Mononuclear phagocytes and MHC class II+ dendritic cells (DC) were identified in frozen sections of skeletal muscle using a panel of pan-specific antimacrophage (MOMA-2, SER-4, Mac-1, F4/80), anti-major histocompatibility complex (MHC) class II (M5/114) and anti-DC (NLDC-145, N418, M342) monoclonal antibodies. Uninjured and regenerating skeletal muscle were investigated in SJL/J and BALB/c mice, strains with known differences in muscle regenerative capacity. Resident tissue macrophages and MHC class II+ DC were present within uninjured mouse muscle. A subpopulation of DC were positive for the pan-DC markers, N418 and M342, and negative for the lymphoid DC marker NLDC-145. Following crush injury, the macrophage population increased by day 2, became marked by day 3, and had decreased by day 6. In contrast, the number of MHC class II+ cells around the injury site increased steadily after injury and remained high at day 6. The numbers of macrophages and DC detected by immunohistochemical staining were consistently higher in SJL/J than BALB/c muscles. This study confirms that macrophages are a significant component of normal murine skeletal muscle and that these cells increase dramatically after injury. Furthermore, the data also reveal for the first time that DC are present in normal skeletal muscle and that MHC class II+ cells, including DC, increase after injury. The presence of DC in muscle has important implications for the understanding of the immunobiology of muscle and immune-mediated processes such as the host versus graft responses following muscle transplants and autoimmune diseases affecting this tissue.


Key words: skeletal muscle • regeneration • macrophages • dendritic cells • immunohistochemistry

MACROPHAGES AND DENDRITIC CELLS IN NORMAL AND REGENERATING MURINE SKELETAL MUSCLE

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Normal mature skeletal muscle is among a growing list of tissues and organs now known to contain a rich resident population of cells of the mononuclear phagocytic system. In normal rat muscle, macrophages are particularly widely distributed throughout the connective tissue of the perimysium and endomysium between muscle fibers. Resident tissue immune cells, such as macrophages, may either play a modulatory role in maintenance of local immunological homeostasis, possibly by down-regulation T cells and dendritic cells (DC) as occurs in the respiratory tract, or perform a proinflammatory role by secreting a range of cytokines in response to injury or exposure to endotoxins, thus aiding the recruitment of other inflammatory cells into sites of damage or infection. Macrophages also serve a variety of additional functions, including phagocytosis of tissue and cell debris during local injury, inflammation, and immune responses (see Ref. 32).

Studies from our laboratories have highlighted the role of macrophages in the phagocytosis of tissue debris following injury to skeletal muscle. There is debate as to the role of resident tissue macrophages versus newly blood-borne monocyte/macrophages recruited during the inflammatory response of various tissues, including muscle, to injury.
limited immunohistochemical study of normal muscle in the rat suggested that blood-borne monocytes/macrophages were responsible for the phagocytosis of a damaged muscle fiber.25 The situation in major muscle injuries is unknown. Evidence from bone-marrow irradiation studies supports the hypothesis that blood-borne macrophages and nonresident macrophages play a vital role in phagocytosis of tissue debris.36 Activated macrophages at the site of muscle injury also facilitate myofiber repair by producing cytokines and growth factors, such as fibroblast growth factor (FGF)32 which are chemotactic for muscle precursor cells and other leukocytes,37 and stimulate proliferation and differentiation of vascular endothelial cells and muscle precursors (reviewed in Refs. 13, 16).

Antigen presenting cells (APCs) include macrophages, B cells, and DC. Cells of the monocyte-macrophage series can express major histocompatibility complex (MHC) class II glycoproteins (Ia antigens) and activate memory T cells and B cells but not naive T cells; however, the efficiency of the activation of T cells by macrophages is negligible compared to DC which, more importantly, are potent accessory cells for induction of primary immune responses44 and thus are particularly important in autoimmune responses and as “passenger” cells in transplantation. DC are characterized in immunomorphological studies by their dendriform shape, constitutive expression of MHC class II antigens, and lack of colocalization of pan-macrophage markers.44 These bone-marrow–derived cells function as immune sentinels (see review, see Ref. 44) and are especially numerous at sites of high antigenic exposure such as the epidermis and dermis,21 respiratory tract,18 and gut,34 where their antigen trapping, internalization, and processing functions are optimized. DC are a dynamic population whose turnover time seems to vary depending on the tissue, from 3 days in lymphoid organs to up to 7 weeks in skin.10 DC have been identified in several species in the connective tissue of quiescent nonlymphoid tissues not in immediate contact with the external environment, such as cardiac muscle and liver.15,42 Rich populations of these cells have recently been demonstrated in the uveal tract of the eye.9,26,27 It is currently unclear whether DC are present in normal skeletal muscle, where they could play an important role as APCs in autoimmune responses, in inflammatory myopathies, and in the outcome of muscle transplantsations.

The overall aim of the present study was to investigate the distribution of resident tissue macrophages and DC in normal and regenerating skeletal muscle in SJL/J and BALB/c mice. We chose to compare these strains as it is known that old SJL/J mice develop spontaneous myositis and several studies have implicated macrophages as effector cells in models of this autoimmune process.31,39 Furthermore, new muscle formation following transplantation of minced muscle grafts or after crush injury is far more effective in SJL/J than in BALB/c mice.31,12,14,29

**MATERIALS AND METHODS**

**Animals.** Inbred 8-week-old SJL/J and BALB/c female mice (Specific Pathogen Free) were obtained from the Animal Resource Centre (Murdoch, WA). Animals were kept under routine laboratory conditions. All animal procedures were approved by the Animal Welfare Committee and carried out in strict accordance with the guidelines of the National Health and Medical Research Council of Australia.

**Surgical Procedures and Tissue Preparation.** The midregion of the tibialis anterior muscles in both hindlimbs were injured by a transverse crush injury as previously described.29 Muscles were removed from uninjured mice or at various times (1–8 days) after crush injury, bisected longitudinally, and frozen in isopentane cooled in liquid nitrogen. Samples of spleen collected from each mouse served as positive control tissue. Blocks were stored at −70°C until required. Longitudinal frozen sections (10 μm) were cut from each muscle at three levels (50–60 μm apart). A section of spleen was placed on each slide as a positive control. Both legs from duplicate mice were studied at each time point.

**Monoclonal Antibodies (mAbs).** The rat antimonouse mAbs (culture supernatant or ascites) used in this study were a generous gift from Dr. Pat Holt at the Institute for Child Health Research (Subiaco, WA) and the hamster antibodies were generously donated by Dr. Ralph Steinman (Rockefeller University, New York, NY). The specificity of the mAbs is summarized in Table 1.

**Immunohistochemical Procedures.** Sections were fixed for 15 min in cold absolute ethanol, air-dried for 1 h, and placed in a 2% solution of hydrogen peroxide in cold absolute ethanol for 15 min which served to reduce endogenous or pseudoperoxidase background staining. Primary antibodies were used at predetermined optimal dilutions. A standard indirect immunoperoxidase procedure using biotinylated sheep anti-rat antibodies (Amersham, UK) and
strepavidin–horseradish peroxidase (S-HRP) (Amersham International) was used to detect binding of the rat mAbs in this study. Binding of hamster anti-mouse mAbs was visualized with biotinylated goat anti-hamster secondary antibodies (Sigma Chemical Co., St. Louis, MO) and streptavidin–horseradish peroxidase (S-HRP) (Amersham International) was used to detect binding of the rat mAbs in this study. Binding of hamster anti-mouse mAbs was visualized with biotinylated goat anti-hamster secondary antibodies (Sigma Chemical Co., St. Louis, MO) and S-HRP. The peroxidase was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB: Sigma Chemical Co., St. Louis, MO) plus 5.5 µL of hydrogen peroxide (30% wt/vol). Slides in which the primary mAb was omitted and replaced by phosphate-buffered saline (PBS) plus 1% bovine serum albumin (Commonwealth Serum Laboratories, Australia) acted as negative controls. Sections were lightly counterstained with hematoxylin before mounting.

Quantitative Analysis of Immunohistochemical Staining. Due to the large numbers of immunopositive cells in regenerating muscles the boundaries between individual cells were often indistinct and, therefore, enumeration of individual cells was impractical. To overcome this problem a point-counting method was used to determine the percentage of tissue area occupied by immunoperoxidase staining. Projected images of the sections (Zeiss Mop 1) were placed onto a random-dot graticule (magnification ×600). The dots superimposed on the stained cells were counted in 20 random fields per section and the mean percentage area of each section covered by positively stained cells was calculated. Data from the three sections of each muscle were pooled. The mean values for both limbs were to provide a mean value for each animal. Two mice were studied at each time point making statistical analysis inappropriate.

RESULTS

Muscle from SJ L/J and BALB/c Mice. Immunohistochemical Studies of Normal Uninjured Muscle

Negative control slides showed very low background staining (less than 0.8% of the tissue section). The staining pattern on positive control tissue (spleen) was consistent with previous descriptions of the antibodies (Table 1). The spleens and muscles of SJL/J mice were negative when stained with M5/114 antibody as expected, since this antibody only recognizes cells from mice with the H-2 haplotypes b (C57Bl/6J), d (BALB/c), and q, but not s (SJL/J). In normal uninjured muscle from SJL/J and BALB/c mice, macrophages were scattered regularly in the perimysium and epimysium. They often displayed an elongated morphology in longitudinal sections (Fig. 1a and b), and smaller rounder/pleomorphic morphology in transverse section (not shown), reflecting their location in the interstitial space between muscle fibers. The location, distribution, and morphology of cells stained with the various known anti-macrophage markers (MOMA-2, SER-4, F4/80, and Mac-1) were similar. These cells occupied 1–2.6% of the tissue section area (Fig. 2a and b). Pleomorphic or bipolar MHC class II+ cells were dispersed widely in the perimysium and endomysium among the muscle fibers of BALB/c mice in a similar pattern to macrophages and were especially numerous in connective tissue around larger vessels (Fig. 1c and d). Quantitative analysis revealed that these cells occupied about 1–1.2% of the sectional area in normal BALB/c mouse skeletal muscle (Fig. 2c). MHC class II+ cells with a dendriform or bipolar morphology in sections are interpreted by many authors as positive evidence of DC. These putative DC in mouse skeletal muscle were similar to those described in rat cardiac muscle and other peripheral connective tissues. The DC population in mouse muscle failed to stain with NLDC-145, a recognized marker of subpopulation of lymphoid DC, and the cytoplasm of mononuclear phagocytes in the bone marrow and blood tissues.

Table 1. Monoclonal antibody specificities.

<table>
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<tr>
<th>mAb</th>
<th>Specificity</th>
<th>Reference</th>
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<tr>
<td>MOMA-2</td>
<td>Most mononuclear phagocytes including those in T- and B-cell areas of lymphoid organs; also some reaction in dendritic cells.</td>
<td>Kraal et al. 1987²¹</td>
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<td>SER-4</td>
<td>Binds to sialylated glycoconjugates on the surface of stromal macrophages of hematopoietic and lymphoid organs, but not on monocytes and macrophages in serous cavities; also components of the extracellular matrix.</td>
<td>Crocker and Gordon 1989⁷</td>
</tr>
<tr>
<td>Mac-1</td>
<td>Identifies macrophages and polymorphonuclear leukocytes, recognizes CD11b; receptor for complement factor C3b.</td>
<td>Springer et al. 1979⁶³</td>
</tr>
<tr>
<td>F4/80</td>
<td>Binds to the cell surface of most mononuclear phagocytes (blood monocytes and most resident and exudate macrophages) and Langerhans cells; also the cytoplasm of mononuclear phagocytes in the bone marrow and blood tissues.</td>
<td>Austyn and Gordon 1981³</td>
</tr>
<tr>
<td>MS/114</td>
<td>Recognizes cells expressing class II antigens (I-A b, d, &amp; q, but not k), e.g., dendritic cells and activated macrophages, some endothelial and muscle cells, etc.</td>
<td>Lemke et al. 1979²⁴</td>
</tr>
<tr>
<td>NLDC-145</td>
<td>Identifies nonlymphoid DC, e.g., veiled cells, interdigitating cells, Langerhans cells.</td>
<td>Kraal et al. 1986⁵⁰</td>
</tr>
<tr>
<td>N418</td>
<td>β2 (CD11c) integrin on isolated splenic DC and DC in tissue sections.</td>
<td>Metlay et al. 1990²⁴⁷</td>
</tr>
<tr>
<td>M342</td>
<td>Antigen within intracellular granules in DC.</td>
<td>Agger et al. 1992²⁵</td>
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ing with these hamster anti-mouse mAbs, N418 and M342, revealed a small population of pleiomorphic or bipolar cells in the connective tissue (Fig. 1e and f), thus confirming that some of the MHC class II+ cells in skeletal muscle are likely to be of the DC lineage.

Distribution and Density of Immunopositive Cells in Regenerating Skeletal Muscle. Conventional histology of regenerating muscles from SJL/J and BALB/c mice during the 6 days after crush injury revealed similar changes to those described in a previous publication. The qualitative and quantitative results of the present immunohistochemical study revealed a similar pattern of staining with both anti-macrophage mAbs (MOMA-2 and Mac-1) (Fig. 2a and b); therefore, only the staining pattern of one of these mAbs (Mac 1) is illustrated (Fig. 3A, B, and C). In both strains there was a marked increase in macrophages at the crush injury site by days 2 and 3 (Figs. 2a and b; 3A). In SJL/S mice almost one third of the

**FIGURE 1.** Immunohistochemical demonstration of the distribution of macrophages (Mac-1+) (a, low power; b, high power) and MHC class II (Ia)+ cells (M5/114) (c, low power; d, high power) in longitudinal and oblique sections of normal uninjured skeletal muscle (BALB/c mouse). (e, f) High-power fields of skeletal muscle cut in longitudinal sections stained with the pan-DC mAbs N418 (e) and M342 (f). Arrows indicate examples of some of the immunopositive cells. Original magnifications: a ×100, b ×240, c ×100, d ×240, e ×180, f ×180.
tissue section area displayed immunopositive reaction product (Fig. 2a and b). Although cell boundaries were indistinct around the injury site, individual cells were discernible distant from the site on examination at high magnification (Fig. 3B). The extent of staining around the injury site had decreased both qualitatively (Fig. 3C) and quantitatively (Fig. 2a and b) by day 6. At all time points studied the area of immunopositive cells in the muscle of SJL/J was approximately double that of BALB/c mice.

The area occupied by MHC class II+ cells in regenerating muscle tissue remained less than 5% of the tissue section area even at day 6 (Fig. 2c); however, the up-regulation of MHC class II expression could be resolved on single cells present in increased numbers around the injury site or distributed throughout the muscle distant from the injury site at greater than normal density (Fig. 3D, E, and F). In the present study it was not resolved whether this pattern of increased MHC class II expression was due to the accumulation/migration of local DC, up-regulation of the molecule on local DCs, an influx of blood-borne DC precursors, or the expression of MHC class II by macrophages.

DISCUSSION

The immunohistochemical demonstration of a population of resident tissue macrophages within the perimysium and endomysium of normal skeletal muscles in the present study confirms and substantially extends previously published descriptions. Furthermore, the present study reveals for the first time with the aid of anti-mouse DC-specific mAbs that DC are present in normal rodent skeletal muscle. This has important implications for our understanding of the pathogenesis of a variety of diseases, including inflammatory myopathies with an autoimmune etiology (dermatomyositis and polymyositis) and inherited disorders (Duchenne dystrophy). Furthermore, both macrophages and DC are crucial to the outcome of transplantation and the response of muscle to various forms of injury.

**Macrophages in Skeletal Muscle.** Preliminary evidence from a limited study describing low-grade muscle fiber repair in a “normal” rat suggested that ED2+ resident tissue macrophages played no role in phagocytosis of damaged muscle fibers. Our study showed that in response to crush injury macrophages increased in number both near the injury site and more generally throughout the muscle by day 2, peaked at day 3, and decreased by day 6. Similar dynamics in macrophage numbers were reported in a study of regeneration following bupivacaine-induced muscle injury in the rat. In the present study, staining patterns with the mAbs MOMA-2 and SER-4, which are reported to recognize mature murine tissue macrophages not identified by F4/80,

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**FIGURE 2.** Results of quantitative analysis of immunostained uninjured (day 0) and regenerating muscles from SJL/J and BALB/c mice showing the mean percentage area of the section immunopositive for (a) MOMA-2, (b) Mac-1, and (c) M5/114 (Ia). Regenerating muscles were sampled at 2, 4, and 6 days after crush injury. The control background level of staining has been subtracted from the data. *The mean values per animal, calculated from both legs, of duplicate mice are indicated.
were very similar. The fact that cells immunopositive for both of these mAbs increased following crushing suggests that either resident tissue macrophages (equivalent to ED2\textsuperscript{+} cells in rat) participate in the injury response or that new monocyte/macrophages (exudate macrophages) are also positive for these markers.

The Mac-1 mAb recognizes the C3b receptor (CD11b), present not only on macrophages but also on neutrophils. This may explain the greater increase in immunopositive stained tissue area at 2 days following injury when compared to pan-specific macrophage markers such as MOMA-2 (Fig. 2a) and SER-4 (data not shown). During the early phase of the muscle injury response, neutrophils are a conspicuous component of the cellular infiltrate in common with other acute inflammatory responses.\textsuperscript{45} Previous studies of injured muscles of SJL/J mice have

FIGURE 3. Immunohistochemical demonstration of macrophages and Ia\textsuperscript{+} cells in regenerating muscles of normal mice at 3–6 days after crush injury. (A, B) Low- and high-power views of Mac-1 stained SJL/J muscle at day 3. (C) Low-power field of Mac-1 stained SJL/J muscle at day 6. (D, E) Low- and high-power fields of the distribution of Ia (M5/114) staining in BALB/c muscle at day 3 and (F) day 6. Arrows indicate examples of some of the immunopositive cells. Original magnifications: A ×40, B ×150, C ×40, D ×40, E ×150, F ×40.
shown neutrophils are present at 6 h and peak at 12 h, but by 24 h macrophages are the predominant cell type.33 Very similar results were reported in regenerating rat muscles34 where neutrophils were first seen within 30–60 min, increased rapidly in number by 6 h, and peaked around 12 h. Thereafter they were gradually replaced by actively phagocytic macrophages. Macrophages have also been identified as the predominant cell type in the inflammatory infiltrate in experimentally induced myositis.39

In vitro studies have shown that factors produced by damaged skeletal muscle [e.g., FGF and platelet-derived growth factor (PDGF)] are highly chemoattractant for both neutrophils and macrophages37 and this activity was apparent within muscle tissue 3 h postinjury and had increased by 24 h. This correlates with commencement of the influx of macrophages in vivo shown in the present study. Cytokines produced by resident tissue macrophages, newly recruited monocyte/macrophages, and neutrophils will in turn attract additional leukocytes to the wound site.6 Furthermore, it has been shown that activated macrophages (equivalent to those which accumulate at the site of muscle damage) but not neutrophils produce soluble factors (FGF and PDGF) which are highly chemoattractant and also mitogenic for muscle precursor cells.37 Thus the activated macrophages which accumulate in response to muscle damage will not only phagocytose necrotic tissue but also facilitate the repair of damaged myofibers. The present study revealed a more marked macrophage response following crush injury in SJL/J mice compared to BALB/c. This confirms a previous conventional histological study29 in which a comparison of sequential cellular events in regenerating muscles in the two strains revealed twice as many mononuclear cells in SJL/J muscles at 2 and 3 days after crush injury. In light of our present immunophenotypic analysis, it is likely that many of these cells are macrophages. Irradiation bone-marrow chimeras experiments have shown that this difference between strains is not a consequence of the blood-borne infiltrating cell population, but is a consequence of “host” tissue microenvironment, which must affect macrophage function.30 One of the factors in the host microenvironment of these two strains may include the high numbers of resident tissue macrophages which are known to have long half-lives. Alternatively, the accentuated response to skeletal muscle damage in SJL/J mice may be an early manifestation of the low-grade autoimmune myositis that develops in older animals (40 weeks and over).38,39 The number of macrophages within muscles may also play a role in other pathological conditions. For example, in Grave’s ophthalmopathy, an organ-specific autoimmune disease affecting orbital tissues, the extraocular muscles most affected by the dysthyroidism are those which contain the highest number of macrophages.40 

The use of one specific hindlimb muscle, the tibialis anterior, throughout the present study is unlikely to have biased results, as a recent investigation has shown that macrophage numbers were similar in both slow twitch (soleus) and fast twitch (extensor digitorum longus) muscle.41

### Presence of DC in Skeletal Muscle

The demonstration in the present study of irregularly shaped MHC class II+ cells in the connective tissue of normal skeletal muscle is strong evidence that these cells are DC according to the generally accepted standards of immunomorphological criteria.44 Studies with the recently described hamster antimouse DC mAbs, N418 and M342, confirmed that these important immune “sentinels” are indeed a component of normal murine skeletal muscle. The DC in normal muscle do not stain with the mAb NLDC-145, which recognizes lymphoid DC and DC subpopulations in some peripheral tissues.22 The predominantly perivascular location of the N418+ and M342+ cells and the reduced density in comparison to MHC class II+ cells suggests that a subpopulation of the MHC class II+ cells may be resident tissue macrophages. Alternatively, the anti-DC mAbs may not recognize all MHC class II+ DC in peripheral tissues. Resolution of this issue will require further double immunohistochemical and functional studies. Evidence from double immunohistochemical studies performed in our laboratory on rat extraocular muscle strongly supports the contention that la+ DC populations coexist with resident tissue macrophages in normal muscle (McMenamin, unpublished data). Furthermore, it is known that DC are present in cardiac muscle and connective tissues.15,42 Therefore, the present study is the first firm evidence that this important lineage of cells are present in normal rodent skeletal muscle. The only previous studies include limited analyses of normal human skeletal muscle which were performed as controls in immunohistochemical investigations of myopathies.2,20 MHC class II+ perivascular cells, resembling the DC of the present study, were briefly described and illustrated, although the staining pattern was not ascribed specifically to cells of the DC lineage by these authors.

The persistence of MHC class II positive cells in regenerating mouse muscle even at day 6 (when macrophage numbers had generally decreased) may
be accounted for by increased numbers of newly arrived DC. Other groups have suggested that nonleukocyte cell types such as endothelial cells,39 muscle precursor cells, and myotubes may be induced to express MHC class II antigens. Our data did not support this contention.

The present study has provided basic immunophenotypic analysis of resident tissue macrophages and DC in normal murine skeletal muscle and demonstrated the dynamics of these cells in response to an acute injury. These data should provide a valuable baseline for investigating the role of these cells in other forms of muscle injury and disease. The observation that MHC class II+ DC are present in normal skeletal muscle should be of clinical relevance in the context of allogeneic transplantation studies, where these cells may act as a source of MHC class II antigen in graft versus host reactions. The importance of DC in primary immune responses makes them likely local APCs in a variety of organ-specific autoimmune responses. The observations that they are present in skeletal muscle make them a possible candidate as the local APC in autoimmune myopathies.

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