Histol Histopathol (2011) 26: 433-442

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Alterations in the dynamics of inflammation, proliferation and apoptosis in subcutaneous implants of lupus-prone mice

Paula P. Campos[1], Anilton C. Vasconcelos[2], Mônica A.N.D. Ferreira[2] and Silvia P. Andrade[1]

[1]Departments of Physiology and Biophysics and [2]General Pathology of the Institute of Biological Sciences, Federal University of Minas Gerais/Brazil

Summary. Wound repair is a complex process that involves inflammation, proliferation, extracellular matrix deposition/remodeling and apoptosis. Autoimmune diseases profoundly affect the healing process. We have used histological parameters to characterize the recruitment of mast cells and the proliferative activity and apoptosis in the fibrovascular tissue induced by subcutaneous polyether-polyurethane sponge implants in lupus-prone New Zealand White (NZW) and in control Balb/c mouse strains at days 10 and 21 post implantation. Fibrovascular tissue infiltration (hematoxylin and eosin staining), mast cell number (Dominici staining) and cellular proliferation (AgNOR staining) peaked early (day 10) but collagen deposition (picrosirius red staining) and apoptosis remained high in implants of NZW mice during the experimental period. In contrast, implants of Balb/c animals showed a progressive increase in mast cell recruitment and cellular proliferation but apoptosis fell from day 10 to 21 post-implantation. This divergent response early mast cells recruitment, excessive collagen deposition and disturbed removal of apoptotic cells from the site of injury in NZW mice implies that the genotype trait of NZW mice is a determining factor in abnormal healing response.

Key words: Mast cells, Collagen deposition, AgNOR, Fibrovascular tissue

Offprint requests to: Silvia Passos Andrade, Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Av. Antônio Carlos, 6627, Campus Pampulha, CEP 31.270-901, Belo Horizonte - Minas Gerais – Brazil. e-mail: andrades@icb.ufmg.br

Introduction

Tissue repair involves a series of overlapping events (hemostasis, inflammation, proliferation and remodeling). In each of these phases a rapid increase in specific cell populations occur, and after performing their roles leave the wound site to allow the next event to take place (Greenhalgh, 1998; Diegelmann and Evans, 2004). Thus, when disruption of blood vessels occurs after injury, a leakage of blood components into the wound space triggers a well established pattern (platelet activation, clot formation, leukocyte recruitment, activation/proliferation of endothelial cells and fibroblasts). Among the inflammatory cells, mast cells play a relevant role in both acute and chronic inflammation, as well as in allergy and autoimmunity. Specifically, this cell population produces a plethora of molecules such as tumor necrosis factor-α (TNF-α), interleukins, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and vasoactive mediators. These molecules exert profound effects on inflammatory cell activation, recruitment and proliferation (Robbie-Ryan and Brown, 2002; Martin and Leibovich, 2005). Specific cell types that predominate in each phase must be substituted in the wound site by apoptosis, before the beginning of the subsequent phase. This is the main process responsible for decreasing cellularity during the various phases of wound healing (Savill, 1997; Greenhalgh, 1998). Wounds that remain open or that present deficient apoptotic clearance result in chronic inflammatory response (Greenhalgh, 1998).

While many phases of wound repair and specific mechanisms regulating these activities are common to the various types of wounds, multiple causative factors (inflammation, trauma, operations, systemic diseases)

can lead to severe healing deficiencies/alterations (Ttsirogianni et al., 2006; Koch and Distler, 2007). Particularly, auto-immune diseases such as Erythematosus Lupus greatly affect the integrity of many tissues as a result of excessive inflammation, autoantibody production and impairment in the resolution of inflammation (Kono and Theofilopoulous, 2000; Potter et al., 2003). The presence of mast cells or their products in the synovial fluid of patients with rheumatoid arthritis and systemic lupus erythematosus (SLE) is a marked cellular event in these conditions (Levi-Schaffer and Weg, 1997; Trautman et al., 2000). Another altered event in SLE is associated with impaired clearance of apoptotic cells by macrophages, which might be responsible for initiation and maintenance of auto-immune responses in lupus-prone strains (Potter et al., 2003). Analysis of the wound healing trait in autoimmune-prone inbred mouse strain (MRL/+) has shown accelerated and extensive healing to ear punch wound (Clark et al.,1998), rapid wound healing in burned cornea (Ueno et al., 2005) and reduced scarring in the heart (Leferovich et al., 2001). All these fast healing events have been attributed to a lack of a robust proinflammatory gene expression, characteristic of this mouse strain (Ueno et al., 2005). In a recent study, we have shown that the sponge-healing process in NZW mice (another strain of lupus-prone mice) differed importantly from a non-lupus prone strain regarding the intensity and temporal aspects of inflammation, angiogenesis, cytokine production and fibrosis (Campos et al., 2008). Collectively, these data indicate that this autoimmune susceptibility modifies the natural healing process described in mammals. In view of the fact that mast cells and/or their products and altered apoptotic process have been associated with some manifestations of systemic lupus erythematosus in various organs and tissues we decided to use the chronic inflammatory model of sponge implantation to further characterize the influence of a lupus-facilitating susceptibility in mast cell recruitment, cellular proliferation and apoptosis in this model. The type of host reaction to synthetic matrix is a non-specific type of inflammation that occurs in an environment of defined dimensions with no influence by epithelial cells or wound contraction, allowing therefore the assessment of relevant components of the newly formed fibrovascular tissue (Kyriakides et al., 2001; Bradshaw et al., 2001; Campos et al., 2006).

Material and methods

Male NZW and Balb/c mice seven to eight weeks old (20-30 g body weight) provided by the Central Animal Facility at the ICB, UFMG were used in these experiments. The animals were housed individually and provided with chow pellets and water *ad libitum*. The light/dark cycle was 12:12 hours with lights on at 7:00 a.m. and lights off at 7:00 p.m. Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia and post-operative care concurred with the guidelines established by our local Institutional Animal Care and Use Committee that also reviewed and approved this work.

Preparation of sponge discs and implantation

Polyether-polyurethane sponge (Vitafoam Ltd., Manchester, UK) was used as the implanted material. The implants were discs, 5 mm thick x 8 mm diameter and were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 minutes before implantation. The animals were anaesthetized with 2,2,2-tribromoethanol (1 mg kg^{-1}; i.p. Aldrich, USA), the dorsal hair shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. Post-operatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress; any showing such signs were immediately humanely killed.

Histological staining and analysis

The implant-bearing mice were killed by cervical dislocation at 10 and 21 days post-implantation and the sponge discs carefully removed, dissected free from adherent tissue, weighed and fixed in formalin (10% w/v in isotonic saline). For each time point 4 to 5 implants from different animals were examined. Sections (5 μm) were stained and processed for light microscopic studies and morphometric analysis. Hematoxylin and eosin staining (HE) was used for determining the implant fibrovascular area. AgNOR, a silver staining technique, commonly used to detect changes in nucleolar organizer regions (Aubele et al., 1994; Campos et al., 2006) was employed as a marker for cellular activity/proliferation. Picrosirius staining followed by polarized-light microscopy was used to visualize and determine collagen fibers (Campos et al., 2006). Picrosirius Red is an anionic composite that distinguishes the thickness and density of collagen fibers through coloration emitted under polarized light. While the fine dissociated fibers typical of type III collagen are greenish, the thickest and strong associated fibers of type I collagen emit colors with larger wave-length such as red and yellow.

Mast cells were detected by Dominici staining and apoptotic cells were detected by the terminal deoxynucleodidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method (Gavrieli et al., 1992).

All stainings were performed in paraffin-embedded sponge implant sections mounted in glass slides. To perform morphometric analysis 10 images of cross sections obtained from whole slides were evaluated for densitometric fibrovascular area determination and collagen quantification (final magnification = 100x). Collagen fibers stained by picrosirius and observed under polarized light microscopy were digitized and then

binarized according to their pixel density (and color) to select only type I and III collagen within the sponge. Densitometric quantification of collagen was then achieved by image processing and the sum of those areas with the same pixel density.

The following formula was used to quantify the areas of types I and III collagen:
Type I or III collagen = (∑ areas with the same density of pixels / total area of sponge within the microscopical field) x100

For the morphometric analysis of number of mast cells, AgNORs, and apoptotic cells, images were obtained from 20 fields per slide (8,533 μm^2/field) - with a planapochromatic objective 40x in light microscopy (final magnification: x400). The results are presented as cell indices (Apoptosis, AgNORs, Mast cells) using the percentage of positive labeled cells by the total number of cells per field. The images were digitized through a JVC TK-1270/JGB microcamera and transferred to an analyzer (Kontron Eletroniks, Carl Zeiss – KS300, version 2).

Statistical analysis

Results are presented as mean±S.E.M. Comparisons between the groups (two) were carried out using Student's t-test for unpaired data. A P-value less than 0.05 was considered significant.

Results

Subcutaneous implantation of the sponge discs in both strains of mice induced a wound repair-like response, causing the synthetic matrix to be filled with fibrovascular tissue as shown by H&E staining. This granulation tissue was composed of an inflammatory infiltrate with leukocytes, spindle-shaped fibroblast-like cells and endothelial cells forming microvessels (Fig. 1A-D). There were, however, important strain related differences. Thus, the amount of tissue infiltration (area of fibrovascular tissue; μm^2) in NZW implants was denser than in the corresponding implants from Balb/c mice, as assessed by the morphometric analysis at days 10 and 21 post-implantation. The entire matrix was occupied by the new tissue at day 10 in NZW mice but in implants of Balb/c animals the area of fibrovascular tissue growth was significantly less at day 10 post-implantation but continued to increase up to day 21 (Fig. 1E). Densitometric analysis of collagen content (Picrosirius staining) showed a progressive increase in implants of NZW animals whereas no change in collagen deposition was observed from day 10 to 21 in implants of the Balb/c strain (Fig. 2A-E).

In sections stained with Dominici (Fig. 3A-D), mast cells were clearly increased in implants of NZW mice at day 10 post implantation as compared with the number in Balb/c mice. In these animals the number of mast cells comparable to those of NZW occurred at day 21 (Fig. 2E). Cell proliferation and activation in the implants of both types of animals were determined by AgNOR technique (seen as aggregates of dark structures on a grey nuclear background; Fig. 4A-D). AgNOR index (AgNOR/cell) as determined by morphometric analysis was increased in NZW implants at day 10 post-implantation (2.74±0.04) relative to Balb/c implants (2.42±0.04). The values for NZW after 21 days post-implantation (2.77±0.05) did not differ from the balb/c group (2.65±0.05) (Fig. 4E). The turn-over pattern of the cell population within the fibrovascular tissue induced by the sponge implants was determined by the number of TUNEL-positive cells. Representative micro-photographs of apoptotic cells show dark-brown TUNEL positive nuclei with other morphological features of cellular death (anoikia, cellular shrinkage, condensed chromatin). Apoptosis index had parallel courses at day 10 in both types of implants but decreased in Balb/c implants at day 21 while it increased in implants of NZW animals.

Discussion

We have studied cellular events associated with healing response in NZW mice in view of their susceptibility to develop systemic erythematous lupus-like (SLE) disease, either spontaneously or after being exposed to several internal or environmental stimuli (Morel et al., 1997; Rudofsky and Lawrence, 1999). This systemic disease has been shown to alter key components and the temporal pattern of the natural healing process. Previously, we have shown that in this lupus-prone mouse strain inflammation was impaired and angiogenesis was accelerated in the sponge model (Campos et al., 2008). In another strain of lupus-prone mice (MRL/+) rapid healing accompanied by decreased inflammatory response after several injuries has been reported (Leferovich et al., 2001; Ueno et al., 2005). In this study we have characterized the dynamics of mast cell number, proliferative and fibrogenic activities and apoptosis in the fibrovascular tissue in synthetic polyether-polyuretane implanted subcutaneously in NZW and Balb/c mouse strains. We deliberately chose a non lupus-prone strain of mouse in this study because our aim was to determine how animals of two distinct genetic backgrounds would respond to the same inflammatory insult (sponge implant), with key components of wound healing process.

Except for collagen deposition and apoptosis, the cellular events peaked at day 10 in implants of NZW mice, tending to decrease by day 21 post-implantation. In implants of Balb/c animals the dynamics of fibrovascular tissue growth differed from that of lupus-prone mice. In the newly formed tissue only mast cell recruitment increased from day 10 to 21 post implantation and apoptosis fell. The other parameters remained overall unchanged during the experimental period. This pattern of early inflammation, proliferation and progressive collagen deposition in implants of NZW mice is fully compatible with the notion of accelerated

healing response observed in lupus-prone strains in a variety of wound healing models (Clark et al., 1998; Ueno et al., 2005). Interestingly, these changes were observed in the absence of clinical signs of disease, raising the possibility that the genetic trait is a determining factor for these wound healing alterations.

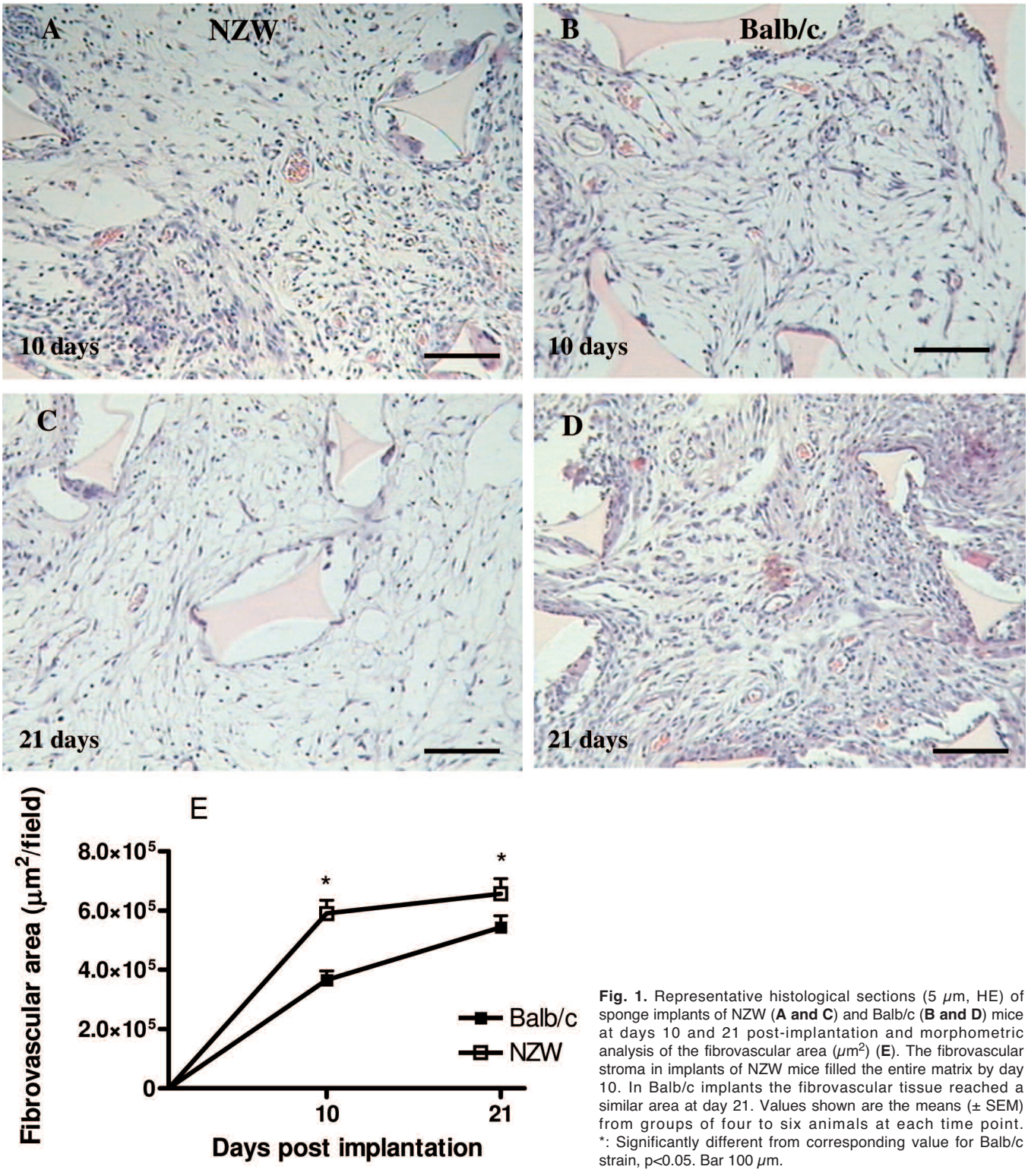


Fig. 1. Representative histological sections (5 μm, HE) of sponge implants of NZW (**A and C**) and Balb/c (**B and D**) mice at days 10 and 21 post-implantation and morphometric analysis of the fibrovascular area (μm^2) (**E**). The fibrovascular stroma in implants of NZW mice filled the entire matrix by day 10. In Balb/c implants the fibrovascular tissue reached a similar area at day 21. Values shown are the means (± SEM) from groups of four to six animals at each time point. *: Significantly different from corresponding value for Balb/c strain, $p<0.05$. Bar 100 μm.

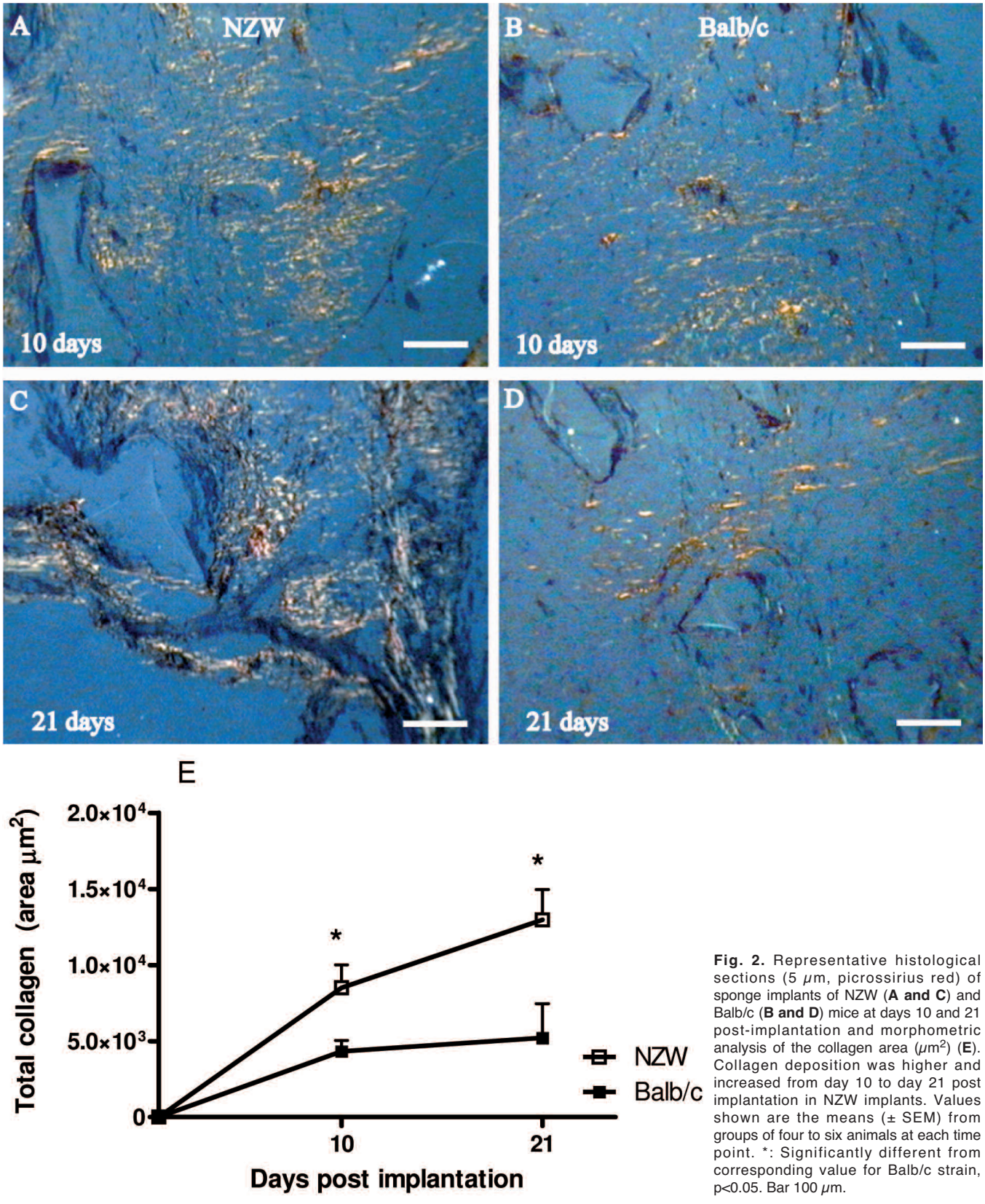


Fig. 2. Representative histological sections (5 μm, picrossirius red) of sponge implants of NZW (**A and C**) and Balb/c (**B and D**) mice at days 10 and 21 post-implantation and morphometric analysis of the collagen area (μm^2) (**E**). Collagen deposition was higher and increased from day 10 to day 21 post implantation in NZW implants. Values shown are the means (± SEM) from groups of four to six animals at each time point. *: Significantly different from corresponding value for Balb/c strain, $p<0.05$. Bar 100 μm.

The fact that mast cells have peaked earlier in implants of NZW mice compared with the increasing mast cell recruitment in Balb/c implants suggests that this cell population plays a critical role in accelerating fibrovascular tissue deposition in the lupus-prone strain. This suggestion has strong experimental evidence since mast cells and their products are increased in a number of autoimmune dysfunctions and immunological skin

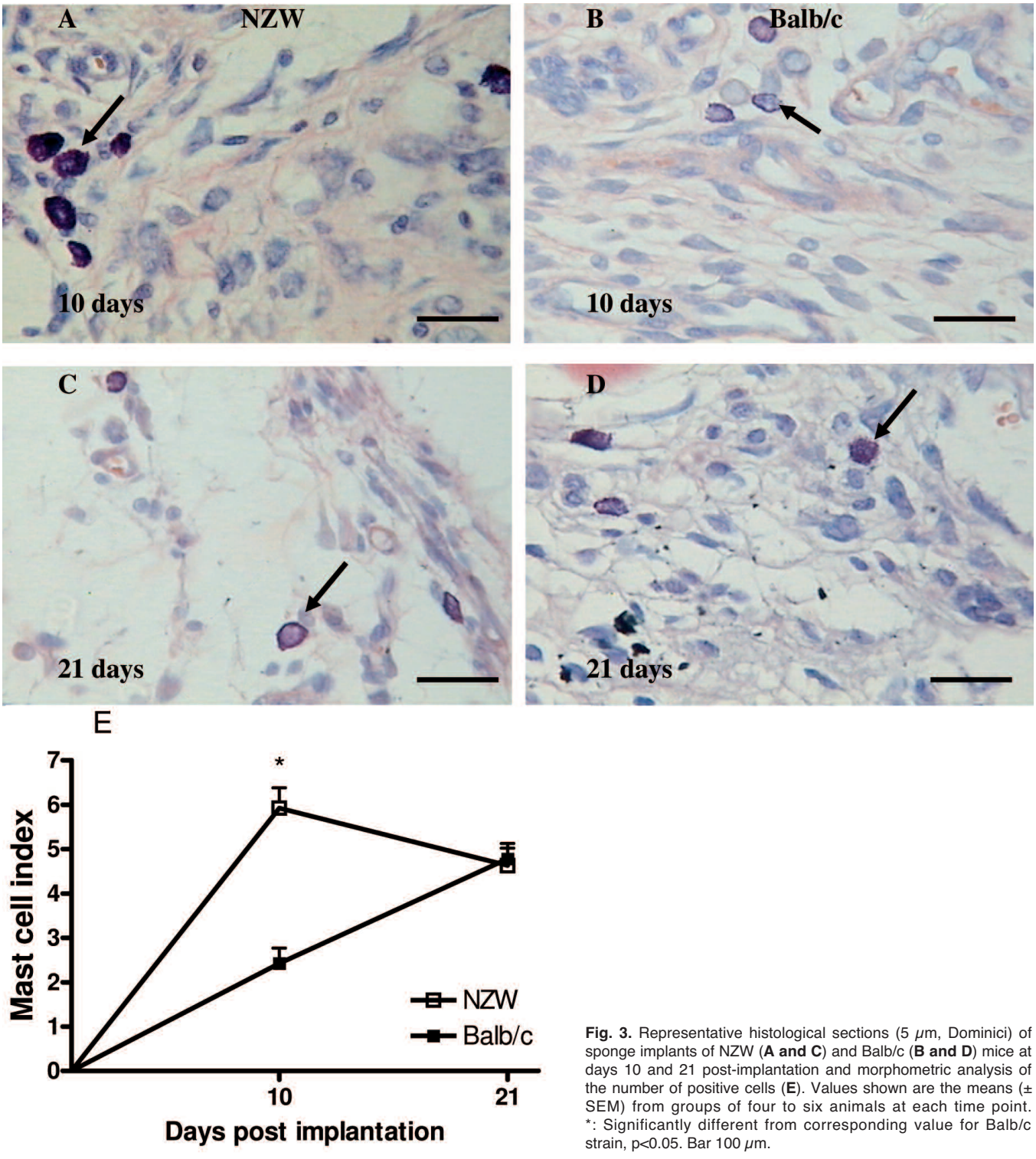
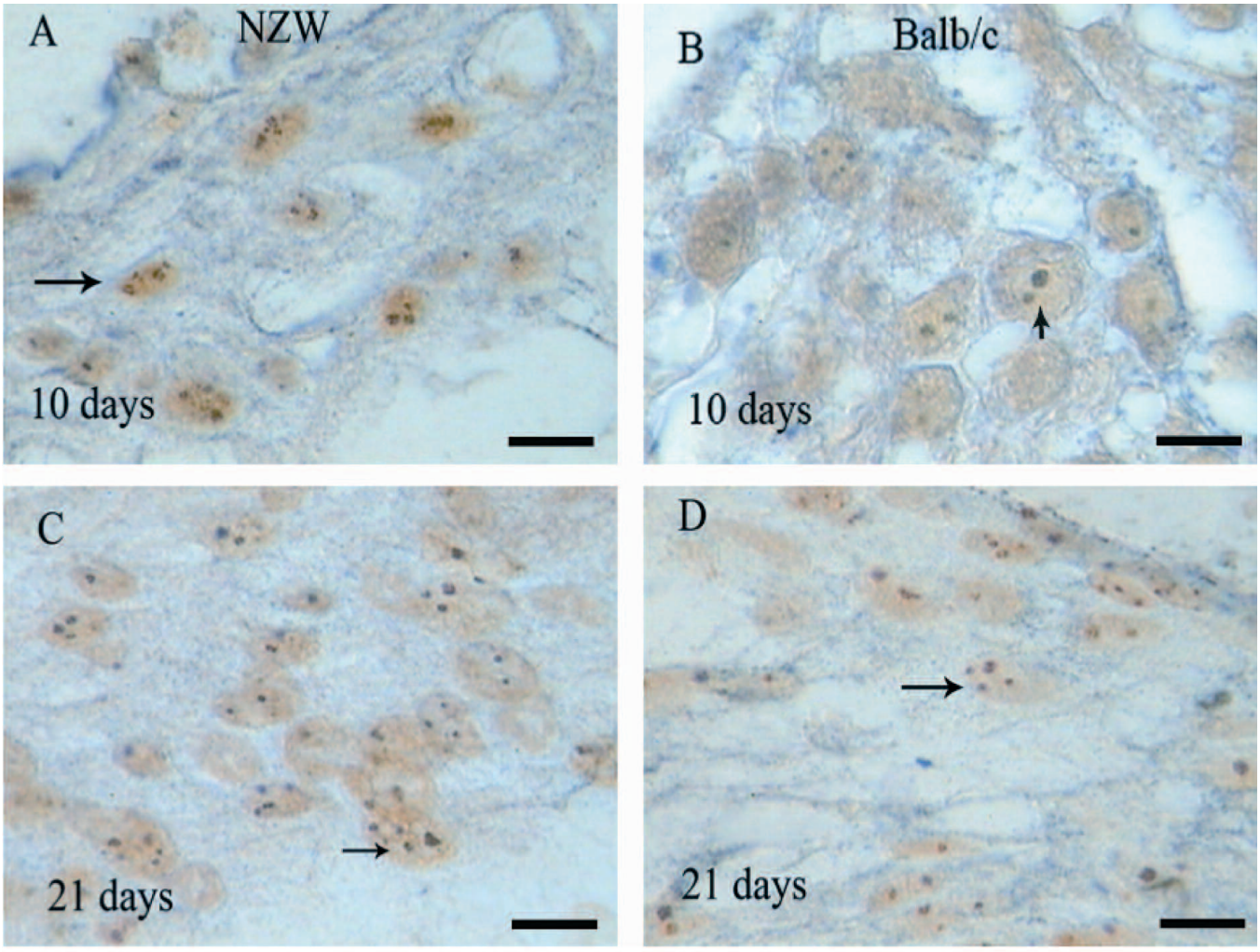


Fig. 3. Representative histological sections (5 μm, Dominici) of sponge implants of NZW (**A and C**) and Balb/c (**B and D**) mice at days 10 and 21 post-implantation and morphometric analysis of the number of positive cells (**E**). Values shown are the means (± SEM) from groups of four to six animals at each time point. *: Significantly different from corresponding value for Balb/c strain, $p<0.05$. Bar 100 μm.



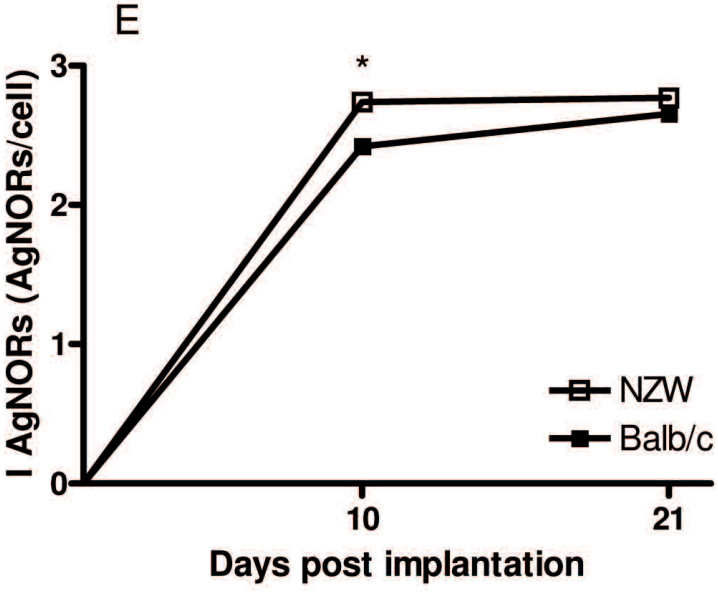


Fig. 4. Representative histological sections (5 μm, AgNOR) of sponge implants of NZW (**A and C**) and Balb/c (**B and D**) mice at days 10 and 21 post-implantation and morphometric analysis of the number of positive cells (**E**). Values shown are the means (± SEM) from groups of four to six animals at each time point. *: Significantly different from corresponding value for Balb/c strain, $p<0.05$. Bar 10 μm.

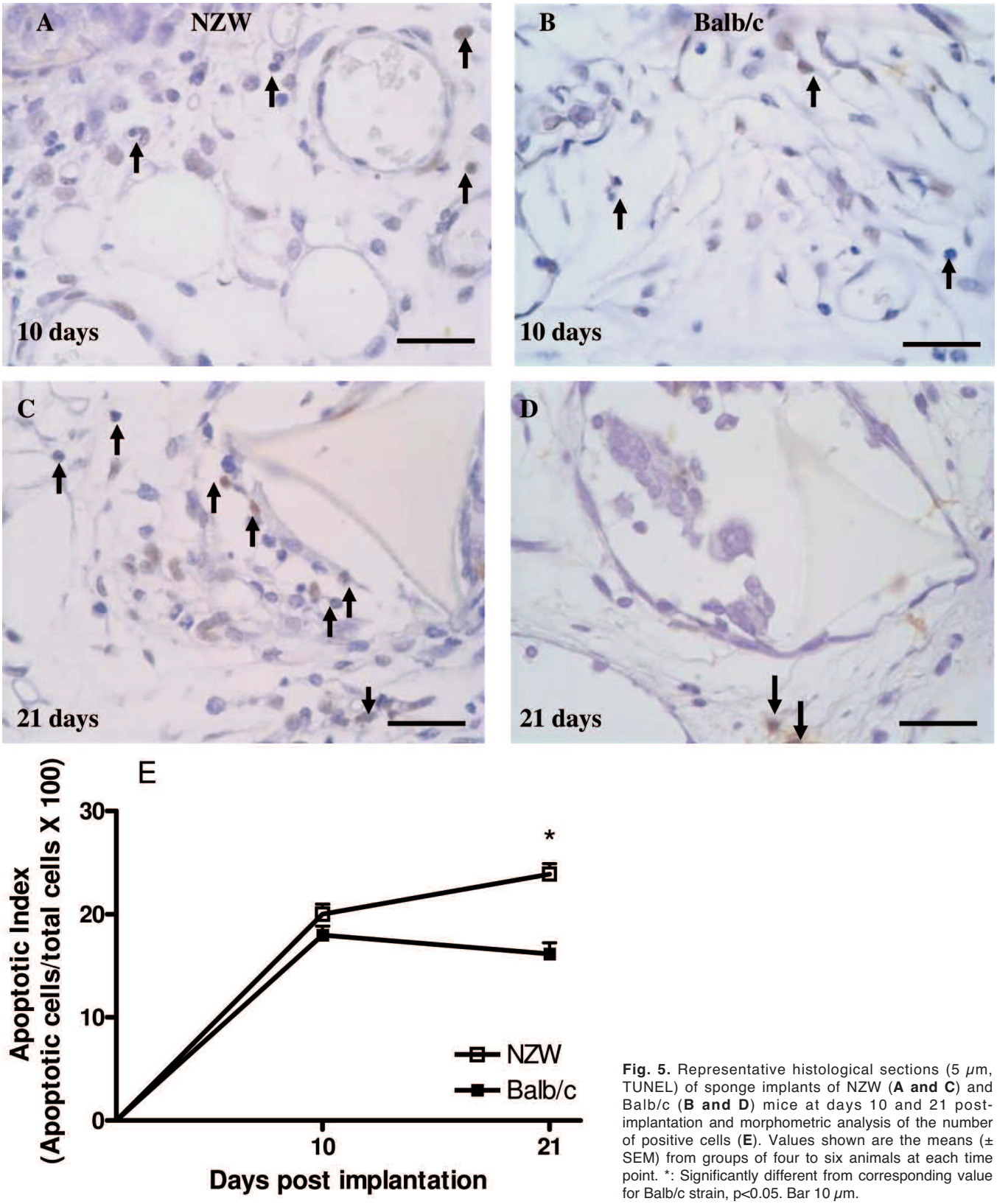


Fig. 5. Representative histological sections (5 μm, TUNEL) of sponge implants of NZW (**A and C**) and Balb/c (**B and D**) mice at days 10 and 21 post-implantation and morphometric analysis of the number of positive cells (**E**). Values shown are the means (± SEM) from groups of four to six animals at each time point. *: Significantly different from corresponding value for Balb/c strain, $p<0.05$. Bar 10 μm.

diseases (Trautman et al., 2000; Navi et al., 2007), and in autoimmune mice the number of this cell population is also enhanced (Ohmori, 1994). In addition, mast cells perform a variety of functions in both early and late phases of the healing process (Egozi et al., 2003; Weller et al., 2006). Thus, during early wound repair mast cells contribute to production of inflammatory mediators (histamine, serotonin), and subsequently these cells promote proliferation of fibroblasts, endothelial cells and keratinocytes and induce the release of their products (angiogenic factors, extracellular matrix components). In the remodeling phase, mast cells produce and release proteolytic enzymes and increase collagen production of fibroblasts and myofibroblasts (Gailit et al., 2001). It is likely, therefore, that early mast cell recruitment was responsible, at least in part, for anticipating the proliferative activity (as determined by AgNOR staining) and for the progressive increase in collagen deposition (picrosirius red) in lupus-prone mouse implants. The pattern of accelerated inflammation, proliferative and fibrogenic activities in implants of NZW mice was followed by sustained apoptosis, in contrast to a fall in apoptosis in implants of Balb/c mice. This form of programmed cell death is considered the main mechanism by which inflammatory cells are removed from injured sites (Brown et al., 1997; Greenhalgh, 1998). It is relevant to mention that in lupus-prone mice defects in apoptotic pathways result in increased levels of apoptotic nucleosomes (Zykova et al., 2007) and that these animals present defects in the resolution of inflammation (Potter et al., 2003). It has been proposed that the persistence of apoptotic and necrotic cells may be harmful to the inflammatory response, as this may lead to a specific immune response to these cells (Potter et al., 2003).

The results that have emerged from this study have revealed for the first time that in NZW mice the dynamics of mast cell recruitment, proliferative cellular activity, collagen deposition and apoptosis in sponge-induced wound healing of NZW mice (lupus-prone strain) differed from the response of the Balb/c strain. This divergent response suggests a link between immune alterations and wound healing and may be explained by the genotype trait of the NZW mice which is in line with an aberrant healing pattern reported in this and in other lupus-prone mouse strains.

Acknowledgements. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil); Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG).

References

Aubele M., Biesterfeld S., Derenzini M., Hufnagl P., Martin H., Ofner D., Ploton D. and Rüschoff J. (1994). Guidelines of AgNOR quantitation. Committee on AgNOR Quantitation within the European Society of Pathology. Zentralbl. Pathol. 140,107-108.

Bradshaw A.D., Reed M.J., Carbon J.G., Pinney E., Brekken R.A. and Sage E.H. (2001). Increased fibrovascular invasion of subcutaneous polyvinyl alcohol sponges in SPARC-null mice. Wound Rep. Reg. 9, 522-530.

Brown D.L., Kao W.W.Y. and Greenhalgh D.G. (1997). Apoptosis down-regulates inflammation under the advancing epithelial wound edge: Delayed patterns in diabetes and improvement with topical growth factors. Surgery 121, 372-380.

Campos P.P., Andrade S.P., Moro L., Ferreira M.A.N.D. and Vasconcelos A.C. (2006). Cellular proliferation, differentiation and apoptosis in polyether-polyurethane sponge implant model in mice. Histol. Histopathol. 21, 1263-1270.

Campos P.P., Bakhle Y.S. and Andrade S.P. (2008). Mechanisms of wound healing responses in lupus-prone New Zealand White mouse strain. Wound Rep. Reg. 16, 416-424.

Clark L., Clark R. and Heber-Katz E. (1998). A new murine model for mammalian wound repair and regeneration. Clin. Immunol. Immunopathol. 88, 35-45.

Diegelmann R.F. and Evans M.C. (2004). Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci. 9, 283-289.

Egozi E.I., Ferreira M.A., Burns A.L., Gamelli R.L. and Dipietro L.A. (2003). Mast cells modulate the inflammatory but not the proliferative response in healing wounds. Wound Rep. Reg. 11, 46-54.

Gailit J., Marchese M.J., Kew R.R. and Gruber B.L. (2001). The differentiation and function of myofibroblasts is regulated by mast cells mediators. J. Invest. Dermatol. 117, 1113-1119.

Gavrieli Y., Sherman Y. and Bem-Basson A.S. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. 119, 493-501.

Greenhalgh D.G. (1998). The role of apoptosis in wound healing. J. Biochem. Cell Biol. 30, 1019-1030.

Koch A.E. and Distler O. (2007). Vasculopathy and disordered angiogenesis in selected rheumatic diseases: rheumatoid arthritis and systemic sclerosis. Arthrits Res. Ther. 9, Suppl 2, S3.

Kono D.H. and Theofilopoulous A.N. (2000). Genetics of systemic autoimmunity in mouse models of lupus. Int. Rev. Immunol. 19, 367-387.

Kyriakides T.R., Zhu Y.H., Yang Z., Huynh G. and Bornstain P. (2001). Altered extracellular matrix remodeling and angiogenesis in sponge granulomas of thrombospondin2-null mice. Am. J. Pathol. 159, 1255-1262.

Leferovich J.M., Bedelbaeva K., Samulewicz S., Zhang X-M., Zwas E.B.L. and Heber-Katz E. (2001). Heart regeneration in adult MRL mice. Proc. Natl. Acad. Sci. USA 98, 9830-9835.

Levi-Schaffer F. and Weg V.B. (1997). Mast cells, eosinophils and fibrosis. Clin. Exp. Allergy 27, 64-70.

Martin P. and Leibovich S.J. (2005). Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol. 15, 599-607.

Morel L., Mohan C., Coker P.B., Tian X-H. and Wakeland E.K. (1997). Functional dissection of systemic lupus erythematosus using congenic mouse strains. J. Immunol. 158, 6019-6028.

Navi D., Saegusa J. and Liu F.T. (2007). Mast cells and immunological skin diseases. Clin. Rev. Allergy Immunol. 33, 144-155.

Ohmori J. (1994). A study of mast cells in autoimmune mice: the proliferative response of autoimmune mast cells to cytokines. Autoimmunity 17, 279-285.

Potter P.K., Cortes-Hernandez J., Quartier P., Botto M. and Walport M.J. (2003). Lupus-prone mice have an abnormal response to thioglycolate and an impaired clearance of apoptotic cells. J.

Immunol. 170, 3223-3232.

Robbie-Ryan M. and Brown M. (2002). The role of mast cells in allergy and autoimmunity. Curr. Opin. Immunol. 14, 728-733.

Rudofsky U.H. and Lawrence D.A. (1999). New Zealand mixed mice: a genetic systemic lupus erythematous model for assessing environmental effects. Environm. Health Perspec. 107, 713-721

Savill J. (1997). Apoptosis in resolution of inflammation. J. Leukocyte Biol. 61, 375-380.

Trautman A., Toksoy A., Engelhardt E., Brocker E.B. and Gillitzer R. (2000). Mast cell involvement in normal human skin wound healing: expression of MCP-1 is correlated with recruitment of mast cells which synthesize IL-4 in vivo. J. Pathol. 190, 100-106.

Tsirogianni A.K., Moutsopoulos N.M. and Moutsopoulos H.M. (2006). Wound healing: immunological aspects. Injury 37, 1, S5-12.

Ueno M., Lyons B.L., Burzenski L.M., Gott B., Shaffer D.J., Roopenian D.C. and Shultz L.D. (2005). Accelerated wound healing of alkali-burned corneas in MRL mice is associated with a reduced inflammatory signature. Invest. Ophthalmol. Vis. Sci. 46, 4097-4106.

Weller K., Foitzik K., Paus R., Syska W. and Maurer M. (2006). Mast cells are required for normal healing of skin wounds in mice. FASEB J. 20, 2366-2368.

Zykova S.N., Seredkina N.E. and Rekviq O.P. (2007). Glomerular targets for autoantibodies in lupus nephritis- an apoptotic origin. Ann. NY Acad. Sci. 1108, 1-10.

Accepted October 20, 2010