisher Scientific). Cells were stained with fixable viability dye Yellow as above.

Transcriptome Analyses. Twenty thousand cells were double-sorted into TRizol (Thermo Fisher Scientific). All samples were generated in duplicate or triplicate. Sample processing and data analysis were performed as previously described (37). Extracted and amplified RNAs were hybridized to GeneChip Mouse Genome M1.0 ST chip arrays V2 (Affimetrix) according to the manufacturer’s protocol described in Mostafavi et al. (40). Cells were sorted and processed according to Immgen protocol for microarray analysis (41). Raw data can be retrieved from NCBI repository (superseries

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Histology. Injured TA muscles were collected and fixed in 10% formalin. Tissues were processed at the Rodent Histopathology Core at Harvard Medical School for H&E (for inflammation) and Gomori’s trichrome (for fibrosis) staining. Two sections, 200 μm apart, were recovered and stained for analysis. Images were acquired with a Zeiss Axio Imager.M1 microscope. Inflammation and fibrosis scorings were done blindly by two independent investigators. Averaged scores from the dual assessments were reported. Scoring scales: 0; none; 1; mild; 2; moderate; 3; moderate; 4; severe.

Statistical Analyses. Data were routinely presented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 from the unpaired t test using GraphPad Prism software. Volcano plot enrichment P values were determined via the χ^2 test using Microsoft Excel. Only significant P values are indicated on the various plots.

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