M2 Polarized Macrophages and Giant Cells Contribute to Myofibrosis in Neuromuscular Sarcoidosis

The etiopathogenesis of sarcoidosis, a systemic granulomatous disease, still remains obscure. A multitude of organs have been described to be affected in systemic sarcoidosis. Skeletal muscles may also be affected, leading to myalgia and weakness. A workup of the specific immune response with emphasis on the macrophage response is provided herein. Affected muscle tissue from seven patients with systemic sarcoidosis was analyzed and compared with that from seven patients with other myopathies containing macrophagocytic infiltration. Monocytes/macrophages and giant cells in granulomas of muscle tissue from patients with sarcoidosis show a status of alternative activation (M2) based on their expression of CD206, CD301, arginase-1, and suppressor of cytokine signaling-1 as a consequence of a functionally type 2 helper T cell (Th2)-biased cytokine profile. Significant fibrosis and up-regulation of CCL18 were associated with the M2 phenotype of macrophages. Conversely, up-regulated Th1 cytokines did not result in significant classical activation of macrophages (M1), with poor inducible nitric oxide synthase and cyclooxygenase-2 production. Giant cell formation was further associated with up-regulated expression of DNAX-activating protein of 12 kDa (DAP12; gene symbol TYROBP). Functionally, alternative activation of macrophages on the basis of a Th2-biased immune response may induce clinical symptoms and chronic evolution of neuromuscular sarcoidosis. This is the first characterization of Th2-mediated immune mechanisms in neuromuscular sarcoidosis suggesting that a specific macrophage activation status leading to myofibrosis may be a key event in the pathogenesis of this disease. (Am J Pathol 2011, 178:1279–1286; DOI: 10.1016/j.ajpath.2010.11.065)
infectious contexts, and therefore macrophages are thought not to be terminally differentiated.19–23

Materials and Methods

Patients and Biopsy Specimens

Biopsied muscle tissue samples from seven patients with muscle sarcoidosis and seven patients with other diseases of the muscle that contain significant macrophage infiltrates [dermatomyositis (n = 2), Becker muscular dystrophy (BMD) (n = 2), necrotizing myopathy (n = 2), and macrophagic myofasciitis (n = 1)] were analyzed retrospectively. In each patient, sarcoidosis of the muscle was part of a generalized chronic sarcoidosis also involving the lungs. Available clinical information is given in Table 1. Informed consent was obtained from all the patients, and all the biopsy specimens had been cryopreserved at −80°C before diagnostic workup.

Histologic, Enzyme Histochemical, and Immunohistochemical Analyses

Cryostat sections, 7 μm thick, were stained with H&E, Elastica van Gieson, modified Gömöri trichrome, and enzyme histochemically for nonspecific esterase and acid phosphatase. For immunohistochemical analysis, 7-μm-thick cryostat sections were prepared for demonstration of CD3, CD4, CD8, CD11b, CD20, CD31, CD45/leukocyte common antigen, CD68, CD206/mannose receptor, CD301/macrophage galactose-type C lectin, fibrinogenic pulmonary and activation-regulated chemokine (CCL18), major histocompatibility complex (MHC) class I, MHC class II, and suppressor of cytokine signaling-1 (SOCS-1) (Table 2). Immunohistochemical analysis was performed using theVIEW-Ventana ABC Kit (Ventana, Illkirch, France), with appropriate biotinylated secondary antibodies and diaminobenzidine visualization of the peroxidase reaction product on a Benchmark XT immunostainer (Ventana). Omission of primary antibodies in control sections resulted in the absence of any cellular labeling.

Quantitative Real-Time PCR

Total RNA was extracted from all muscle tissue samples using the TRIzol/chloroform method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Thereafter, RNA was resuspended in 60 μL of diethylpyrocarbonate-treated water. Concentration of total RNA was determined using a fluorescence plate reader (Tecan Group Ltd, Männedorf, Switzerland). Owing to the uneven distribution of granulomas and of macrophage density in the investigated specimens, tissue that was collected for PCR analysis was reassessed histologically for the presence of the respective pathologic alterations in all investigated cases. The RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the man-
Figure 1. Macrophages, epithelioid cells, and giant cells in granulomas are illustrated by Gömöri stainings (A, B, and C) and H&E stainings (D, E, and F) and nonspecific esterase preparation (G, H, and I) in a patient with sarcoidosis (A, D, and G) and in two control patients: BMD case 1 (B, E, and H) and a patient with necrotizing myopathy (C, F, and I). Monocytes strongly express CD68 (J, K, and L), whereas CD11b is less intensely expressed in the control group (M vs N and O). MHC class I molecules (P) are expressed on monocytes and additionally on the sarcolemma of muscle fibers of patients with muscle sarcoidosis and of those with necrotizing myopathy (P and R), whereas in the BMD case, MHC class I molecules are expressed exclusively on monocytes (Q). Original magnification, ×200.
manufacturer’s protocol using 2 μg of total RNA per sample as described previously.24

The expression levels of gene transcripts, and of the endogenous control gene, phosphoglycerate kinase 1 (PGK1), was analyzed in the specimens by means of quantitative real-time RT-PCR using the 5'-nuclease technology using an ABI PRISM 7900HT Sequence Detection System and the human TaqMan predeveloped assay reagents (Applied Biosystems). The assay identification numbers are as follows: IL-4, Hs00929862_m1; IL-4 receptor (IL-4R), Hs00166237_m1; IL-13, Hs99999038_m1; CD206, Hs00267207_m1; CD301, Hs00197107_m1; SOCS-1, Hs00705164_s1; CCL18, Hs00268113_m1; arginase-1, Hs00968979_m1; DNA-activating protein of 12 kDa (DAP12; gene symbol TYROBP), Hs00182426_m1; inducible nitric oxide synthase (iNOS), Hs01075529_m1; IFN-γ, Hs00989291_m1; IL-18, Hs99999040_m1; IL-27R, Hs00175472_m1; and PGK1, Hs99999906_m1.

The PCR reactions were prepared in a final volume of 20 μL in the presence of the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) containing a cDNA equivalent of 20 ng of RNA. All analyses were performed in triplicate, and the threshold cycle was determined. Gene expression was concomitantly measured in normal muscle tissue as a calibrator to allow comparison between the different samples of muscle tissue containing sarcoidosis granulomas versus other muscle disease samples with inflammation using the 2(ΔΔ Ct) method.25

Statistics

The two-tailed t-test was applied for analysis of quantitative differences of mRNA transcripts. Data are presented as mean ± SD. The level of significance was set at P < 0.05.

Results

Macrophages in Sarcoid Granulomas of Skeletal Muscle Exhibit the M2 Phenotype

Granulomas of all the patients analyzed exhibited macrophages, epithelioid cells, and giant cells, with strong reactivity for nonspecific esterase. The presence of these myeloid cells was associated with severe myopathic changes in the muscle tissue and was found to be distributed throughout the endomysium and perimysium of the entire muscle specimen (Figure 1, A, D, and G). Immunohistochemical analyses consistently revealed the expression of CD68 (Figure 1J) and the pan-macrophage marker CD11b (Figure 1M) on macrophages, epithelioid cells, and giant cells. Although most macrophages were located in the granulomas, some macrophages were also found to be diffusely distributed in the endomysium. Up-regulation of MHC class I molecules was detectable on macrophages, epithelioid cells, and giant cells. Although most macrophages were located in the granulomas, some macrophages were also found to be diffusely distributed in the endomysium. Up-regulation of MHC class I molecules was detectable on macrophages, epithelioid cells, and giant cells and also on the sarcolemma of all muscle fibers (Figure 1P). All these monocytes strongly express the mannose receptor (CD206) (Figure 2A), and they are also SOCS-1 positive (Figure 2D). Focal up-regulation of CD206 was found on macrophages from the control group as illustrated: BMD case 1 (B) and necrotizing myopathy (C). Macrophages from the control group, however, did not express SOCS-1 (BMD case 1 (E) and necrotizing myopathy (F)). Immunohistochemical iNOS staining did not differ significantly in both groups as exemplified by iNOS staining of a muscle sarcoidosis specimen (G) compared with a specimen from the patient with BMD (H) and from a patient with necrotizing myopathy (I). Original magnification, ×200.
dystrophy and necrotizing myopathy specimens (Figure 2, B and C), whereas SOCS-1 was absent or, if at all, hardly detectable compared with specimens derived from patients with sarcoidosis (Figure 2, E and F).

**Expression of Th2 Cytokines in Sarcoidosis**

Lesions of Skeletal Muscle Leads to Alternative Activation of Macrophages

Presence of the common IL-4R is a prerequisite for effective (type 2 helper T cell Th2) signaling via the master cytokines IL-4 and IL-13. All specimens of the muscle sarcoidosis group showed robust expression of IL-4R, whereas IL-4R expression was significantly lower in the control group (Figure 3A). In line with this, IL-4 expression was significantly increased in the muscle sarcoidosis group, and IL-13 expression was restricted to specimens of the muscle sarcoidosis group (Figure 3A). Analyzing alternative activation markers of macrophages revealed that CD206, CD301, SOCS-1, and arginase-1 were significantly up-regulated in muscle biopsies of the muscle sarcoidosis group compared with the control group (Figure 3B). Furthermore, the IL-27R, which is expressed on IL-4– and IL-13–primed M2 macrophages, was also significantly upregulated in this group compared with controls. In addition, DAP12, which regulates macrophage fusion in giant cell formation, was clearly up-regulated in the sarcoidosis group (Figure 3C).

**Expression of Th1 Cytokines in Sarcoidosis**

Lesions of Skeletal Muscle Resulting in Weak Classically Activated Macrophages

Although the expression of IFN-γ and IL-18, known to be essential for a functional Th1 response in pulmonary sarcoidosis, was found to be up-regulated in the muscle sarcoidosis group (Figure 4A), an anticipated subsequent up-regulation of iNOS and cyclooxygenase-2 was not detected (Figure 4B). Conversely, IL-12p40 expression, indicating classical (M1) activation of macrophages, was found to be low on RNA levels in the muscle sarcoidosis group, whereas control specimens exhibited a somewhat more pronounced, however non-significant, expression (Figure 4B). Furthermore, iNOS staining was found in few macrophages and giant cells in granulomas of skeletal muscle sarcoidosis, indicating a certain degree of classical (M1) activation in the lesions (Figure 2G). Muscle specimens from the control group also showed iNOS immunoreactivity in the myophagocytes in BMD and necrotizing myopathy (Figure 2, H and I). In addition, IL-12p40 was highly expressed in the sarcoidosis group (Figure 4C), indicating a functional, but possibly disbalanced, IFN-γ–IL-12 axis in sarcoidosis of the muscle. Along these lines, the anti-inflammatory/regulatory cytokine IL-10 was expressed at significantly higher levels in the sarcoidosis group, lending further support for the M2 polarization of these macrophages (Figure 4C).

![Figure 3](image-url)

**Figure 3.** Log_{10} Relative Quantification (RQ) values of mRNA calibrated against expression of the respective molecules in normal muscle specimens. **A:** Both of the experimental groups show expression of the common IL-4R, IL-4 is expressed significantly higher in the muscle sarcoidosis group, and IL-13 is exclusively expressed in that group. **B:** Markers of alternative activation are expressed at a significantly higher level. **C:** Furthermore, expression of DAP12 is also higher in the muscle sarcoidosis group. \( \ast P < 0.05, \ast\ast P < 0.01, \ast\ast\ast P < 0.001 \).

![Figure 4](image-url)

**Figure 4.** Log_{10} values of mRNA calibrated against expression of the respective molecules in normal muscle specimens. Expression of IFN-γ and IL-18 was significantly higher in the muscle sarcoidosis group than in the control group (**A**), whereas the expression of iNOS and cyclooxygenase-2 (COX2), as markers of classical macrophage activation, is not up-regulated in either groups (**B**). **C:** Expression of IL-10 and IL-12 was significantly higher in the muscle sarcoidosis group than in the control group. NS, not significant. \( \ast\ast P < 0.01, \ast\ast\ast P < 0.001 \).
These results indicate that Th2 activation via IL-4 and IL-13 in muscle sarcoidosis is more important and effective than is Th1-mediated activation via IL-18 and IFN-γ, which results in an M2 rather than an M1 polarization of inflammatory macrophages.

**Alternative Activation of Macrophages Induces Myofibrosis in Muscle Sarcoidosis**

Expression of CCL18, an important chemokine involved in fibrosis in the lung, was significantly up-regulated in the muscle sarcoidosis group at the protein (Figure 5, A vs B and C) and mRNA (Figure 5M) levels. In line with this observation, severe endomysial and perimysial fibrosis in the muscle tissue from patients with sarcoidosis was visualized by elastica van Gieson staining (Figure 5, D vs E and F). Furthermore, neovascularization, as illustrated by immunohistochemical stainings for CD1 (Figure 5G) and smooth muscle actin (Figure 5J), was detectable in granulomas of patients with muscle sarcoidosis. Fibrous tissue was associated with CCL18 expression, which, although at significantly lower levels, was also evident in control specimens (Figure 5M). Fibrosis as detected in this specimen (Figure 5, B and E) is a well-known phenomenon in patients with BMD. However, these specimens and all other control muscles with even less fibrosis lacked cell-rich granulomas or neovascularization (Figure 5, H, I, K, and L). These data illustrate an essential chronic granulomatous tissue reaction in the sarcoidosis muscle specimens, which is localized to the macrophage/giant cell infiltrates.

**Discussion**

According to the generally accepted concept, sarcoidosis is thought to be a Th1-mediated disease. Because sarcoidosis is a chronic disease in approximately 20% of patients, severe tissue damage and active repair mechanisms are evolving throughout the course of the disease. Herein, we show via analysis of macrophage function, and expression of their marker profiles in light of Th1 and Th2 activation, that macrophages and giant cells strongly express markers of alternative activation in muscle sarcoidosis. Furthermore, despite a strong Th1 stimulus, iNOS and cyclooxygenase-2 production as key mediators of classical activation of macrophages are only weakly expressed, whereas severe myofibrosis, clinically linked to muscle weakness and myalgia, is associated with M2 macrophages. Because of the dominant Th2 response, we conclude that M2-mediated chronic macrophage activity in concert with associated DAP12-induced giant cell formation is the basis for adverse and chronic evolution of the disease in the affected skeletal muscle.

In pulmonary sarcoidosis, a Th1-mediated immune response is documented by the presence of CD4+ T cells producing high levels of IFN-γ, IL-12, IL-2, and other Th1 cytokines, which are considered the main immuno-
logic drivers in granuloma formation. The Th2 cells are thought to be inactive in such granulomas, also because low levels of the Th2-associated cytokines IL-4 and IL-13 were described. Because some patients develop sarcoidosis during treatment with IFN-α (mainly due to hepatitis C), it has been argued that this may be due to exacerbation of a Th1-mediated immune response.

The concept of an imbalanced immune response implicates that in the chronic disease course of sarcoidosis, there may be a shift toward increased Th2-type cytokines. However, Möllers et al found fewer Th2 cytokines in the alveolar compartment compared with in the peripheral blood. The Th2 cytokines may be of clinical relevance, especially in patients who develop lung fibrosis, given that increased IL-13 expression was described in bronchoalveolar lavage cells of patients with sarcoidosis. Because, to our knowledge, no studies on Th1/Th2 immunopathogenesis in muscle sarcoidosis have been reported, the present findings, apart from explaining the pathogenesis of the morphologically typical lesions in the muscle, may also give insights into the principal pathogenetic stages of sarcoidosis, which also may apply to sarcoidosis lesions (eg, in the lungs or other tissues).

In muscle specimens from patients with sarcoidosis, macrophages with an M2 phenotype are by far the most abundant cell population. These M2 macrophages represent an alternative status of activation beside classical M1 activation, which is required for acute killing of microbial products via production of nitric oxide and oxygen-free radicals. Both iNOS and arginase-1 share arginine as a substrate, and expression of either molecule is crucial for the functional properties of these macrophages and the ensuing immunopathologic condition. Because iNOS expression was absent in fibrotic parts of pulmonary lesions but was found to be increased in areas with strong inflammation, one can conclude that either fibrosis in muscle sarcoidosis is a more prominent feature or muscle involvement reflects a more chronic and stable stage of sarcoidosis. Because arginase-1, in contrast to iNOS, is known to induce collagen production via the production of prolines and the reconstruction of damaged extracellular matrix, accompanied by a switch of macrophages to an M2 “repair” phenotype, the herein reported significant arginase-1 expression and clear M2 marker profile with high levels of CD206 and CD301 on macrophages and giant cells in muscle specimens from patients with sarcoidosis are striking.

CD206+ macrophages have only recently been described in the context of muscle diseases (Duchenne muscular dystrophy) by Desguerre et al, and the relevance of these cells in myofibrosis has been pointed out. However, we did not detect any increased angiogenesis in our dystrophy patients’ specimens. Conversely, important neovascularization in the granulomas and the adjacent inflammatory muscle tissues may also be the consequence of a sustained IL-4 effect on macrophage activity. We identified a significant up-regulation of SOCS-1 in the muscle of patients with sarcoidosis, which is known to induce CD206 and arginase-1 expression, whereas SOCS-3 is essential for the development of M1 macrophages in vitro and in vivo. Functionally, the highly significant expression and up-regulation of CCL18 in the muscle tissue, which was identified as a central mediator of fibrosis in the affected muscle specimens from patients with sarcoidosis, has also been described in alveolar macrophages obtained by means of bronchoalveolar lavage from patients with idiopathic lung fibrosis.

The agent driving the formation of giant cells in sarcoidosis and its functional relevance remain unclear, although the fundamental feature of granuloma formation on a “nidus of stimulating antigens” on the one hand and of the characteristic clinical course with special features in neurosarcoidosis on the other hand is underscored. In a more general approach, the molecular mechanisms of macrophage fusion have recently been intensely studied, and the link between an alternatively activated phenotype of macrophages and giant cell formation has been established. Furthermore, in parasitic infection with Schistosoma mansoni, giant cell formation depended on the presence of IL-4, and IL-4 is also required in foreign body giant cell formation. Giant cell and granuloma formations are striking morphologic hallmarks in patients with muscle sarcoidosis. We identified DAP12 as being significantly up-regulated, which further argues for its functional significance in the context of M2 macrophage polarization. We, therefore, hypothesize that the granuloma-inducing stimulus may procure a special milieu during muscle sarcoidosis, which causes sustained macrophage stimulation, leading to an alternatively activated (M2) phenotype.

In conclusion, analysis of a comprehensive set of Th1/Th2 mediators and cytokines, which regulate M1/M2 polarization of macrophages, investigated in human muscle specimens of patients with sarcoidosis have identified a dominant Th2 response with expression of M2 markers on macrophages and giant cells. The chronic inflammatory evolution of the disease is accompanied by important fibrosis of the muscle tissue, which, consequently, leads to an adverse clinical outcome of the disease in affected patients.

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References
