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Functional Tolerance of CD8+ T Cells Induced by Muscle-Specific Antigen Expression

Sébastien Calbo,2* Héloïse Delagrèverie,* Christophe Arnoult,* François-Jérome Authier,* François Tron,* and Olivier Boyer2*

Skeletal muscles account for more than 30% of the human body, yet mechanisms of immunological tolerance to this tissue remain mainly unexplored. To investigate the mechanisms of tolerance to muscle-specific proteins, we generated transgenic mice expressing the neo-autoantigen OVA exclusively in skeletal muscle (SM-OVA mice). SM-OVA mice were bred with OT-I or OT-II mice that possess a transgenic TCR specific for OVA peptides presented by MHC class I or class II, respectively. Tolerance to OVA did not involve clonal deletion, anergy or an increased regulatory T cell compartment. Rather, CD4+ T cell tolerance resulted from a mechanism of ignorance revealed by their response following OVA immunization. In marked contrast, CD8+ T cells exhibited a loss of OVA-specific cytotoxic activity associated with up-regulation of the immunoregulatory programmed death-1 molecule. Adoptive transfer experiments further showed that OVA expression in skeletal muscle was required to maintain this functional tolerance. These results establish a novel asymmetric model of immunological tolerance to muscle autoantigens involving Ag ignorance for CD4+ T cells, whereas muscle autoantigens recognized by CD8+ T cells results in blockade of their cytotoxic function. These observations may be helpful for understanding the breakage of tolerance in autoimmune muscle diseases. The Journal of Immunology, 2008, 181: 408–417.

Tolerance reflects the ability of the immune adaptive system to mount a highly specific response against invading pathogens while functionally ignoring self-molecules. The physical elimination of autoreactive T cells during thymocyte development is the primary mechanism used by the immune system to establish such self-tolerance (1, 2). Because many self-proteins are not expressed in the thymus (3), additional peripheral mechanisms are needed to prevent the activation of autoreactive T cells. For instance, the immune system can either ignore tissue-specific self-Ags (4) or develop active mechanisms of self-tolerance that rely on suppression (5, 6), physical elimination (7, 8), or functional inactivation of mature autoreactive T cells (9).

Skeletal muscles account for 30–50% of the human body. However, little is known about the mechanisms of immunological tolerance to this tissue. Skeletal muscle fibers do not express detectable levels of MHC class I (10–12), preventing direct presentation of autoantigenic peptides to self-reactive T cells, although regenerative myogenic cells may express a low level of MHC class I transiently during muscle repair (12). Therefore, ignorance is generally considered as the main tolerance mechanism for muscle Ags, although this concept has not been formally demonstrated. In support of this assumption, overexpression of MHC class I is an early event in many autoimmune diseases, in particular in tissues that express low or no MHC class I molecules like muscle (13, 14), pancreatic β cells (15), or thyroid (10). In human inflammatory myositis (16) or a group of autoimmune muscle diseases such as polymyositis, dermatomyositis, or inclusion-body myositis, the early and widespread appearance of MHC class I on the surface of muscle fibers, even at sites distant from leukocyte infiltration, is a striking feature (17, 18). Moreover, in patients with polymyositis, endomysial inflammatory infiltrates consisting mainly of oligoclonal expansions of CD8+ T lymphocytes are found in muscle biopsy specimens (19, 20), suggesting the recognition of muscle autoantigens upon expression of MHC class I molecules. The muscle tissue can also be the target of pathogenic autoantibodies in diseases such as myasthenia gravis in which they bind to the acetylcholine receptor (AChR)3 and block neurotransmission (21).

To investigate the immunological consequences of skeletal muscle-specific Ag expression and to get insight into the mechanisms of tolerance to muscle-specific proteins, we have generated transgenic (Tg) mice expressing the neo-autoantigen OVA exclusively in skeletal muscle (SM-OVA mice). SM-OVA mice were bred with OVA-specific TCR Tg mice, OT-I or OT-II, restricted by MHC class I or class II, respectively. In F1 offspring, no signs of inflammatory myositis were detected. Interestingly, neither central nor peripheral deletion nor anergy was evidenced. Instead, we showed on the one hand that autoantigen immunization induced OVA-specific CD4+ T cell response, and in contrast that muscle-specific autoantigen expression dramatically impaired the capacity of OVA-specific CD8+ T cells to mount a cytotoxic response.

3 Abbreviations used in this paper: AChR, acetylcholine receptor; PD-1, programmed death-1; PD-L1, PD-L1 ligand 1; Tg, transgenic; MCK, muscle creatine kinase; DTH, delayed-type hypersensitivity; Treg, regulatory T cell; LN, lymph node; β-gal, β-galactosidase.

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Moreover, the frequency of OVA-specific CD8\(^+\) T cells expressing programmed death 1 (PD-1) was increased in SM-OVA mice, suggesting a role for PD-1 in this functional tolerance. Therefore, our results demonstrate that tolerance to muscle autoantigens is asymmetric, involving ignorance for CD4\(^+\) T cells and the loss of cytotoxic function for CD8\(^+\) T cells.

**Materials and Methods**

**Mice**

C57BL/6J/d mice were purchased from Charles River Breeding Laboratories. OT-I (22) and OT-II (23) TCR Tg mice were purchased from Iffa Credo. CD3\(^-\)/deficient mice were purchased from Centre de Développement des Techniques Avancées. SM-OVA Tg mice were created using a construct containing the short form of the muscle creatine kinase (MCK) enhancer or promoter sequence (Mammides \(\sim\)1256 to \(\sim\)1 bp; GenBank accession no. AF188002), which was a gift of B. Derijard (University of Nice, Nice, France) and the full-length chicken OVA cDNA (GenBank accession no. V00383.1), which was a gift of N. Grabi (Harvard Medical School, Boston, MA). Mice were cloned upstream to H-2D\(^+\) transmembrane region, a gift of M. Jenkins (University of Minnesota, Minneapolis, MN) in pBuser script II (Strategene) (24). To abolish expression in the cardiac muscle, a triple mutation in the E-box (25) was introduced by PCR mutagenesis (QuikChange; Stratagene). The construct was then fully sequenced. A sulf-HindIII fragment containing these elements was microinjected into C57BL/6 fertilized eggs by the Service d’Expe rimentation Animale et de Transgénese (Centre National de la Recherche Scientifique). To detect the transgene, genomic DNA was analyzed by PCR to identify a 170-bp fragment using OVA-specific primers (5'GGGAAGAAGGGCGATGAGG-3' and 5'H11032/3GATGTCATCAGGCAACAGCA). Mice were kept in specific pathogen-free housing and were analyzed between 6 and 15 wk of age. All mice were used in accordance with Regional Animal Care and Use Committee procedures.

**Immunohistochemistry**

Tissues were treated as described (13). Sections were incubated with biotin-conjugated anti-H-2K\(^b\) Ab (clone AE3-88.5; BD Pharmingen) followed by streptavidin HRP (DakoCytomation) and 3-amino-9-ethylcarbazole substrate (Vector Laboratories) or by biotin-conjugated anti-OVA Ab followed by streptavidin HRP (DakoCytomation) and 3-amino-9-ethylcarbazole substrate (Vector Laboratories) or by biotin-conjugated anti-OVA Ab followed by streptavidin Alexa Fluor 568 red (Molecular Probes).

**Flow cytometry**

Abs anti-CD8 (clone 53-6-7), anti-CD4 (clone GK1.5), anti-V\(\alpha\)2 (clone B20.1), anti-FoxP3 (clone FJK-16S), anti-CD122 (clone TM-b1), anti-CD44 (clone IM7), anti-IFN-\(\gamma\) (clone XMG1.2), anti-perforin (ebioMake D), anti-granzyme B (clone 1666), anti-CD279 (clone J43) and secondary Abs were purchased from eBioscience. Cells were analyzed with a FACSCalibur apparatus (BD Biosciences) and Flowjo software (Tree Star).

**Proliferation assays**

Lymph node (LN) cells from immunized mice (5 \(\times\)10\(^4\) per well) and spleen cells from OT-I or OT-II mice (10\(^5\) per well) were cultured in triplicate in 96-well round-bottom plates in DMEM with 10% FCS for 2–3 days, in the presence of [\(^3\)H]thymidine (1 \(\mu\)Ci/well) for the last 20 h. Level of radioactivity was determined by liquid scintillation counting.

**Immunization and delayed-type hypersensitivity (DTH) response**

Mice were s.c. immunized with 100\(^6\) CD8\(^+\) T cells from Rag2KO/OT-I were transferred i.v. in Rag2KO or Rag2KO/SM-OVA mice, and analyzed 6 wk later for PD-1 expression.

**Tumor cells and tumor protective immunity**

E.G7-OVA cells was a gift from B. Combadiere (Hospital Pitié-Salpêtrière, Paris, France). These cells were already described (27). Fourteen days after immunization, five mice per group were challenged with a lethal dose (10\(^6\)) of viable E.G7-OVA cells injected s.c. in 0.1 ml of PBS in the right flank. Tumor growth was monitored three times a wk for 30 days, to evaluate tumor-protective immunity. Mice were euthanized at day 30.

**Results**

**Muscle-specific OVA transgene expression**

We developed Tg mice expressing a membrane-bound form of OVA (24) under the control of the MCK promoter (28). To avoid the potential development of myocarditis, we used a mutated form of the MCK promoter named MCK-3E with no cardiac expression (25). SM-OVA Tg mice were generated using a Tg construct comprising the leader sequence from the H-2K\(^b\) gene, the full-length OVA cDNA, and the H-2D\(^b\) transmembrane sequence under the control of the MCK-3E promoter (Fig. 1A). OVA mRNA was detected by RT-PCR only in skeletal muscle and was not found in thymus, spleen, heart, brain, kidney, intestine, lung, and liver (Fig. 1B). The presence of OVA protein was verified by western blot from a muscle protein extract (data not shown) and by immunohistochemistry (Fig. 1C), revealing OVA protein expression at the membrane of most muscle fibers.

**SM-OVA mice are tolerant to OVA**

SM-OVA mice were healthy and bred normally. To study T cell tolerance to a muscle-expressed autoantigen, SM-OVA mice were bred to OT-I or OT-II TCR Tg mice. The TCR from OT-I mice (V\(\alpha\)2 V\(\beta\)5) is specific for OVA257–264 (SIINFEKL) peptide presented by H-2K\(^b\) (22) while the TCR from OT-II mice (V\(\beta\)5 V\(\beta\)8) recognizes OVA\(_{223–239}\) (ISQAIVAAHAEHINAGR) peptide associated with I-A\(^b\) (23). OT-I/SM-OVA and OT-II/SM-OVA double Tg mice were healthy and histological analysis of muscle section did not reveal any sign of inflammation such as leukocyte infiltrates (Fig. 2A) or MHC class I expression on muscle fibers (Fig. 2B). Therefore, T cells from SM-OVA mice are tolerant to OVA. We then determined at which level the mechanism of tolerance was taking place.
whether thymic clonal deletion participated to OVA tolerance. The frequency and number of cells in each thymic developmental subset were indistinguishable in single and double Tg mice (Fig. 2, C and D). Moreover, there was no decrease in the TCR expression level on single positive or double positive cells (data not shown). Therefore, there is no central negative selection against OVA protein in SM-OVA mice.

In the periphery, presentation of autoantigens can lead to the deletion of autoreactive T cells (32, 33). We thus analyzed the frequency and number of CD4+ and CD8+ subsets in spleen and LN of single and double Tg mice. Again, no difference was observed in the fraction (data not shown) and number of cells in single and double Tg mice (Fig. 2E). Therefore, there is no deletion of OVA-specific T cells in SM-OVA mice, and central or peripheral deletion is not the main tolerance mechanism operating toward muscle-expressed autoantigens.

Because OVA-specific T cells are not deleted and that MHC molecules are not expressed on muscle fibers, it is possible that a mechanism of tolerance by ignorance, defined by the nondetection of Ag by the immune system, is prominent in this model. However, and interestingly, we observed a statistically significant increase in the frequency of memory-type CD8+ T cells in OT-I/SM-OVA double Tg mice as compared with OT-I mice (Fig. 2F, \( p < 0.00001, n = 20 \)), whereas such augmentation was not found in the CD4+ T cell subset in OT-II/SM-OVA double Tg mice as compared with OT-II mice (data not shown). Indeed, \( \alpha \nu 2^+ \) CD8+ memory T cells defined as CD122highCD69high and Ly6C+ were readily detectable from the age of 6 wk (Fig. 2F). In contrast to CD4+ T cells, this increase in CD8+ memory T cells indicates that CD8+ T cells are not ignorant toward muscle-expressed autoantigens and that these cells encounter their cognate Ag in vivo. As MHC class I molecules are not detected on muscle fibers, one could postulate that OVA presentation to CD8+ T cells is due to re-expression of MHC class I on regenerating muscle fibers (12) or is mediated through a mechanism of cross-presentation (33). However, in absence of costimulation, this in vivo interaction could render OVA-specific T cells anergic and prevent them from proliferating (34). We thus investigated this possible mechanism of tolerance by determining T cell reactivity against OVA.

**OVA specific CD4+ T cells can respond to Ag**

Clonal anergy has been defined as a reversible, induced tolerance state, following the in vivo systemic exposure to the Ag in which T cells can neither produce IL-2 nor proliferate in response to their cognate Ag (35). We thus measured the proliferative response of LN T cells to OVA peptide in vitro. As shown in Fig. 3A, there was no difference in the dose-response curve to OVA between OT-II and OT-II/SM-OVA mice. No difference in IFN-\( \gamma \) production was observed either between OT-II and OT-II/SM-OVA mice after in vitro activation (data not shown). Moreover, 14 days after immunization with OVA protein and adjuvant, the proliferative response to OVA_{323-339} in vitro by cells harvested from the draining LN was similar in control and SM-OVA mice (Fig. 3B). Therefore, CD4+ T cells from SM-OVA mice are not anergic and can respond to OVA upon antigenic recognition.

We then determined whether OVA expression in muscle could affect the in vivo T cell response by analyzing the DTH. Classical DTH is a memory T cell response in which CD4+ T cells are recruited at the site of Ag injection and release inflammatory cytokines that cause a visible swelling after 24–48 h (36). Mice were immunized with CFA-OVA, and challenged 7 days later by injecting OVA in PBS in the right footpad. Footpad swelling was measured 24 and 48 h after the challenge. No difference in the DTH response was observed between SM-OVA and control mice.

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**Analysis of T cell subsets in TCR/SM-OVA double Tg mice**

Negative selection occurs in the thymus and results in the deletion of thymocytes with high affinity for self proteins. Due to its extreme sensitivity, even minute traces of OVA could induce OVA-specific T cell deletion (29). This sensitivity is why, to avoid OVA circulation and localization in the thymus, a membrane-bound form of OVA was used to generate SM-OVA mice. However, other mechanisms could still account for presentation of OVA to developing T cells in the thymus. For instance, OVA could be presented by macrophages or dendritic cells recruited during muscle regeneration (30) that could migrate into the thymus (31), be cut off by proteases at the surface of muscle fibers, or expressed at a low level in the thymus due to autoimmune regulator-dependent promiscuous expression of some tissue-restricted self-Ags (3). Considering those possibilities, we did not detect OVA mRNA in the thymus of SM-OVA mice by RT-PCR (Fig. 1B), nor OVA protein in the serum by ELISA (data not shown). In addition, no proliferation was observed when OVA-specific TCR Tg T cells were used as responder cells either in vitro in presence of APC from SM-OVA mice or in vivo after adoptive transfer of CFSE-labeled OVA-specific T cells in SM-OVA mice (data not shown).

Besides, we studied the thymocyte cellularity and the developmental cell subsets from single and double Tg mice to determine
Another hallmark of Th cell response in vivo is the capacity to provide B cell help for the production of isotype-switched Abs. Twenty days after immunization, there was no difference between SM-OVA and control mice in the IgG anti-OVA humoral response (Fig. 3D). It should be noted that we did not detect anti-OVA IgG in the serum of SM-OVA mice before immunization (Fig. 3D), consistent with the state of ignorance of CD4+ T cells in this system. Altogether, these results indicate that CD4+ T cells can respond normally to neo-autoantigen following immunization, suggesting that tolerance is maintained by ignorance. Moreover, muscle-specific membrane-bound OVA expression did not lead to elimination or inactivation of OVA-specific B
cells, suggesting that membrane skeletal muscle Ags are not detected by the preimmune B cell repertoire. However, even if immunization can induce CD4+ T cells and B cells to respond to OVA, this effect was not sufficient to induce pathology as no sign of inflammation was detected in skeletal muscle after immunization (data not shown).

**Functional tolerance of OVA-specific CD8+ T cells**

CD8+ T cells can proliferate upon antigenic activation and differentiate into cytokine-secreting cytolytic effectors. Thus, to examine the functional properties of CD8+ T cells specific for muscle-expressed autoantigens, we analyzed in vitro proliferative response of LN T cells to OVA_{257-264} peptide. No difference was observed between OT-I and OT-I/SM-OVA mice (Fig. 4A), indicating that the proliferative capacity of CD8+ T cells in OT-I/SM-OVA mice is preserved. Therefore, similarly to CD4+ T cells, CD8+ T cells from OT-I/SM-OVA mice are not anergic.

To evaluate the functional activity of CD8+ T cells in SM-OVA mice, we performed an in vivo cytotoxic assay. Immunization of control mice resulted in an anti-OVA cytotoxic response that was able to clear almost half of OVA_{257-264} peptide-coated target cells, labeled with CFSE within 20 h (mean cytotoxic activity: 43 ± 8%, n = 5). Importantly, SM-OVA mice failed to mount a cytotoxic response in the same experimental conditions (mean cytotoxic activity: 2 ± 3%, n = 5, p < 0.002) (Fig. 4B). Therefore, although they can respond by proliferating in vitro to their cognate Ag (Fig. 4A), CD8+ T cells from SM-OVA mice are functionally deficient in their capacity to differentiate into cytotoxic effectors in vivo.

CTL activity is required for antitumor immune responses in vivo (37). For instance, OVA-specific CTL primed by immunization can protect against a challenge with E.G7-OVA cells, i.e., an EL4 mouse thymoma cells stably expressing OVA (27). To further evaluate how muscle expression of an autoantigen impairs the cytotoxic response, SM-OVA and control mice were immunized and further challenged by s.c. E.G7-OVA injection. Immunized control mice remained tumor free for up to 30 days (Fig. 4C). In marked contrast, immunized SM-OVA mice failed to mount an antitumoral response as tumors developed with similar kinetics than in control immunized mice (median tumor free: 13 days). This phenomenon was OVA-specific because no difference in tumor development kinetics was observed when OVA-negative parental EL4 cells were injected in control or SM-OVA mice immunized or not by CFA-OVA (Fig. 4D). These results confirm the existence of a CTL functional deficiency toward an Ag that is specifically expressed in muscle. These results also suggest that shared expression by tumors cells of Ags normally present in muscle tissue may facilitate tumor growth by limiting the CTL immune response.

**No increase of FoxP3+ regulatory T cells (Tregs) in SM-OVA mice**

The lack of antitumoral response observed in SM-OVA mice demonstrates a blockade of CTL differentiation that may be intrinsic to...
CD8+ T cells, but may also suggest a suppressive mechanism. CD4+CD25+ Tregs expressing the lineage marker FoxP3 represent an important population of suppressor T cells that are essential to prevent autoimmunity (38), as well as antitumor immunity induced by therapeutic vaccines (39). Although the precise signals that induce thymic development of Tregs are unknown, agonist ligands expressed specifically in thymic epithelial cells promote de novo generation of Tregs (40). Moreover, Tregs can be generated in the periphery by continuous supply of subimmunogenic doses of agonist peptides (41). The growth of tumor in SM-OVA mice is unlikely to depend upon a higher number of autoantigen-specific FoxP3+ Tregs because no differences were observed in the percentage of FoxP3+CD4+ T cells (Fig. 5A) and in the total number of FoxP3+CD4+ T cells (Fig. 5B) between OT-I and OT-I/SM-OVA mice. Moreover, there was no increase in FoxP3+CD8+ T cells in SM-OVA mice, as 0.5% of CD8+ cells were FoxP3+ in mice expressing the OT-I TCR (data not shown). No difference was observed either in the percentage or the number of FoxP3+CD4+ Vα2+ or Vα2− cells (Fig. 5A) in the total number of FoxP3+CD4+ T cells (Fig. 5B) between OT-I and OT-I/SM-OVA mice. Moreover, there was no increase in FoxP3+CD8+ T cells in SM-OVA mice, as <0.5% of CD8+ cells were FoxP3+ in mice expressing the OT-I TCR (data not shown). No difference was observed either in the percentage or the number of FoxP3+CD4+ Vα2+ or Vα2− in OT-II or OT-II/SM-OVA mice (data not shown). Therefore, it is unlikely that OVA muscle-specific expression significantly contributes to the generation of OVA-specific Tregs in the present model.

Increase of PD-1+ CD8+ T cells in OT-I/SM-OVA mice

We then analyzed the classical cytotoxic pathways but we did not detect any defect in Fas ligand, granzyme B, or perforin expression between CD8+ T cells from OT-I and OT-I/SM-OVA mice after in vitro activation with OVA257–264 peptide (data not shown). We immunized with CFA-OVA (filled symbols) or with CFA alone (open symbols). Results show immunized control mice remained tumor-free. D. EL4 cell line was not rejected in control or SM-OVA mice immunized or not immunized. These experiments are representative of two different experiments (n = 5 mice per group).
FIGURE 6.  PD-1 up-regulation on OVA-specific CD8\(^+\) T cells in SM-OVA mice.  A, The level of PD-1 expression is shown on CD8\(^+\) V\(\alpha\)2\(^+\) T cells from OT-I (thick line histogram) and OT-I/SM-OVA (dashed line histogram) mice. Isotype control is shown (gray shaded histogram). B, Summary scatter plots of the percentage of CD8\(^+\) V\(\alpha\)2\(^+\) PD-1\(^+\) in OT-I and OT-I/SM-OVA mice, with each data point representing an individual mouse. C, CD8\(^+\) T cells from Rag2KO/OT-I were transferred i.v. in Rag2KO or Rag2KO/SM-OVA mice. The percentage of CD8\(^+\) V\(\alpha\)2\(^+\) PD-1\(^+\) is shown 6 wks after transfer. D, The percentage of CD8\(^+\) V\(\alpha\)2\(^+\) PD-1\(^+\) in recipient Rag2KO (\(n = 5\)) mice and Rag2KO/SM-OVA mice (\(n = 7\)) is shown (\(p < 0.001\)). E, Control mice were s.c. immunized with 100 \(\mu\)g of CFA-OVA or with CFA alone. Purified CD8\(^+\) T cells from OT-I or OT-I/SM-OVA mice were transfer by i.v. injection. At 3 days later, a mixture of CFSE\(\text{high}\)-labeled unpulsed and CFSE\(\text{low}\)-labeled OVA\(257\text{--}264\) peptide pulsed as cytotoxic targets was injected i.v. At 20 h later, spleen cells were examined by FACS to detect and quantify CFSE-labeled cells. Histograms represent the amount of CFSE-labeled cells from one representative experiment of two performed. The number in each plot indicates the percentage of specific lysis of peptide-pulsed CFSE\(\text{low}\) cells.

AS well observed no difference in the capacity of CD8\(^+\) T cells from OT-I and OT-I/SM-OVA mice to secrete IFN-\(\gamma\) (data not shown).

During chronic infection by viruses, such as lymphocytic choriomeningitis virus in mice or HIV in humans, CD8\(^+\) T cells can lose their cytotoxic activity due to a mechanism involving expression of the inhibitory receptor PD-1 (42–45). In such conditions, CD8\(^+\) T cells express classical memory or activation markers (CD44\(\text{high}\), CD62L\(\text{low}\)) and proliferate in vivo like regular memory cells (46, 47). Because of the similarities between our OVA-specific CD8\(^+\) T cells and the functionally deficient CD8\(^+\) T cells observed during virus chronic infection (48), i.e., expression of activation markers and loss of cytotoxic activity, we sought for PD-1 expression on OVA-specific T cells in OT-I/SM-OVA. We found a significant increase of CD8\(^+\) T cells expressing PD-1 in OT-I/SM-OVA (6 ± 4%, \(n = 15\)) as compared with OT-I (2 ± 1.5%, \(n = 12\), \(p < 0.02\)) (Fig. 6, A and B). Therefore, the CTL deficiency associated with muscle Ag expression correlates with up-regulation of the inhibitory molecule PD-1 within the CD8 compartment.

To further evaluate how PD-1 is up-regulated in the tolerogenic SM-OVA environment, we transferred CD8\(^+\) T cells from OT-I/Rag2KO mice into Rag2KO or SM-OVA/Rag2KO mice. After 6 wks, we measured the percentage of CD8\(^+\) T cells expressing PD-1. Although <2% of CD8\(^+\) V\(\alpha\)2\(^+\) T cells expressed PD-1 before i.v. injection (data not shown), up to 24 ± 4.5% of CD8\(^+\) V\(\alpha\)2\(^+\) T cells expressed PD-1 in Rag2KO/SM-OVA recipient mice (\(n = 7\)) as compared with 10 ± 3.5% in Rag2KO recipients (\(n = 5\), \(p < 0.001\)) (Fig. 6, C and D). Therefore, even if homeostatic proliferation up-regulates PD-1 on some CD8\(^+\) T cells, we observed a significant increase in PD-1\(^+\) T cells when OVA is expressed in muscle. This suggests that a peripheral mechanism continuously induces the up-regulation of PD-1 on muscle autoantigen-specific CD8\(^+\) T cells.

To investigate whether continuous expression of OVA is necessary to maintain peripheral tolerance, we performed adoptive transfer of purified CD8\(^+\) T cells from OT-I and OT-I/SM-OVA mice in normal Bl6 mice. Recipient mice were immunized or not with CFA-OVA, and the cytotoxic activity of CD8\(^+\) T cells was measured by performing an in vivo killing assay, analyzed as early as 3 days after immunization before recipient mice could mount a primary cytotoxic response. Results showed that a very potent cytotoxic activity could be restored after transfer of CD8\(^+\) T cells from OT-I/SM-OVA mice to immunized recipient mice because nearly all OVA-pulsed T cells rapidly disappeared (Fig. 6E). Interestingly, CD8\(^+\) T cells from OT-I/SM-OVA mice were even able to lyse target cells in unimmunized mice probably because of the high percentage of memory CD8\(^+\) T cells in OT-I/SM-OVA mice. Therefore, in recipient mice that did not express OVA in skeletal muscle, OVA-specific CTL regained their cytolytic function. This result suggests that OVA muscle-specific expression is not only necessary to induce but also to maintain CD8\(^+\) T cell OVA-specific tolerance.
Discussion

Muscle is the contractile tissue of the body and its function is to produce force and cause motion. There are three types of muscle: skeletal, smooth and cardiac muscle. An average adult male is made up of 40–50% of skeletal muscle and an average adult female of 30–40%. Whereas muscle has an important role in survival of species, little is known about the mechanisms of immunological tolerance to this tissue. Ignorance is defined as the undetection of Ag by the immune system due to absence of presentation (49) or the lack of appropriate T cell activation conditions (4). Because skeletal muscle fibers do not express MHC molecules, it has been tempting to consider that ignorance is one of the main mechanisms that confer tolerance to this tissue.

In this study, we examined the mechanisms of tolerance to muscle-specific Ags by generating OVA-specific muscle Tg mice that were crossed with OT-I or OT-II mice. Several major observations were made: 1) no central or peripheral deletion or anergy to OVA were observed; 2) immunization of CFA-OVA was sufficient to trigger CD4+ T and B cell responses because we could induce DTH, CD4+ T cell proliferation, and cytokine production, as well as isotype switch resulting in anti-OVA IgG production; 3) an increased number of OVA-specific memory CD8+ T cells was observed which constitutes a strong argument against ignorance; 4) OVA-specific CTL lost their cytotoxic function in SM-OVA mice; 5) PD-1 was up-regulated on OVA-specific CD8+ T cells when OVA was expressed in muscle; 6) adoptive transfer of OVA-specific CD8+ T cells to lymphopenic SM-OVA mice induced up-regulation of PD-1 expression; and 7) OVA expression in muscle was necessary to maintain cytotoxic blockage. Therefore, tolerance to muscle specific Ag can be referred as asymmetric because CD4+ T cells and B cells are tolerant by ignorance, whereas CD8+ T cells are tolerant by the loss of their cytotoxic activity. Nevertheless, OVA is artificially expressed in muscle and it cannot be formally excluded that other tolerance mechanisms might operate for natural muscle autoantigens.

To study the pathogenesis of myasthenia gravis, Tg mice that express β-galactosidase (β-gal) or a heterologous AchR under the control of chicken AchR α-chain promoter sequences have been developed (50, 51). This resulted in muscle but also thymic expression of the transgene. Using different forms of the AchR promoter, two β-gal-Tg lines were obtained with low (β-gal-Tg1) or high (β-gal-Tg2) muscle and thymic expression (51). CD4+ T cells of both β-gal-Tg and AchR-Tg mice appeared to be anergic because they could not respond in vitro to Ag restimulation after in vivo immunization. This could be due to muscle Ag expression but also to thymic expression. In SM-OVA mice, in which the Ag is only expressed in muscle but not in thymus, no such CD4+ T cell anergy was observed. The discrepancy between β-gal-Tg and AchR-Tg mice as compared with SM-OVA mice suggests that CD4+ T cell anergy in the first two models is due to thymic rather than muscle autoantigen expression. Regarding B cell tolerance, a specific Ab response was observed in β-gal-Tg1, AchR-Tg, and SM-OVA mice after autoantigen immunization in the presence of CFA, whereas β-gal-Tg2 mice did not respond (50, 51). This indicates that, despite muscle and eventually thymic expression, it remains possible to induce a specific B cell response except when the autoantigen is expressed at very high levels in muscle such as the case in β-gal-Tg2 mice. Together, these results indicate that concomitant expression in the thymus and level of expression may contribute to additional tolerance mechanisms toward otherwise muscle-expressed autoantigens. In addition to β-gal-Tg and AchR-Tg mice, the SM-OVA model described in this study further revealed that muscle expression induces a strong CD8+ T cell tolerance in the absence of thymic expression.

It has been shown that during chronic virus infection, expression of PD-1 on CD8+ T cells correlates to a loss of their cytotoxic activity (42–45). Similarly, we show in this study that PD-1 expression is also associated with the loss of cytotoxic activity against muscle Ag, which may play a pivotal role in tolerance to muscle autoantigens. In this regard, it is interesting to note that PD-1-deficient mice develop spontaneously a lethal dilated cardiomyopathy on BALB/c background (52), due to autoantibodies directed against the cardiac muscle autoantigen, troponin I (53). Moreover, PD-1 ligand 1 (PD-L1), one of the two known ligands of PD-1, was recently shown to regulate CD8+ T cell mediated injury in a mouse model of CD8+ T cell-mediated myocarditis (54). In humans, PD-L1 was also shown to be expressed in muscle biopsy specimens of patients with polymyositis, dermatomyositis, or inclusion body myositis, especially in areas of strong inflammation (55). These data highlight a possible feedback protective mechanism in which PD-1/PD-L1 interaction may represent an inhibitory mechanism induced upon inflammatory stimuli and aimed at protecting muscle fibers from autoimmune aggression. Recently, PD-1/PD-L1 pathway has also been involved in fetal-maternal tolerance (56) and in maintaining immune privilege in the eye (57, 58), as well as regulating autoreactive CD8+ T cells response (59–61), suggesting a major role in mediating tissue tolerance.

By adoptive transfer experiments, we observed that CD8+ T cells could up-regulate PD-1 in a lymphopenic environment when OVA was expressed in muscle. Because muscle fibers do not express MHC molecules, OVA needs to be cross-presented in the periphery, a mechanism whereby extracellular Ags, which are normally presented in association with MHC class II molecules, are also presented by MHC class I molecules. The principal cells cross-presenting Ags are dendritic cells, but under some circumstances various other cell types like macrophages, B cells, neutrophils, or endothelial cells also display this activity (62). Macrophages are involved in skeletal muscle repair and they have been shown to infiltrate massively the site of injury after muscle damage (30). Moreover, recruited macrophages display anti-inflammatory properties and appear to enhance myogenic cell growth (63, 64). Therefore, in our model, macrophages or others APC could capture OVA following muscle fiber death and induce tolerance to CD8+ T cells in the periphery. Interestingly, we could restore OVA-CTL activity by adoptive transfer of CD8+ T cells from OT-1/SM-OVA into normal mice, suggesting that continued cross-presentation of OVA by tolerogenic APC was responsible for this peripheral tolerance mechanism. However, direct presentation of OVA by regenerative myogenic cells, that express low level of MHC class I, could also be accountable for the OVA-CTL blockage.

Rejection of cancer cells results from a series of events that include T cell activation, expansion, infiltration of the tumor, and cytotoxic activity. Our model highlights the role of CTL in tumor rejection and has unexpectedly shown that antitumor response may be affected by Ags shared between tumor and muscle fibers because OVA-expressing tumor cells were not rejected in SM-OVA mice.

Autoantibodies directed against cell surface molecules cause the pathogenesis of a variety of organ-specific autoimmune diseases. However, ubiquitous expression of membrane-bound Ags induces deletion of self-reactive B cells (65, 66). Still, membrane-bound Ag specifically expressed in thyroid did not eliminate or inactivate reactive B cells (67), as we observed in SM-OVA mice. Interestingly, production of autoantibodies after immunization did not trigger signs of thyroid autoimmunity either. We observed the
same phenomenon in SM-OVA mice because a B cell response could be induced by OVA immunization without signs of muscle destruction. It would be interesting to further study what prevents circulating preimmune B cells to be tolerized against organ-specific membrane-bound Ags and, once autoantibodies are generated, what prevents them from being pathogenic.

In conclusion, we demonstrate for the first time to our knowledge that, despite the large amount of muscle fibers in the organism, muscle Ag expression does not provoke negative selection or anergy of autoantigen-specific T cells but rather induces a novel mechanism of immunological tolerance in which CD4\(^+\) T cells are tolerant by ignorance, whereas CD8\(^+\) T cells become refractory to induction of a cytotoxic response. These results imply that the break of tolerance observed in inflammatory myositis restores the cytotoxic activity of autoreactive CD8\(^+\) T cells through a mechanism that needs to be further defined. Notably, it would be interesting to analyze the functional properties of the PD-1/PD-L1 pathway in these disorders. Advances in the understanding of tolerance mechanisms to skeletal muscle Ag may help develop novel therapeutic approaches in muscle-specific autoimmune diseases.

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Disclosures
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