Monocyte chemoattractant protein-1 (MCP-1) transgenic mice: lessons from cardioprotection against ischemia to autoimmune inflammatory diseases

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for who really loves me

1. Introduction

1.1. MCP-1

MCP-1 is a glycoprotein of 76 amino acids that was isolated and identified for the first time from human tumor cell lines as related factor with monocyte chemotactic activity (Van Damme, Decock et al. 1989) (Fig. 1).

It belongs to chemokines (chemotactic cytokines), a family of low-molecular-weight (8-10 kD) proteins with four conserved cysteins. Chemokines are divided into two subfamilies, called C-X-C and C-C chemokines, depending on whether the two cysteins nearest the N-terminals are separated by one amino acid or not (Miller and Krangel 1992; Baggiolino, Dewald et al. 1994). Recently, two others subfamilies have been identified: the C chemokines, which lack the first and third of the conserved cysteine, and transmembrane chemokines with an active C-X-3C motif. (Bazan, Bacon et al. 1997). Most C-X-C chemokines specifically attract neutrophils, whereas most C-C attract monocytes, T lymphocytes and in less degree eosinophils and basophils. MCP-1 belongs to the C-C group together with regulated on activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory proteins-1α/1β(MIP-1α/1β), MCP-2 and MCP-3. Members of C-X-C group are interleukin (IL)-1, IL-8. Lymphotactic is a member of C chemokines.

The biological effects of MCP-1 and all members of the subfamilies are mediated by the interaction with members of the superfamily of seven transmembrane domain G protein-coupled receptors.

All members of the chemokine subfamily share the ability to induce directional migration, growth and possible activation of specific leukocytes (Baggiolino, Dewald et al. 1994). *In vitro,* MCP-1 is chemoattractant and activating for monocytes, T lymphocytes (Jiang, Beller et al. 1992; Lukacs, Chensue et al. 1997; Carr, Roth et al. 1994), and natural killer cells (Taub, Sayers et al. 1995). The *in vivo* data are obtained using local injection of MCP-1 in different animal models or by transgenic mice generation overexpressing MCP-1 in a variety of organs. Experiments using intradermal injection of human MCP-1 showed monocyte infiltration at the site of injection in rats (Zachariae, Anderson et al. 1990), and rabbit (Van Damme, Proost et al. 1992). In contrast, intradermal injection of pure murine MCP-1 did not induce infiltrates in mouse skin (Ernst, Zhang et al. 1994).

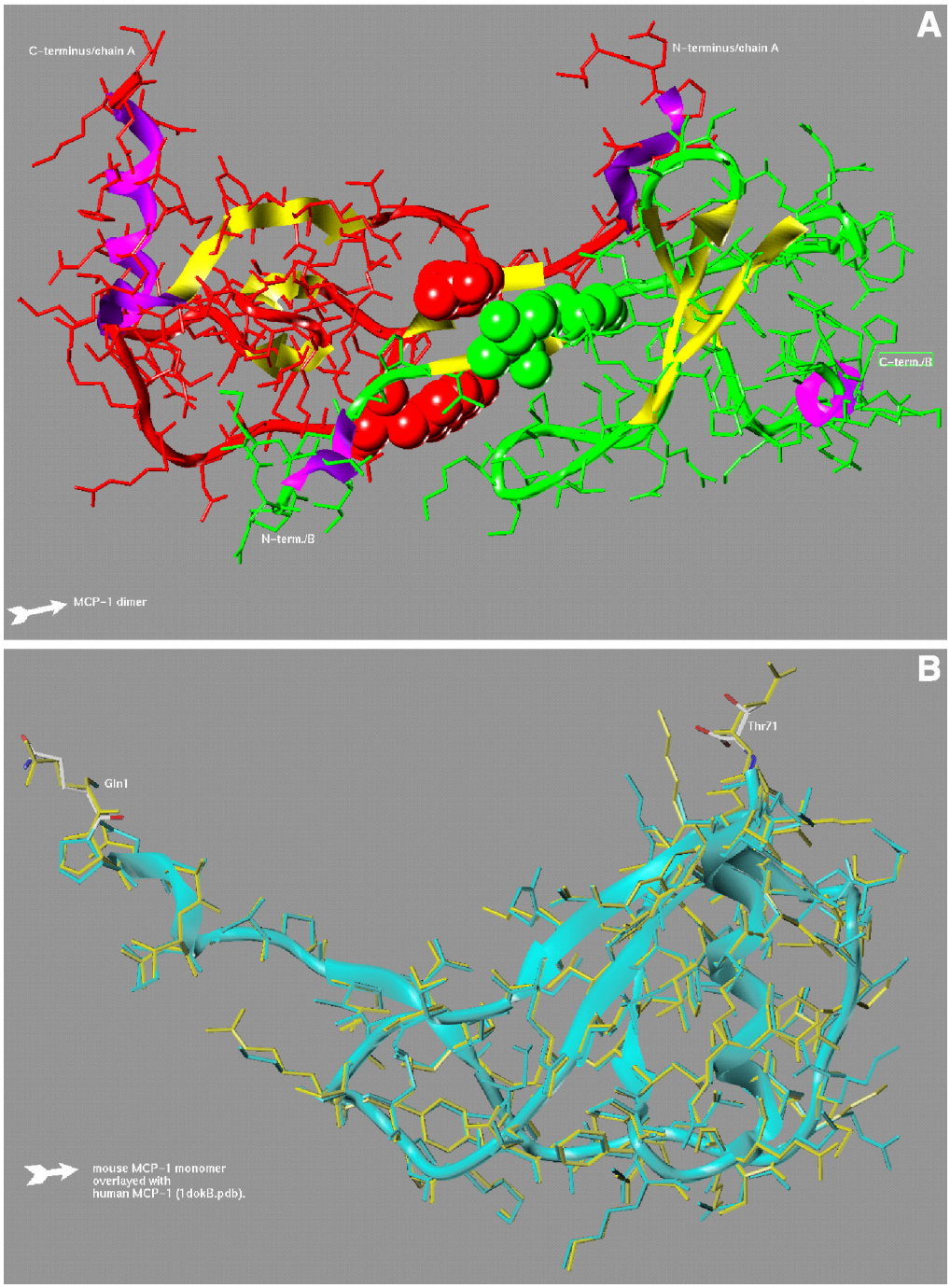

Fig. 1. *(A) The dimeric crystalline form of human MCP-1 as derived from X-ray-spectroscopy (1 dok.pdb, 1.85 A resolution). (B) The possible structure of mouse MCP-1 (yellow) as obteined by homology modeling using Composer / Biopolymer (Tripos force field, Kollman charges, ε= 4) with 1 dok.B.pdb as template (cyan). The quaternary structure of MCP-1 is established for human MCP-1. Murine MCP-1 differs from human mainly by its molecular weight with additional 49 amino acids at the C-terminus with O-linked oligosaccharide chains terminated by sialic acid. The molecular weight of the monomer (mouse) is approximately 25 kDa. If one considers residues 1 to 71 the sequence identity between human and mouse MCP-1 is 59% leading to the homologous structure of mouse MCP-1.*

Rutledge *et al.* using transgenic mice that constitutively express MCP-1 in a variety of organs could not show any monocyte infiltrates in MCP-1-expressing organs in any animals and at any age (Rutledge, Rayburn et al. 1995). Transgenic mice, expressing human MCP-1 in type II alveolar epithelial cells showed accumulation of monocytes and lymphocytes into the bronchoalveolar space (Gunn, Nelken et al. 1997). In this animal model, MCP-1 alone did not induce inflammatory activation of cells but it led to an enhanced inflammatory response by treatment with other *stimuli* (Gunn, Nelken et al. 1997). Transgenic mice overexpressing MCP-1 in the thymus and brain showed modest infiltrates of monocytes but not lymphocytes and pronunced mononuclear infiltrates, respectively (Fuentes, Durham et al. 1995). Transgenic mice, overexpressing MCP-1 specifically in the heart, exhibit in young animals (from neonatal to 2 months old) focal accumulation of macrophages but not lymphocytes in the myocardial interstitium (Kolattukudy, Quach et al. 1998). In addition, no sign of leukocyte activation was detected at this early age (Kolattukudy, Quach et al. 1998), but MCP-1 overexpression leads in old animals to ischemic cardiomyopathy and a gradual loss of myocytes (Kolattukudy, Quach et al. 1998; Moldovan, Goldschmidt-Clermont et al. 2001). MCP-1-deficient mice were also generated by targeted gene disruption (Lu, Rutledge et al. 1998). The animals develop normally, show normal hamatologic profiles, and have normal number of resident macrophages but they develop serious abnormalities in several inflammatory and immunological models. Upregulation of MCP-1 mRNA has been shown to be responsible for leukocyte recruitment following ischemia/reperfusion of the liver in rats (Yamaguchi, Matsumura et al. 1998).

At the mRNA and protein level, MCP-1 is expressed by a variety of cells such as monocytes, macrophages, fibroblasts, chondrocytes, keratinocytes, melanocytes, mesangial cells, osteoblasts, astrocytes, lipocytes, mesothelial cells, epithelial cells, endothelial cells, smooth muscle cells, and some tumor cell lines (for review see Proost, Wuyts et al. 1996).

MCP-1 can be produced constitutively in many different tumor cells (Graves, Jiang et al. 1989) or in normal cells after stimulation with cytokines such as IL-6, tumor necrosis factor–α (TNF-α), interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), or mitogens, viruses, and endotoxins. In addition, lipopolysaccharide (LPS), low-density lipoprotein, thrombin and shear stress can induce MCP-1 expression by endothelial cells (Proost, Wuyts et al. 1996). Unstimulated and non-malignant cells express low levels of MCP-1.

MCP-1 is upregulated at mRNA and protein levels in several diseases, in which an inflammatory response occurs, e.g., cancers, rheumatoid arthritis, atherosclerosis, inflammatory skin diseases (psoriasis, lichenoid dermatitis, spongiotic dermatitis), bronchial infections (idiopathic pulmonary fibrosis, spongiotic dermatitis), liver diseases (chronic acute hepatitis, fulminant hepatic failure), ischemia and ischemia/reperfusion injuries (Koch, Kunkel et al. 1994; Strieter, Koch et al. 1994; Yla-Herttuala, Lipton et al. 1991; Birdsall, Green et al. 1997; Kumar, Ballantyne et al. 1997; Kakio, Matsumori et al. 2000). In ischemia and ischemia/reperfusion injuries recruitment of leukocytes is a crucial step in the physiological response of the infarcted tissue. During reperfusion, leukocytes infiltrate and accumulate in the infarcted area and upon activation they initiate the inflammatory process. The main role of MCP-1 expression during myocardial ischemia has been attributed to its chemoattractant capacity for monocytes, leading to the initiation of the healing process after ischemic myocardial injury. It has been shown that MCP-1 mRNA is induced in the endothelium of small veins in the ischemic area within the first hour of reperfusion and peaked at 3 hours (Kumar, Ballantyne et al. 1997; Kakio, Matsumori et al. 2000), while MCP-1 expression starts after 3 hours coinciding with the infiltration of leukocytes (Birdsall, Green et al. 1997). Neutralization of MCP-1 significantly reduces myocardial reperfusion injury in a rat model of ischemia/reperfusion (Ono, Matsumori et al. 1999). MCP-1 expression has also been shown in focal cerebral ischemia after middle cerebral artery occlusion in mouse (Che, Ye et al. 2001) and rat (Yamagami, Tamura et al. 1999).

1.2. Mitogen-activated protein kinases

The mitogen-activated protein kinases (MAPKs) are a superfamily of Pro-directed Ser/Thr cytoplasmic protein kinases involved in the signal transduction pathway from extracellular *stimuli* to the nucleus (Lufen and Michael 2001) (Fig. 2). MAPKs respond to chemical and physical stresses through plasma membrane or cytoplasmic receptors (such as G-protein-receptors, tyrosin kinase-receptors) thereby controlling cell death, cell survival and adaptation. The activation of these receptors from extracellular *stimuli* induces the activation of a signaling cascade with final translocation of a terminal kinase to the nucleus, where target proteins are modified by phosphorylation to regulate gene expressions and other cell functions. Each kinase is activated through multistep protein kinase cascades by dual phosphorylation on a tyrosine and a threonin residue (Fig. 2). The cascades are composed by a MAPK, MAPK kinase (MAPKK, MKK, or MEK)

and a MAPKK kinase or MEK kinase (MAPKKK or MEKK) (English, Pearson et al. 1999).

MAPKKKs, MEKKs	MAPKK kinase
MAPKKs, MKKs, MEKs	MAPK kinase
MAPKs	MAP kinase

Each MAPKs is activated by specific MAPKKs. However, they can be activated by more than one MAPKKs, increasing the complexity and diversity of MAPKs signalling. In general, there is a considerable specificity in MAPKs activation (Fig. 2).

Downstream effectors of the MAPKs pathway include the so-called transcription factors. Phosphorylation of transcription factors is the trigger for transactivation. The transactivation is the process by which genes are activated by means of a *trans*-activating domain that is contained in a transcription factor. This domain enables transcription factors to interact with proteins that are involved in binding RNA polymerase to DNA in a sequence-specific manner that are favorable to the initiation of transcription. The genes that are induced by transcription factors are called immediate-early gene for their capacity to be induced rapidly and transiently without a need for new protein synthesis. Many immediate-early genes control the transcription of other genes.

Transcription factors such as c-Jun, related-to-serum-response factor (RSRF), myocyte enhancer factor 2 (MEF2), and activating transcrption factor-1/2 (ATF-1/2), control *jun* family (*c-jun*, *junB*, and *junD*) gene expression. Transcription factors such as activator protein-1 (AP-1), ternary complex element (TCF), Elk-1, serum related factor (SRF), nuclear factor-kB (NF-kB), Janus kinase/signal transducer and activator of transcription (JAK/STAT) system, and cAMP-response-element-binding protein (CREB) induce *fos* family (*c-fos*, *fosB*), *Egr-1*, *gadd45 β,* and *xiap* gene expression (Kyriakis and Avruch 1996; Hazzalin and Mahadevan 2002; Force, Pombo et al. 1996 ; Kyriakis and Avruch 2001).

The MAPKs include different protein kinase subfamilies: the extracellular signal-regulated protein kinases1/2 (ERK1/2 or p44 and p42 MAP kinase), 10 or more splice variants of the stress-activated protein kinases/c-Jun-NH2-terminal protein kinases (SAPK/JNKs), 4 isoform of p38-MAPkinases (p38-MAPKs) designated α, β, γ and δ, 3 forms of ERK3, ERK4, ERK5, and ERK7.

MAPKs are activated by different *stimuli* (Fig. 2). The ERKs are mostly activated by growth factors such as fibroblast growth factor (FGF), insulin growth factor (IGF), epidermal growth factor (EGF), angiotensin-II (Ang-II), and endothelin-1 (ET-1) (Fisher, Singh et al. 1998; Htun, Barancik et al. 1998). SAPK/JNKs and p38-MAPKs are activated by cellular stress such as heat shock, ultraviolet radiation, protein synthesis inhibitors, osmolarity changes, okadaic acid, antibiotics, ischemia, ischemia/reperfusion, pro-inflammatory cytokines such as TNF-α and IL-1 and to a lesser degree by growth factors (Moriguchi, Kawasaki et al. 1995; Clerk, Harrison et al. 1999; Winston, Chan et al. 1997).

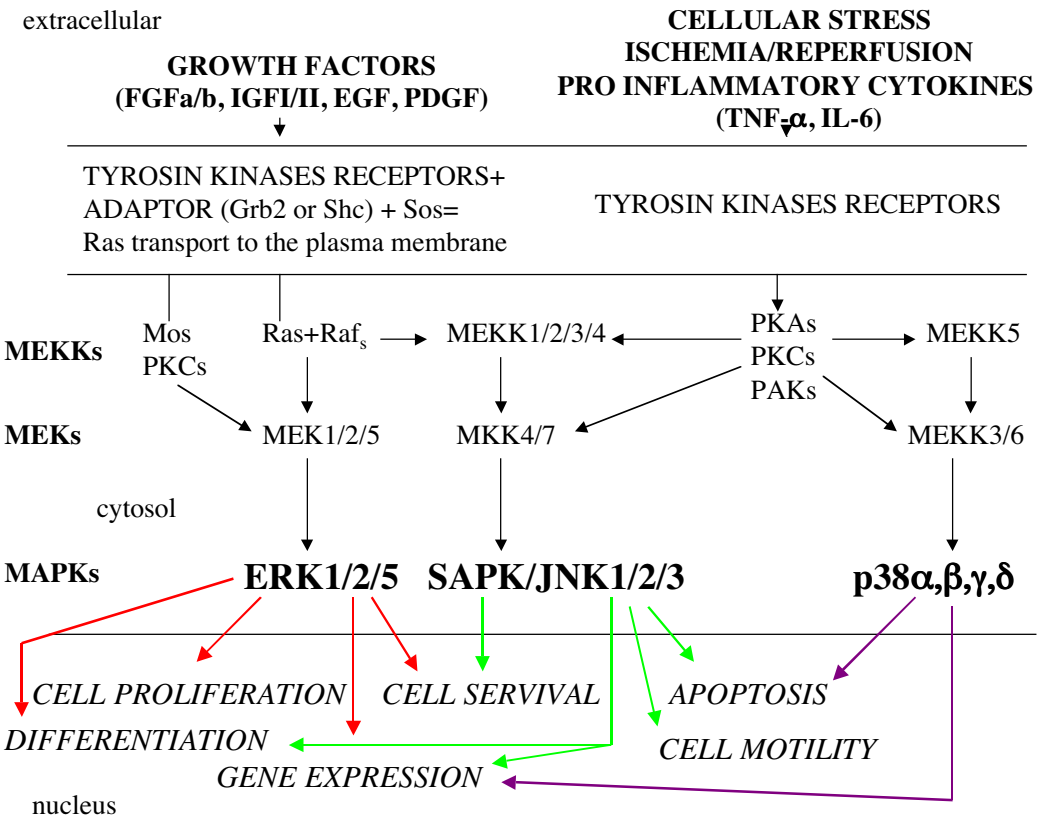

Fig. 2. *MAPKs SIGNAL TRANSDUCTION PATHWAY*

The activation of ERK1/2/5 pathways is associated with the onset of survival, cell proliferation, transformation, cell cycle control, DNA synthesis, differentiation, long-term potentiation in neurons, the production of insulin in pancreatic β cells (Fig. 2). ERK1/2 may also induce growth factors production (Lewis, Shapiro et al. 1998; Cobb 1999; Kato, Tapping et al. 1998; Chang and Karin 2001). ERK7 function has been associated with a negative regulation of growth (Abe, Kuo et al. 1999). The activation of ERK1/2 pathway leads to phosphorylation (activation) of transcription factors such as Elk-1, serum response factor (S) accessory protein

(SAP-1), MAPK interacting protein kinase-1/2 (Mnk-1/2), mitogen-and stress-activated protein kinase (MSK1), and MAPK-activated protein kinase 1 (MAPKAP-K1), NF-kB, JAK/STAT system (Treisman 1996; Hazzalin and Mahadevan 2002; Chakraborti and Chakraborti 1998; Chang and Karin 2001; review in Brivanlou and Darnell 2002). ERK5 ‘s substrates is MEF 2 (Hazzalin and Mahadevan 2002).

SAPK/JNKs and p38-MAPKs activation has been demonstrated to be involved in apoptosis, cell transformation, proliferation, differentiation, cytokine biosynthesis, and stress responses (Kyriakis 2001; Dong, Yang et al. 1998; Craxton, Shu et al. 1998) (Fig. 2). Recently, activation of SAPK/JNKs pathway has been associated with the induction of cell survival in different cell types and animal models (Yue, Ma et al. 1998; Barancik, Htun et al. 1999; Andreka, Zang et al. 2001). The activation of SAPK/JNKs pathway induce the phosphorylation (activation) of transcription factors such as c-Jun, ATF-2, Elk-1, NF-kB, JAK/STAT system (Treisman 1996; Chakraborti and Chakraborti 1998; Chang and Karin 2001; Brivanlou and Darnell 2002; review in Hazzalin and Mahadevan 2002). p38 activates the transcription factors ATF-2, Elk-1, SRF accessory protein (SAP-1), C/EBP homologous protein (CHOP), MEF-2, Mnk 1/2, MSK1, and p38-related/activated protein kinase (PRAK) (Treisman 1996; Chakraborti and Chakraborti 1998; Chang and Karin 2001; Brivanlou and Darnell 2002; review in Hazzalin and Mahadevan 2002). In general, with the exception of transcription factors, the MAPKs substrates that regulate processes initiated by extracellular *stimuli* are not known and their identification is under study.

1.3. Ischemic preconditioning

Ischemic preconditioning is the endogenous mechanism of the myocardium to protect itself against infarction. Ischemic preconditioning consists of short transient periods (~5) of sublethal ischemia (5 min) and reperfusion (5 min) that confer myocardial adaptation and resistance against cardiomyocyte death after a subsequent prolonged coronary occlusion (Murry, Jennings et al. 1986; Cohen and Downey 1995; Yellon, Baxter 1998). This phenomenon of marked limitation of infarction was described for the first time in 1986 (Murry, Jennings et al. 1986) and has been already demonstrated in every animal species studied (Yellon, Baxter et al. 1998). The cardioprotection is only induced when the duration of prolonged ischemia insult is 30 to 90 min, but is ineffective when this period is more than 3 hours and the protection is only observed when the prolongated ischemia is followed by reperfusion (Murry, Jennings et al. 1986; Yellon, Baxter

et al. 1998). This immediate adaptation represents an early phase of protection and is defined “classical preconditioning” (or “early preconditioning”). Secondary, a late (or delayed) phase of ischemic preconditioning has been described and called “delayed preconditioning” (or “second window of protection”). The “delayed preconditioning” begins 12 to 24 hours after the *stimulus* and lasts 3 to 4 days (Kuzuya, Hoshida et al. 1993; Marber, Latchman et al. 1993). It has been shown to protect the myocardium not only from lethal ischemia/reperfusion injury (as early preconditioning), but at least also from postischemic disfunction (Bolli 1996; Sun, Tang et al. 1995), and arrhythmias (Kaszala, Vegh et al. 1996).

The mechanism of protection achieved by classical preconditioning is composed of a cellular cascade and starts with adenosine and reactive oxygen species released by ischemic myocardium, which act as triggers. Reactive oxygen species are highly unstable molecules, capable of attacking cell membranes and subcellular structures to induce metabolic and structural changes, that can be detrimental to cell survival (Kukreja and Hess 1992). They include superoxide anion, hydrogen peroxide, and hydroxyl radical, which are abundantly produced during reperfusion (Kukreja and Hess 1992). The adenosine and reactive oxygen species mediate the activation (phosphorylation) of protein kinase C (PKC) and ATP-dependent K+ (KATP) channels. PKC has been described as integral and essential intermediate intracellular messenger in the cardioprotection program (Ytrehus, Liu et al. 1994; Speechly-Dick, Grover et al. 1995). In fact, the inhibition of PKC activation has been shown to abolish ischemic preconditioning in both, rabbit hearts and isolated cardiomyocytes (Ytrehus, Liu et al. 1994; Armstrong, Downey et al. 1994). Slight oxidative modification of the regulatory domain of PKC predisposes the protein to being easily activated (Gopalakrishna and Anderson 1989). In addition, when PKC is exposed to oxidative stress, it is translocated from the cytoplasm to the cell membrane, where the protein can be easily activated (Von Ruecker, Han-Jeon et al. 1989). PKC down stream signaling cascades include MAPKs and transcription factor activation (see below). KATP channels have been also described as end-effector of protection in the preconditioning program (Gross and Auchampach 1992; Yao, Mizumura et al. 1997). KATP channel blockers reverse the beneficial effects of ischemic preconditioning (Toombs, Moore et al. 1993; Gross and Auchampach 1992). The link between adenosine and reactive oxygen species and KATP channel activation has also been already established. In fact, KATP channels are opened by various species of oxigen radicals (Jabr and Cole 1993; Tokube, Kiyosue et al. 1994). Liang (Liang 1996) demonstrated a direct preconditioning of ventricular myocytes involving adenosine-receptors (A1) and KATP channels. Although many

studies have hypothesized that KATP channels are potential end-effector of protection, new evidences are emerging regarding the role of the opening of these channels in the triggering of ischemic preconditioning rather than in the end-effecting (Pain, Yang et al. 2000; Downey and Cohen 2000). In fact, it has been proposed that the opening of the KATP channels during ischemic preconditioning generates free radicals that lead to a preconditioned state and activation of MAPKs (Pain, Yang et al. 2000).

As mentioned above, delayed preconditioning becomes apparent 12 to 24 hours after the preconditioning *stimulus* and lasts 3 to 4 days (Kuzuya, Hoshida et al. 1993; Marber, Latchman et al. 1993). Although early ischemic preconditioning requires only activation of preexisting proteins, late ischemic preconditioning needs increased synthesis of new cardioprotective proteins (Rizvi, Tang et al. 1999). A wide variety of *stimuli* can activate the late ischemic preconditioning, and they can be classified as nonpharmacological and pharmacological. Initiators (or triggers) of the late ischemic preconditioning discovered until now are adenosine (Dana, Baxter et al. 1998), nitric oxide (Qiu, Rizvi et al. 1997), reactive oxygen species (Sun, Tang et al. 1996), and may be opioid receptor agonists (Fryer, Hsu et al. 1999). Mediators (or effectors) of the protection (cardioprotection proteins) include nitric oxide synthase (in the late ischemic preconditioning nitric oxide plays a role as a trigger as well as effector) (Bolli, Dawn et al. 1998), cycloxygenasa-2 (Shinmura, Tang et al. 2000), aldose reductase (Srivastana, Chandra et al. 1998), antioxidant enzymes (Hoshida, Kuzuya et al. 1993), heat shock proteins (Radford, Fina et al. 1996), and perhaps KATP channels (Takano, Tang et al. 2000). The cellular pathway of late ischemic preconditioning includes activation of signaling cascade through PKC (Ping, Takano et al. 1999), and Src protein tyrosin kinases (Imagawa, Baxter et al. 1997) leading to transcription factors activation, as NF-kB, (Xuan, Tang et al. 1999) that control the expression of the cardioprotective genes responsible for late ischemic preconditioning (Fig. 2).

Currently, the search for the identification of the underlying intracellular signaling pathways and endogenous or pharmacological substances able to protect the myocardium against infarction, is one of the most important challenges in cardiovascular research.

1.4. Ischemic preconditioning and MAPKs

It is already known that the ERKs pathway is activated during ischemia and reperfusion (Htun, Barancik et al. 1998; Ping, Zhang et al. 1999), and that

activation leads to protect the myocytes against cell death (Henaff, Hatem et al. 2000; Yue, Wang et al. 2000; Strohm, Barancik et al. 2000).

Ischemia and reperfusion activate the p38-MAPKs pathway, and this activation has been associated with the induction of apoptosis or cell injuries (Bogoyevitch, Gillespie-Brown et al. 1996; Gu, Jiang et al. 2000; Ozawa, Shioda et al. 1999).

The SAPK/JNKs pathway is activated during ischemia and is maintained during reperfusion (Bogoyevitch, Gillespie-Brown et al. 1996; Yue, Wang et al. 2000; Gu, Jiang et al. 2000). However, the activation of SAPK/JNKs pathway during ischemia and reperfusion has been associated with the induction of both, apoptosis or cytoprotection in different cell types (Ozawa, Shioda et al. 1999; Yue, Ma et al. 1998; He, Li et al. 1999; Yue, Wang et al. 2000; Mielke, Damm et al. 2000).

Recently, several groups have shown that different members of the MAPKs superfamily are activated during ischemic preconditioning suggesting their role in the protection achieved by ischemic preconditioning (Weinbrenner, Liu et al. 1997; Barancik, Htun et al. 1999; Nakano, Baines et al. 2000; Sato, Cordis et al. 2000). Several studies have shown activation of the MAPKs pathways after a preconditioning procedure and infarct size reduction by ischemic preconditioning (Maulik, Watanabe et al. 1996; Maulik, Yoshida et al. 1998; Weinbrenner, Liu et al. 1997). However, the precise role of each member of the MAPKs superfamily in the induction of cardioprotection during ischemic preconditioning is still controversial. After ischemic preconditioning, the activity of ERKs (Ping, Zhang et al. 1999b), SAPK/JNKs (Ping, Zhang et al. 1999a), and p38-MAPKs (Nakano, Baines et al. 2000) increase in rabbit and rat (Sato, Cordis et al. 2000). In general, activation of ERKs pathway during ischemic preconditioning is associated with the cytoprotection against necrosis or apoptosis (Henaff, Hatem et al. 2000; Yue, Wang et al. 2000; Strohm, Barancik et al. 2000). In fact, specific inhibition of ERKs pathway by PD98059 and UO126 abolishes ischemic preconditioning-induced cardiac protection in pig myocardium (Strohm, Barancik et al. 2000). The activation of the p38 pathway has been associated with the induction of apoptosis or cell injury (Bogoyevitch, Gillespie-Brown et al. 1996; Ozawa, Shioda et al. 1999). The activation of the SAPK/JNKs pathway was originally associated with the induction of cell death (He, Li et al. 1999; Lin, Weinberg et al. 2000; Yue, Ma et al. 1998) but recently, a cardioprotective role of SAPK/JNKs in different organs and animal model has been reported (Sato, Cordis et al. 2000; Barancik, Htun et al. 1999; Baines, Cohen et al. 1999; He, Li

et al. 1999; Lin, Weinberg et al. 2000; Yue, Ma et al. 1998; Andreka, Zang et al. 2001).

1.5. TNF-α

Cytokines have a broad and overlapping range of cell regulatory activity. Cytokines binding with cellular membrane receptors activates different intracellular cascade of transduction signals, thereby altering the cell's pattern of gene expression and leading to different biological effects such as cell growth, proliferation, differentiation, migration, and cell survival or death. Different members of the cytokine family are also important mediators of the immune system and have critical functions in regulating immune responses.

TNF-α is a multifunctional cytokine (trimeric polypeptide) of 157 aminoacids. It was discovered as an anti-tumor agent, when cancer patients who have a severe infection sometimes had a regression of their tumor (Nauts 1989). It is produced as a 26 kD integral transmembrane precursor protein from which a 17 kD mature TNF-α protein is released after proteolytic cleavage (Kriegler, Perez et al. 1988) catalyzed by a metalloproteinase called TNF-converting enzyme (Moss, Jin et al. 1997). The active form exists in soluble or membrane-bound form (Decoster, Vanhaesebroeck et al. 1995). Membrane insertion may be an effective way to keep the action of TNF-α restricted to specific locations.

TNF-α is mainly produced by activated macrophages (Chensue, Remick et al. 1988; Takeichi, Saito et al. 1994), but many different cell types can produce it under appropriate stimulation. These cell types include lymphocytes, fibroblasts, neutrophils, smooth muscle cells, mast cells, adipocytes and adult cardiomyocytes (Steffen, Ottmann et al. 1988; Hart, Vitti et al. 1989; Sidhu and Bollon 1993; Larrick and Kunkel 1988; Kapadia, Lee et al. 1995). TNFs secretion appears after 2 hours of cultured macrophage stimulation, peaks at 4-8 hours and disappears within 12 hours. This cytokine is synthesized on demand, and it is not stored in the cytoplasm. Production of TNF-α is tightly regulated, both at the transcriptional and at the post-transcriptional level. LPS, viral, fungal, and parasital agents, enterotoxin, immune complexes, IL-1, IL-2 can induce the synthesis of TNF-α. Glucocorticoids and prostaglandin E_2 inhibit TNF-α synthesis at the transcriptional and post-transcriptional level (Beutler, Han et al. 1992). Physiological antagonists such as transforming growth factor–β (TGF–β), IL-4 and IL-10 regulate TNF-α synthesis (Flynn and Palladino 1992; Hart, Vitti et al. 1989; Fiorentino, Zlotnik et al. 1991).

Two TNF-receptors have been described, present on almost all nucleated cell types: TNFR1 (or TNFRI) and TNFR2 (or TNFRII), with a molecular mass of 55 kD and 75 kD, respectively (Vandenabeele, Declercq et al. 1995). Both the receptors belong to the TNFR superfamily, also including Fas, CD40, CD27, and RANK (Idriss and Naismith 2000). The receptors have a cytoplasmic domain and a transmembrane helix, which recognizes TNF-α through a N-terminal extracellular ligand-binding domain (Idriss and Naismith 2000). Recently, two soluble circulating TNFRs, 55 and 75 kD respectively, have been described (Ferrari, Bianchetti et al. 1995). These are membrane-bound TNFR that have been proteolytically cleaved from the cell membrane, and they can bind and inactivate TNF-α (Ferrari, Bianchetti et al. 1995).

TNF-α has different biological activities mediating various physiologic and pathologic processes, such as inflammation, cancer, cachexia, septic shock, cell proliferation and differentiation (Fiers, Beyaert et al. 1996; Wallach, Boldin et al. 1997; Vilcek and Lee 1991). It has also been implicated in the pathogenesis of different human cardiac diseases, such as myocarditis, dilated cardiomyopathy, heart failure, myocardial infarction, cardiopulmonary bypass, allograft rejection, ventricular remodeling, apoptosis and heart failure (Levine, Kalman et al. 1990; Krown, Page et al. 1996; Satoh, Nakamura et al. 1997; Kurrelmeyer, Michael et al. 2000; Sivasubramanian, Coker et al. 2001).

TNF-α transgenic and deficient mice from different strains were generated in the last years. Transgenic mice generated from C57BL/6J mice overexpressing TNF-α specifically in the heart have been shown to develop left ventricular dilatation and remodeling during the first three months of age (Sivasubramanian, Coker et al. 2001). Other transgenic mice generated from FVB mice showed dilated cardiomyopathy leading to congestive heart failure (Kubota, McTiernan et al. 1997). C57BL/6J mice lacking TNF-α gene have been reported to reduce infarct size, chemokine and adhesion molecules expression and leukocyte infiltration (Maekawa, Wada et al. 2002). In marked contrast, recent studies indicate that TNF-α production can also have a beneficial role protecting the myocardium against viral myocarditis and ischemic injury (Wada, Saito et al. 2001; Kurrelmeyer, Michael et al. 2000; Sack, Smith et al. 2000). Wada et al. (Wada, Saito et al. 2001) using C57BL/6J mice generated TNF-α deficient mice. They showed that after induction of acute myocarditis by infection with encephalomyocarditis virus the lack of this cytokine resulted in failure of elimination of infectious agents. In addition, the use of deficient mice lacking both TNFR1 and TNFR2 receptors showed that endogenous TNF-α protects

cardiac myocytes against apoptosis after acute ischemic injury (Kurrelmeyer, Michael et al. 2000).

1.6. TNF-α and MAPKs

The SAPK/JNKs pathway can be activated by TNF-α (Liu, Hsu et al. 1996; Ip and Davis 1998; Kyriakis and Avruch 1996; Modur, Zimmermann et al. 1996) (Fig. 3).

TNF-α binds to two receptors: TNFR1 and TNFR2. The stimulation of these receptors activates different signaling transduction pathways that result either in apoptosis or cell survival.

Ligand-induced trimerization of the TNFR1 results in the recruitment of the cytosolic TNFR-associated death domain (TRADD) proteins (Fig. 3). After its binding with TNFR, TRADD induces the binding of two additional and necessary proteins that act as signal transducers: TNFR-receptor-associated factor-2 (TRAF2) and receptor interacting protein (RIP), which contain a death domain that participates in both activation of NF-kB (through SAPK/JNKs activation) and promotion of apoptosis (through p38 activation) (Hsu, Xiong et al. 1995; Hsu, Shu et al. 1996; Natoli, Costanzo et al. 1997) (Fig. 3). This signaling cascade induces activation of SAPK/JNKs, NF-kB, p38-MAPK, and AP-1 complex through MEKK phosphorylation, which in turn regulate genes involved in inflammatory response, cell survival, and adaptation (Roulston, Reinhard et al. 1998; Wulczyn, Krappman et al. 1996; Liu, Hsu et al. 1996; Natoli, Costanzo et al. 1997). Activation of TNFR1 by way of TRADD has been also shown to stimulate apoptosis by activating the caspase 8 cascade through a member of FADD/MORT-proteins (which also participates in the transduction of Fas-induced apoptotic signals), which selectively induce apoptosis through SAPK/JNKs activation (Hsu, Xiong et al. 1995; Hsu, Shu et al. 1996; Liu, Hsu et al. 1996; Darnay and Aggarwal 1997; Natoli, Costanzo et al. 1997) (Fig. 3)

TNFR2 has been only associated with the induction of survival activating SAPK/JNKs, NF-kB, through TRAF2, that is directly bound to TNFR2 (Liu, Hsu et al. 1996; Darnay and Aggarwal 1997).

1.7. Innate immunity

The immune system is composed by two components: the innate and the adaptative immune system (Medzhitov and Janeway 1998). The innate immune

system is a preprogrammed nonspecific host defense mechanism against pathogens. It is based on non-clonal (or germ-line encoded) receptors that recognized non-self molecular patterns. It is characterized by activation of myeloid-derived cells (such as natural killer cells, neutrophils, and monocytes/ macrophages), which secrete pro-inflammatory cytokines and chemokines controlling recruitment and activation of leukocytes at the sites of infection (Medzhitov and Janeway 1998). The adaptative immune system uses somatically generated antigen receptors, which are cloned and distributed on T cells and B cells (Medzhitov and Janeway 1998). It has been proposed that the innate immune system not only has a role in the early phase of defense but also in the activation of the subsequent, clonal response of adaptative immunity controlling the expression of costimulatory molecules (cytokines and chemokines) (Hoffmann, Kafatos et al. 1999).

The signal transduction for the activation of the innate immune cytokine cascade stars with extracellular pattern-recognition molecules (e.g. LPS) that activate membrane receptors called Toll-like receptors (Hoffmann, Kafatos et al. 1999). The Toll-like receptors interacting with adapter molecules and kinases through their “death domains” direct intracellular signaling events that lead to TRAF6 activation, MAPKK kinases phosphorylation with final NF-kB translocation into the nucleus. The activation of NF-kB lead to the expression of kB binding site genes encoding IL-1β, IL-6, IL-8 necessary for initiating the adaptative immune response (Hoffmann, Kafatos et al. 1999).

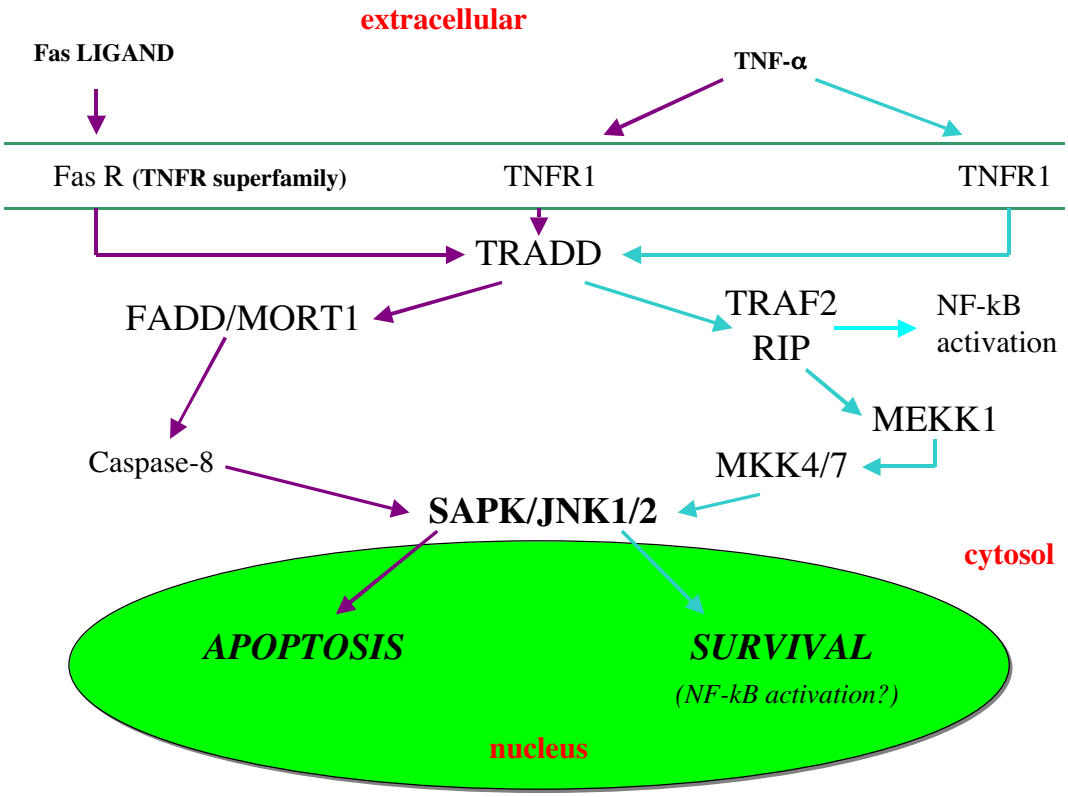

Fig. 3. *TNF-α SIGNAL TRANSDUCTION PATHWAY THROUGH TNFR1Y*

1.8. Innate immunity and ischemic preconditioning

Very recently, the innate immune system has been proposed as an integral part of ischemic preconditioning (Smith, Lecour et al. 2002). Smith et al. (Smith, Lecour et al. 2002) have postulated that ischemic preconditioning could activate an innate (intrinsic) cytoprotection program leading to promote cardiomyocyte survival through Toll-like receptor activation by ischemia and/or reperfusion. Intracellular generation of reactive oxygen species by ischemia and/or reperfusion could facilitate NF-kB translocation to the nucleus with subsequent transactivation of cytokines (Smith, Suleman et al. 2002). TNF-α, a component of the innate immune system plays a crucial role in ischemia/reperfusion injury. The role of TNF-αin ischemia/reperfusion has been associated with the induction of NF-kB activation, thereby inducing chemokines and adhesion molecules production and promoting leukocyte infiltration (Frangogiannis, Lindsey et al. 1998; Maekawa, Wada et al. 2002). Smith et al. proposed that the recruitment and activation of inflammatory cells and the production of cytokines play a role not only in the healing and remodeling process after myocardial infarction but also in the cardioprotective effect promoted and initiated by

ischemic preconditioning. This group and others have proposed an association between TNF-α production and ischemic preconditioning in different animal models (Smith, Lecour et al. 2002; Smith, Suleman et al. 2002). The possible cardioprotective pathway activated by TNF-α during ischemia or ischemia/reperfusion is poorly understood but it has been proposed to be mediated by MAPKs activation (Baines, Cohen et al. 1999; Bogoyevitch, Gillespie-Brown et al. 1996; Martire, Fernandez et al. 2001).

1.9. Ubiquitin

Ubiquitin is a highly conserved small globular protein of 76 amino acids and it belongs to the heat-shock proteins (Mayer, Arnold et al. 1991; Lowe and Mayer 1990). It is present in all eukaryotic cells and occupies a pivotal role in the regulation of protein turnover and degradation in both, normal metabolic processes and in diseases where degenerative processes occur (for review see Weissman 2001). Its covalent conjugation to other proteins is essential for proteasomal degradation but ubiquitin itself does not degrade proteins. It serves only as a tag marking proteins for degradation. Ubiquitin conjugation (ubiquitylation or ubiquitination) to proteins occurs in a multistep process, involving at least three types of enzymes (Weissman 2001). First, an ubiquitin-activating enzyme called E1 binds and actives ubiquitin in an ATP-dependent process forming a thiol-ester bond with the carboxy-terminal glycine of ubiquitin. Second, an ubiquitin-conjugating enzyme, E2, accepts ubiquitin from E1 by a trans-thiolation reaction, involving the carboxyl terminus of ubiquitin. Finally, a ubiquitin protein ligase, E3, catalyses the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue of the substrate leading to its ubiquitylation (Weissman 2001). Only a chain of four or more ubiquitin (multiubiquitylation) targets a protein for proteasomal degradation being adequate as a proteasome-targeting signal, whereas a single ubiquitin (monoubiquitylation) tag does not. Ubiquitylated proteins are recognized by 26S proteasomes. The 26S proteasomes are responsible for ATP-dependent degradation of the target proteins and release of ubiquitin. They are formed by two different components, one is the proteolytic unit (20S), that catalyses the protein degradations, and the other is a multisubunit regulatory complex (19S), that has a role in unfolding the proteins, guiding them into the degradative tunnel formed by 20S subunits (Weissman 2001). The result is a collection of small peptides from which ubiquitin is released and recycled (Field and Clark 1997).

Ubiquitin is involved in many cell processes, such as cell-cycle progression, signal transduction, receptor-mediated endocytosis, transcription, cell proliferation and differentiation, quality control in the endoplasmic reticulum, organelle biogenesis, spermatogenesis, protein transport, antigen processing and embryonic development (Weissman 2001; Mayer, Lowe et al. 1989; Thompson 1995; Hu, Martone et al. 2000; Field and Clark 1997). In addition, ubiquitin is an important protein induced by cell stress (Weissman 2001; Mayer, Arnold et al. 1991; Thompson 1995; Hu, Martone et al. 2000; Field and Clark 1997). In fact, it has been shown that an upregulation of the ubiquitin gene occurs in inflammation, DNA repair, apoptosis, ischemic neuronal death, and heart failure (Weissman 2001; Mayer, Arnold et al. 1991; Thompson 1995; Field and Clark 1997).

1.10. Complement 9

Complement (a member of the immune system) is a cytotoxic host defense system that was identified initially because of its cytolytic effects. It is composed by ≈20 intravascular plasma proteins, which are subdivided into two cascade systems, the classic and alternative complement pathways. The classic pathway is achieved with the involvement of immunoglobulins in the activation of the complement, while the alternative does not (Muller-Eberhard 1988). The activation of the complement system by the classical or the alternative pathway produces complement fragments that have important roles in the inflammatory response. Activation of the complement system produces direct tissue injury through the formation of the membrane attack complement (MAC) by complement 5b-9, followed by its insertion into host cell membranes (Morgan 1989). Rapid cell death can occur through disruption of cellular integrity (Morgan 1989).

Complement has a role in some physiological processes, such as the induction of the humoral immune response, in the elimination of immune complexes and in the protection against bacterial and viral infections. Complement activation and deposition occurs in dermatomyositis (Kissel, Halterman et al. 1991), myasthenia gravis (Tsujihata, Yoshimura et al. 1989), X-linked vacuolated myopathy (Villanova, Louboutin et al. 1995), and inflammation (Mulligan, Schmid et al. 1996). Activation of the complement system has been also implicated in the pathogenesis of myocardial ischemia/reperfusion injury (Weiser, Williams et al. 1996; Vakeva, Morgan et al. 1994; Weisman, Bartow et al. 1990; Hill, Lindsay et al. 1992). Components of the complement system such as complement 3,

complement 4, complement 5, complement 9 and the MAC have been identified in experimentally infarcted tissue as well as in human ischemic myocardium (Mathey, Schofer et al. 1994). Complement 5b-9 accumulates rapidly in ischemic myocardium during reperfusion (Mathey, Schofer et al. 1994). The reperfusion plays a critical role in mediating complement deposition because in its absence the complement accumulation occurs only as a late event (Mathey, Schofer et al. 1994). In a rabbit model of ischemia/reperfusion it has been shown that local production of complement proteins may contribute significantly to the degree of ischemic injury to the myocardium and that complement expression is augmented by reperfusion (Yasojima, Kilgore et al. 1998).

Recently, the use of complement 9 immunostaining has been proposed for the identification of cardiomyocyte necrosis after myocardial ischemia (Robert-Offermann, Leers et al. 2000; Lazda, Batchelor et al. 2000).

1.11. Major histocompatibility complex II

Major histocompatibility complexes (MHCs) were discovered as proteins causing rejection when skin (graft) from a mouse (donor) strain was implanted (grafted) to a back of another strain mouse (recipient) and the graft soon died (Klein 1990; Darnell, Lodish et al. 1990). The graft implanted is destroyed by the recipient's immune system because the donor's cells express a fair number of proteins on their surfaces that are different from those expressed by the recipient and cause the rejection (Klein 1990; Darnell, Lodish et al. 1990). Those molecules act as antigens, in the same way as molecules from invading virus or bacterium. They act as histocompatibility antigens, encoded in a set of clustered loci of histocompatibility genes. The basis for the ability of MHCs to elicit graft rejection is that each animal of a given species has MHCs genes that differ in sequence from the MHCs genes of other individuals. For this reason the MHCs are said to be polymorphic.

The MHCs are divided into two classes, I and II (Klein 1990; Darnell, Lodish et al. 1990). The MHC class I are glycoproteins that can be divided in three functional domains: external, transmembrane, and cytoplasmic (Klein 1990). The external region consists of two-chain proteins with one highly polymorphic component composed by three domains α 1, α 2, α 3, and one constant chain called β_2-microglobulin (β_2-m) (Klein 1990). The transmembrane region is composed by mostly hydrophobic amino acids arranged in an α-helix. The cytoplasmic region composed by the COOH terminal has the potential of interacting with elements of the cytoskeleton (Klein 1990; Darnell, Lodish et al. 1990).

The MHC class II are heterodimers consisting of one α and one β chain. Both the α and β chains consist of an external region, a connecting peptide, a transmembrane region, and a cytoplasmic tail (Klein 1990). The external region is composed of two domains, α1 and α2 or β1 and β2. The connecting peptide connects the external domains with the transmembrane region arranged in both α and β chains. The cytoplasmic tail is arranged also in both, α and β chains (Klein 1990).

The MHC I is expressed on all somatic cells in adults at high concentration in some cells such as lymphocytes, and at low concentration in other cells such as fibroblasts or neurons. MHC II is constitutively high expressed only in certain cells such as macrophages, B lymphocytes and activated T lymphocytes (in human but not in mice), and virally infected human T lymphocytes. Its expression can be also induced by stimulation (for example with interferon-γ) in many different cell types such as macrophages, dendritic cells, melanoma cells, some epithelial cells, and same parenchymal cells such as thryroid and pancreatic cells (Klein 1990).

The function of MHCs is to expose foreign antigens to T lymphocytes on the surface of an antigen-presenting cell (Klein 1990). The T-cell receptor (TCR) recognizes foreign antigens (usually peptides) together with the MHC molecule: nonself is recognized in the context of self. Class I MHC binds peptides from intracellular proteins, while class II MHC binds peptides from extracellular proteins.

1.12. Objective of the present study

For the realization of the present Thesis we used a transgenic (TG) strain of mice that overexpress murine MCP-1 in the heart (Kolattukudy, Quach et al. 1998). Already published data regarding the MCP-1 TG phenotype showed that young (0-2 months old) TG mice exhibit focal accumulation of monocytes/macrophages in the myocardial interstitium leading to ischemic cardiomyopathy and a gradual loss of myocytes in elderly animals (Kolattukudy, Quach et al. 1998; Moldovan, Goldschmidt-Clermont et al. 2001). Therefore, we have postulated the hypothesis that activated leukocytes expressing TNF-α in the heart of MCP-1 TG mice might confer myocardial protection against ischemia by activating the MAPKs pathway. To test this hypothesis we investigated the cardiac resistance to ischemia in TG (~ 4 months old) and control (WT) (non-transgenic mice from the same litter) mice with and without injection of the SAPK/JNKs inhibitor D-JNKI1. Histopathological examinations of MCP-1 TG hearts were performed, as well as

quantitative analysis of TNF-α, phosphorylated-SAPK/JNK1/2, phosphorylated-ERK1/2, and phosphorylated-p38 protein levels. Finally, in order to establish a pattern of temporal effects of MCP-1 overexpression, electron microscopy analyses of hearts from 1 day, 1 months, 2 months, and 6 months old TG mice were performed and TG mice from 6 months old were used for quantitative histological analysis for ubiquitin, complement 9, and MHC II antibodies.

2. Material and Methods

2.1. Generation of MCP-1 TG mice

The MCP-1 TG mice have been generated in the Ohio State University, Columbus, Ohio (Kolattukudy, Quach et al. 1998). The construct consists of the cDNA of the murine MCP-1 (JE) gene, that is linked downstream to the α–cardiac myosin heavy chain promoter in order to confer cardiac specific expression. Briefly, a *Myhca* clone containing the 5.5-kb promoter region of the α-cardiac myosin heavy chain (MHC) was double digested by *Sa*/*l* and *Kpnl*. Then, 1.9-kb DNA element of the mouse JE-MCP-1was generated by PCR amplification and cloned into pUC 19 plasmid. The MCP-1-pUC 19 plasmid was amplified by PCR tecniques using a nucleotide primer containing *Sa*/*l* and *Kpnl* restriction sites. The PCR products cleaved with *Sa*/*l* and *Kpnl* were ligated to the *Sa*/*l*-*Kpnl*-digested *Myhca* clone to obtain the construct pMHC-MCP-1. The generated plasmid pMHC-MCP-1 was then digested with *Notl*/*Kpnl* to obtain a DNA fragment of 7.4-kb. This DNA fragment was microinjected into one-cell FVB/N embryos. The surviving embryos were implanted into pseudopregnant C3B6F1 foster mothers. The TG and WT mice were selected by PCR and Northern blot analysis.

2.2. Animals

The total number of mice used for all the experiments was 43 WT, 3-10 months old and 57 TG 0-9 months old.

Housing and handling of the animals was in accordance with the American Physiological Society guidelines for animal welfare and the Bioethical Committee of the District of Darmstadt, Germany.

2.3. Fourty-five-min coronary artery occlusion (CAO)

2.3.1. Experimental protocol

We performed 45-min of experimental coronary artery occlusion (CAO) in WT (n=5) and TG (n=5) mice. The mice were anaesthetized with an intraperitoneal injection of 10%Ketamine (0.1 mg/g body weight) and 2%Xylazin (0.005 mg/g

body weight) in 9% NaCl solution. For each mouse we used approximately 0.16 ml of Ketamin solution, 0.16 ml of Xylazin solution and 0.32 ml of NaCl solution. When necessary half dose of anaesthetic was re-injected intraperitoneally. After the induction of anaesthesia, the chest coat was shaved and the trachea was intubated with a 1.1 mm steel tube connected to an animal ventilator. Heparin solution (125 IU, 0.025 ml) was injected intraperitoneally to prevent microthrombus formation during occlusion and reperfusion. The body temperature was kept at 37°C. Heart function was continuously recorded with a 3-lead surface electrocardiogram (ECG) inserted under the skin of thorax, abdomen, and flank. After opening the skin overlying the left part of the thoracic cage the pectoral muscles were prepared free and retracted with 6-0 silk suture. Subsequently, the chest was opened through the third or fourth intercostal space by dissecting the intercostal muscles. Lungs were retracted with a moistened sponge, and a retractor was introduced to gain access to the thoracic cage. Ligation was performed proximal to the main branch of the left coronary artery, using a 6-0 prolene suture and a PE-10 tube was also used to allow removal of the ligation. When perfusion was stopped by ligation, changes in the ECG and tissue color were assessed. After 45 min, the tube was removed and the reperfusion of the coronary artery visually assessed and confirmed by the changes in the ECG. A 0.075 ml propidium iodide solution (Sigma, 0.05% w/v in 0.9% NaCl solution) was perfused via a catheter into the right carotid artery and allowed to circulate for 10 min. After the short reperfusion time, the coronary artery was re-occluded and a solution (0.075 ml approximately) of thioflavin S (Sigma, 4% w/v in 0.9% NaCl solution) was injected into the carotid artery for a few seconds. The heart was then removed, the right ventricle excised, and the left ventricle cryopreserved with nitrogen-cooled methylbutane.

2.3.2. Experimental protocol for the SAPK/JNKs inhibitor D-JNKI1

D-JNKI1, a novel SAPK/JNKs inhibitor, was injected in TG and WT mice. A specific cytoplasmic inhibitor of SAPK/JNKs, called JNK interacting protein-1/IB1 (JIP-1/IB1), was characterized and cloned. JIP-1 binding specifically to JNKs causes cytoplasmic retention of JNKs and specific inhibits JNK signal transduction pathway activation (Dickens, Rogers et al. 1997). D-JNKI1 was generated from JIP-1/B1 inhibitor. A 500 μl solution of the SAPK/JNKs inhibitor D-JNKI1 (Alexis Biochemicals, 0.25 mg/body weight in 0.9% NaCl solution/DMSO) was injected into the tail artery in TG (n=5) and WT (n=4) mice 30-min before CAO. Alternatively, 500 μl solution of DMSO (0.5% in O.9% NaCl solution) were also injected into the tail artery of TG (n=2) and WT (n=2) mice 30-

min before CAO. After the inhibitor injections, we performed 45 min CAO as described in *2.3.1*.

2.3.3. Determination of infarct size

Propidium iodide was employed to determine the infarct size for 45-min of CAO. Propidium iodide is routinely used as nuclear marker in cell culture (Laake, Haug et al. 1999; Kang, Haunstetter et al. 2000) and flow cytometry (O´Brain and Bolton 1995) studies to label necrotic cells. During myocardial infarction, the loss of membrane integrity allows the propidium iodide to enter the necrotic cells and stain the nucleus by its specific binding to DNA. This staining results in a red fluorescent signal that delineates infarcted areas. Propidium iodide staining has been successfully employed by other authors to measure infarct size in experimental coronary artery ligation (Ito, Schaarschmidt et al. 1997; Wolff, Chien et al. 2000). These authors demonstrated that in esperiments with a short period of reperfusion, propidium iodide is a more appropriate method to quantify the infarcted area when compared to the more commonly used triphenyl tetrazolium chloride (TTC). TTC reacts with dehydrogenases in the presence of the co-enzyme NADH producing a red colored precipitate (Klein, Pushmann et al. 1981). Necrotic cells lose their ability to retain NADH and show a pale color when incubated with TTC, while viable cells, which contain NADH, react with TTC and are stained in red. The TTC method requires long reperfusion time to wash out dehydrogenases and NADH from the necrotic tissue, and avoid understimation of the infarcted area (Ito, Schaarschmidt et al. 1997; Wolff, Chien et al. 2000; Klein, Pushmann et al. 1981). As propidium iodide requires only a few minutes of reperfusion to appropriately stain necrotic cells, this was the method of choice for 45-min CAO, where the effect of reperfusion on ischemic injury was not desired.

The endothelial cell marker thioflavin S was employed to quantify the non-risk area of the left ventricle.

The risk area was measured and defined as the non-perfused area of the left ventricle (thioflavin S-negative).

Left ventricular cryosections 14 μm thick were cut and photographed under fluorescent light with a DM-RB Leica microscope. The total left ventricle, risk area and infarct area were determined using computer assisted planimetry software NIH Image. Finally, three *ratios* were obtained: infarct area/risk area (IA/RA), infarct area/left ventricle (IA/LV), and risk area/left ventricle (RA/LV).

2.4. Fourty-five-min CAO followed by 3 days of reperfusion

2.4.1. Experimental protocol

We performed 45-min CAO followed by 3 days of reperfusion (45-min CAO+3-day R) in WT (n=4) and TG (n=4) animals. The animal preparation for CAO was the same as for 45-min CAO. Ligation was performed proximal to the main branch of the left coronary artery, using a 6-0 prolene suture and a PE-10 tube was also used to allow removal of the ligation. After 45 min, the tube was removed and the reperfusion of the coronary artery visually assessed and confirmed by the changes in the ECG. The chest was closed in layers using a 4-0 silk suture. The animal ventilator was disconnected when the mice started to wake up, and the mice were kept warm with a heat lamp until complete recovery.

2.4.2. Determination of infarct size

TTC was employed to determine the infarct size after 45-min CAO+3-day R. Three days after 45 min CAO the animals were killed by anesthetic overdose (10%Ketamin, 2%Xylazin) injected intraperitoneally, the heart was excised, the right ventricle removed, and the left ventricle was frozen for 5-10 min to allow to easily cut it in transverse slices. The slices were weighed and incubated at 37°C in 1% TTC (Sigma) in phosphate saline buffer (PBS) solution, pH 7.0, for 20 min.

PBS solution (pH 7.0 and pH 7.4):

NaCl	7.948 g
KCl	0.2 g
KH_2PO_4	0.2 g
$NaHPO_4H_20$	1.775 g
Distilled water	1 L

Adjust the pH with HCl or NaOH.

After incubation with TTC, the upper and the bottom part of each slide were photographed and the pictures used for planimetric analysis. The left ventricle and the infarct area (recognized by the pale color due to lack of TTC staining) were determined using computer assisted planimetry with NIH Image software. These measurements provided the IA/LV *ratio*.

2.5. Three-day CAO

2.5.1. Experimental protocol

The animal preparation for 3-day CAO was the same as for 45-min CAO. Permanent ligation was performed using a 6-0 prolene suture. After assessment for coronary ligation efficiency (changes in the ECG and tissue color), the chest was closed in layers using a 4-0 silk suture. The animal ventilator was disconnected when the mice started to wake up, and the mice were kept warm with a heat lamp until complete recovery.

2.5.2. Determination of infarct size

TTC was employed to determine the infarct size after 3-day CAO.as described in *2.4.2.*.

2.6. Western blot analysis

The hearts of WT (n=12) and TG (n=12) animals were excised, snap-frozen in liquid nitrogen and stored at –80 °C until use. The tissue was homogenized with ice-cold buffer containing 20 mM tris-HCl, 250 mM sucrose, 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM DTT, 0.1 mM sodium orthovanadate, 10 mM NaF, and 0.5 mM PMSF.

Ice-cold buffer preparation (100 ml):

Tris	0.2422 g
Saccarose	8.5575 g
EDTA	0.037224 g
EGTA	0.3804 g
DTT	0.015425 g
Sodium orthovanate	0.001839 g
NaF	0.04195 g
PMSF	0.00871 g dissolved in 500 µl ethanol

Add distilled water until 100 ml and adjust the pH 7.4. The ice-cold buffer has to be frozen for 15 min at –80°C until use.

The homogenate was centrifuged at 4°C and 13,000 *g* for 30 min and the supernatant (cytosolic fraction) was kept for protein analysis. The Bio-Rad Protein Assay (Bio-Rad Laboratories) was used to assess the equivalent amount of proteins for immunoblot analysis following the manufacture's instructions. We used Laemmli Sample Buffer (Bio-Rad Laboratories) (2:1, Laemmli Sample Buffer, cytosolic fraction, respectively) and 5 min denaturation by heating at 95°C for electrophoresis protein preparation.
Laemmli Sample Buffer solution:

Laemmli Sample Buffer	950 μl
Mercaptoethanol	76 μl

Electrophoresis was performed at 120 Volt in 10% and 18% Tris-HCl ready gel (Bio Rad, 10 wells each one for 30 μl of probe solution) in running buffer for 1-3 hours depending on the size of the protein used. Fifteen μl of weight reference marker (MultiMark, Multi-Colored Standard; Invitrogen) was added in every electroforesis.
Running buffer (1 l):

SDS	1 g
TRIS	3.03 g
Glycin	14.41 g
Distilled water	1 l

The proteins were transferred onto nitrocellulose membranes using the electrophoresis machine at 200 mA for 2 hours in transfer buffer.
Transfer buffer (1 l):

TRIS	3.03 g
Glycin	14.41 g
Methanol (20%)	200 ml
Distilled water	800 ml

The membranes were then blocked with 5% nonfat dry milk in tri-phosphate saline buffer (TBS) overnight at 4°C (alternatively, 3 hours at room temperature).
TBS:

TRIS	2.42 g
NaCl	8.0 g
Distilled water	1 l

Adjust the pH 7.4.

After blockage, the membranes were incubated with the first antibody in TBS overnight at 4°C (alternatively, 3 hours at room temperature). Polyclonal antibodies against JNK1 (Santa Cruz Biotechnology, 1:500 dilution), phosphorylated-SAPK/JNK1/2 (Cell Signaling Technology, 1:500 dilution), ERK 2 (Santa Cruz Biotechnology, 1:500 dilution), phosphorylated-ERK1/2 (New England Biolabs, 1:500 dilution), p38 (Santa Cruz Biotechnology, 1:500 dilution), phosphorylated-p38 (Cell Signaling Technology, 1:500 dilution), and TNF-α (Santa Cruz Biotechnology, 1:200 dilution) were used. After washing (6 x10 min) with TBS, the membranes were incubated 2 hours with peroxidase-conjugated anti-rabbit IgG in TBS (Amersham, 1:1000 dilution). The ECL-system (Amersham Pharmacia Biotech) was used for signal detection following the manufacture's instructions and quantification was performed on STORM 860 (Amersham Pharmacia Biotech), using ImageQuan software.

2.7. Terminal dUTP deoxynucleotidyl transferase nick end-labeling (TUNEL) assay

TUNEL staining was performed in three random cryosections from each heart of WT (n=3) and TG (n=5) mice. The *In Situ* Cell Death detection Kit, Fluorescein (Roche Diagnostic) was used according to the manufacture's instructions. Cryosections of duodenum were used as positive control.

2.8. Electron microscopy

The hearts from additional WT (n=2) and TG (n=9) animals were perfusion-fixed with a mixture of 2% paraformaldehyde and 1% glutaraldehyde in PBS solution (pH 7.4) and immersed in 3% glutaraldehyde.
3% glutaraldehyde:

25% glutaraldehyde	12 ml
Cacodylate buffer without saccharose (0.1 M)	88 ml

Cacodylate buffer (0.1 M):

Na-cacodylate	21.4 g
Saccharose	75 g
Distilled water	1l

Adjust the pH 7.4.

The hearts were then post-fixed with osmium tetroxide, dehydrated in a series of ethanol, and embedded in epoxy resin with the following protocol using a Lynx microscopy tissue processor Reichert Jung:

Osmium + Veronal-acetate with 4% saccharose solution	2 hours	4°C
Veronal-acetate with 7.5% saccharose solution	3 x 15 min	4°C
30 % ethanol	15 min	4°C
50% ethanol	15 min	4°C
70% ethanol with 0.5% Uranyl	15 min	4°C
90% ethanol	15 min	4°C
100% ethanol	2 x 15 min	4°C
Propylenoxide	2 x 15 min	4°C
Propylenoxide-Epon 1:1	15 min	4°C
Epon	3 x 60 min	37°C

Osmium(tetroxide) solution:
Stock solution:

OsO_4	1 g
Distilled water	16.4 g

Keep in the refrigerator until used.
Used solution:

Veronal acetate (from stock solution)	2 ml
OsO_4 (from stock solution)	1 ml

Adjust the pH 7.4.

Veronal acetate buffer:
Stock solution:

Na-Acetate ($CH_3COONa_3H_2O$)	9.7 g
Veronal-Na (Na-5.5-Diethyl-barbituracid)	14.7 g
Distilled water	250 ml

Dissolve at room temperature and than add distilled water until 500 ml.
Used solution:

Veronal-Acetate (from stock solution)	20 ml
HCl (0.1 N)	20 ml
Ditilled water	60 ml

Add 4% or 7.5% of saccharose and adjust the pH 7.4.

Ultramicrotome sections were counterstained with uranyl acetate and Reynolds lead citrate with the following protocol:

- 2 min in Uranyl-Acetate solution
- 1 min wash in distilled water
- 2 min in Reynold lead citrate solution
- 1 min wash in distilled water

Uranyl acetate:

3% uranyl acetate in distilled water.

Reynolds lead citrate solution:

Stock solution:

$Pb(NO_3)_2$	1.33 g
Na-Citrate	1.76 g
NaOH (1 N)	8 ml

Dissolve $Pb(NO_3)_2$ and Na-Citrate with 30 ml distilled water shaking for 1 hour, then add NaOH (1 N) and distilled water until 50 ml. The pH has to be 12, if not, the protocol has to be repeated because it is not possible adjust it.

Used solution:

Stock solution	10 ml
Distilled water	10 ml

Add NaOH (1 N) to obtain pH>11. Filter the solution with a Millipore-filter and keep it in the dark and in a closed bottle.

The photographs and evaluations were performed using a Philips CM10 electron microscope.

2.9. Histology and immunohistochemistry

For the analysis of specific structural changes due to MCP-1 overexpression, additional animals (WT, n=7; TG, n=10) were killed by anesthetic overdose (10%Ketamin, 2%Xylazin) injected intraperitoneally. The hearts were dissected and cryopreserved with nitrogen-cooled methylbutane and stored at -80°C until used. Alternatively, the hearts were perfusion fixed, first with 0.01% adenosine in PBS solution (pH 7.4), and then with 4% paraformaldehyde in PBS solution

(pH 7.4). After perfusion/fixation, the hearts were fixed in 4% paraformaldehyde for 48-72 hours. After the fixation, the hearts were embedded in paraffin with the following protocol:

• wash in distilled water, 1 hour and 30 min

• dehydration with:

30% ethanol, 1 hour and 30 min

50% ethanol, 1 hour and 30 min

70% ethanol, 1 hour and 30 min (or overnight)

80% ethanol, 1 hour and 30 min

95% ethanol, 1 hour and 30 min

100% ethanol, 1 hour and 30 min (x2)

Xylol, 1 hour and 30 min (x2)

• bath with xylol+paraffin (1:1), 1 hour and 30 min at 60°C

• bath in paraffin, overnight at 60°C

• re-bath in paraffin, 1 hour and 30 min at 60°C

• preparation of the block at room temperature.

The paraffin blocks or cryopreserved hearts were cut in 5 μm sections and stained with hematoxilin and eosin (H&E).

H&E staining:

• desparaffination in xylol (only for paraffin blocks), 5 min (x3)

• hydration (only for paraffin blocks) with:

95% ethanol, 5 min

70% ethanol, 5 min

50% ethanol, 5 min

• staining with Delafield-hematoxilin, 10-15 min

• staining with Yellow-eosin, 7-10 min

•dehydration with:

95% ethanol, 5-10 seconds

100% ethanol, 5-10 seconds

- Xylol, 10 min
- cover with coverglass using DPX.

For immunohistochemistry, only cryopreserved hearts were used. The following primary antibodies were used, a FITC-conjugated monoclonal antibody against the transferrin receptor CD 71 (Ancell, 1:100 dilution), a polyclonal antibody against TNF-α (Santa Cruz Biotechnology, 1:20 dilution), a polyclonal antibody against ubiquitin (Dako, 1:50 dilution), a rat monoclonal antibody against MHC-II (MHC-II) (BMA, 1:50 dilution), a polyclonal antobody against phosphorylated-SAPK/JNK1/2 (Cell Signaling Technology, 1:100 dilution), and a mouse monoclonal antibody against complement 9 (NOVOCASTRA, 1:50 dilution). Cryosections, 5 μm thick, were air dried and fixed with 4% paraformaldehyde or ice-cold acetone for 10 min. In addition, the sections used for TNF-α and phosphorylated-SAPK/JNKs antibodies were incubated with 3% hydrogen peroxide for 10 min to quench the endogenous peroxidase activity. After the washing steps (3x5 min) in PBS solution (pH 7.4), the slides were immersed in a blocking solution containing 0.1% bovine serum albumin and 0.4% glycine in PBS for 30 min. After incubation with the primary antibodies, the sections were washed (3x 5 min) with PBS solution. The sections were incubated with specific second biotinylated-antibodies for 1 hour: anti-rabbit (1:100 dilution) for ubiquitin, anti-rat (1:100 dilution) for MHC-II, and anti-mouse (1:100 dilution) for complement 9, respectively, and then incubated with Cy_2-streptavidin (1:100 dilution) for 1 hour. All the sections, excluded those used for TNF-α and phosphorylated-SAPK/JNKs antibodies, were incubated with the myocyte marker phalloidin-TRITC labelled (Sigma, 1:500 dilution) for 20 min. Finally, the sections were incubated with the nuclear markers 4‘,6-diamidino-2-phenylindole, dilactate (DAPI) (Molecular Probes, 1:1000 dilution) or propidium iodide (Sigma, 1:1000 dilution) for 10 min. The sections used with TNF-α and phosphorylated-SAPK/JNKs antibodies were incubated with peroxidase-conjugated anti-rabbit IgG (Amersham, 1:100 dilution). Peroxidase was detected by incubation with 3,3‘-diaminobenzidine (Sigma), and the nuclei were counterstained with hematoxilin. Omission of the first antibodies was used as negative control. Finally, the sections were evaluated and photographed under transmitted or fluorescent light with a DM-RB Leica microscope or a TCSNT Leica confocal microscope.

2.9.1. Quantitative immunofluorescence analysis for ubiquitin, complement 9, and MHC II antibodies

Ten randomly chosen cryosections from the apex to the base of hearts from WT (n=2) and TG (N=5) mice were immunolabeled for ubiquitin, complement 9, and MHC II as described in *2.9.*. Positive-myocytes for each antibody were counted in each section as well as the total number of myocytes using computer assistant planimetry with NIH Image software. Finally, we obtained the number of ubiquitin, complement 9, and MHC II positive-cardiomyocytes as a percentage of the total number of myocytes.

2.10. Quantitative immunohistochemical analysis by densitometry

Three randomly chosen cryosections from WT (n=3) and TG (n=4) hearts were immunolabeled for phosphorylated-SAPK/JNK1/2 as described in *2.9*. All sections were stained and photographed using the same conditions. Twenty-four randomly selected phosphorylated-SAPK/JNK1/2-positive myocytes were measured from each section. Quantitative intensity level analysis was performed using computer assistant planimetry software (NIH Image).

2.11. Statistical analysis

Results are reported as mean ± SEM. Unpaired *t*-test or the Mann-Whitney-U-test was used to estimate significant differences between groups. The accepted minimum level of statistical significance was $p<0.05$.

3. RESULTS

3.1. Infarct size

3.1.1. Fourty-five-min CAO

We performed 45-min CAO in TG (n= 5) and WT mice (n=5) in order to investigate the TG cardiomyocyte resistance to ischemia. Previous studies in our group has demonstrated that 45-min of ischemia are enough to obtain a near maximum infarct size in the murine heart. Fig. 4A,B shows a transversal section of the left ventricle from a WT and a TG heart. Dead myocytes are stained in red with propidium iodide and represent the infarct area (IA). Endothelial cells from the non-risk area of the left ventricle (LV) are stained in blue with thioflavin S and represent the viable myocardium not irrigated by the occluded coronary artery. The risk area (RA) not stained appears dark and represents cardiomyocytes irrigated by the ligated coronary artery that are still alive (propidium iodide-negative cells). After 45-min of CAO, the infarct size was 3.7-fold reduced in TG mice as compared to WT (IA/RA: 14.7 ± 2.6% *versus* 52.0 ± 2.4%, IA/LV: 7.8 ± 1.5% *versus* 29.0 ± 1.5%) (Fig. 4C). The risk area did not differ significantly between TG and WT animals (RA/LV: 53.5 ± 1.2% *versus* 50.9 ± 0.9%) (Fig. 4C). Quantification of the ratio IA/RA, IA/LV and RA/LV in TG and WT animals is represented in Fig. 4C.

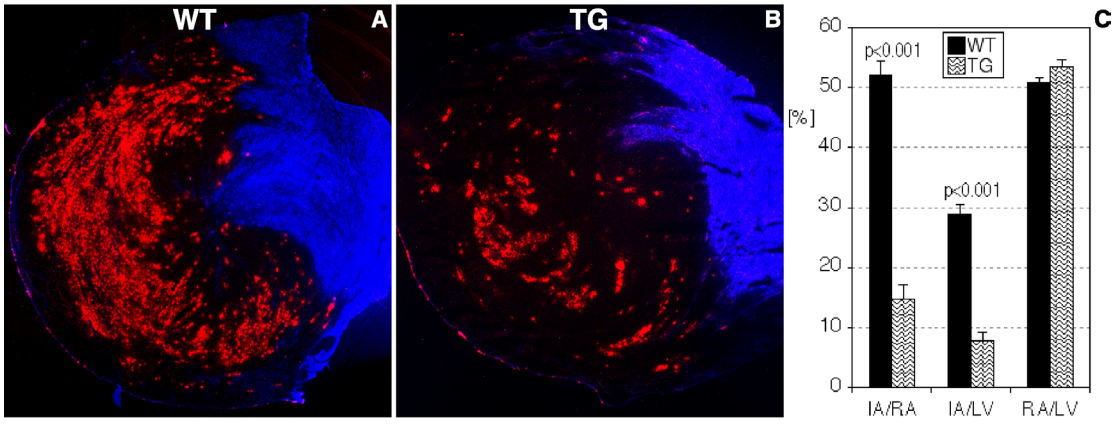

Fig. 4. *(A) and (B) Transverse section (14 μm) of the left ventricle from WT (A) and TG (B) animals after 45-min of CAO. Dead myocytes are stained in red with propidium iodide. Endothelial cells from the non-risk area are stained in blue with thioflavin S. The risk area is not stained and appears dark. x50. (C) Quantification of the ratios IA/RA, IA/LV, RA/LV, in WT (n=5) and TG (n=5) animals after 45-min of CAO. The statistical significance in the infarct size between WT and TG mice is reported.*

3.1.2. Three-day CAO

In order to check if the reduction in infarct size observed after 45-min of CAO in MCP-1 TG mice is still induced after long-term ischemia, we performed long-term (3-day) CAO in WT and TG mice. The Fig. 5A,B shows a transversal section of the left ventricle from a WT and a TG heart after 3-day of CAO. Dead myocytes (IA) are showed in a pale color by the reaction with TTC, while alive myocytes are stained in red with the reaction with TTC. The infarct size did not differ significantly between TG and WT animals (IA/LV: 33.9 ± 4.1%versus 31.8 ± 2.9%) (Fig. 5A,B). The quantification of the ratio IA/LV is represented in Fig. 5C.

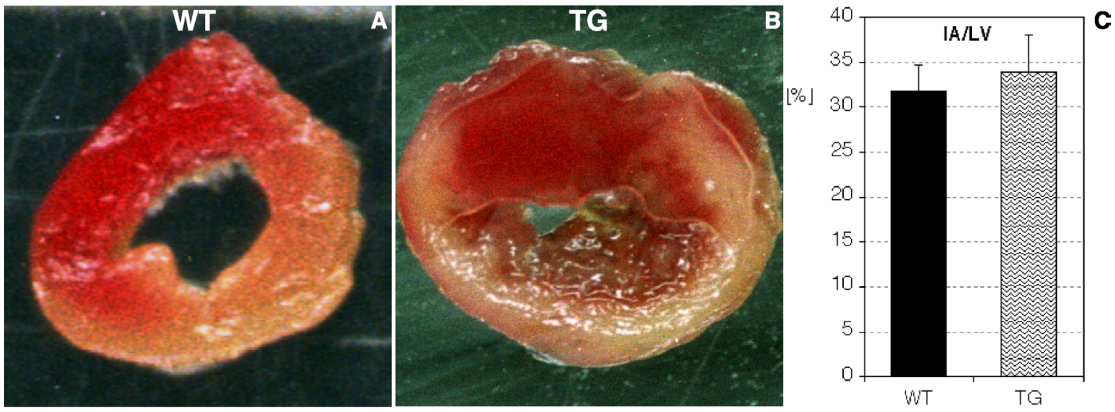

Fig. 5. *(A) and (B) Left ventricle slices from WT (A) and TG (B) hearts after 3-day of CAO. Viable myocardium is stained in red by the reaction with TTC. Infarct area appears pale due to lack of TTC staining. (C) Quantification of the ratio IA/LV in WT (n=3) and TG (n=4) animals after 3-day of CAO. No significant difference in the infarct size can be detected between WT and TG animals at this time point after coronary occlusion.*

3.1.3. Fourty-five-min CAO+3-day R

In order to check if the reduction in infarct size observed after 45-min of CAO in MCP-1 TG mice was induced permanently, we performed 45-min of CAO followed by 3 days of reperfusion in WT and TG mice. The infarct size was significantly decresed in TG mice when compared with WT (IA/LV: 23.2 ± 1.8% vs 30.0 ± 1.8%) (Fig. 6).

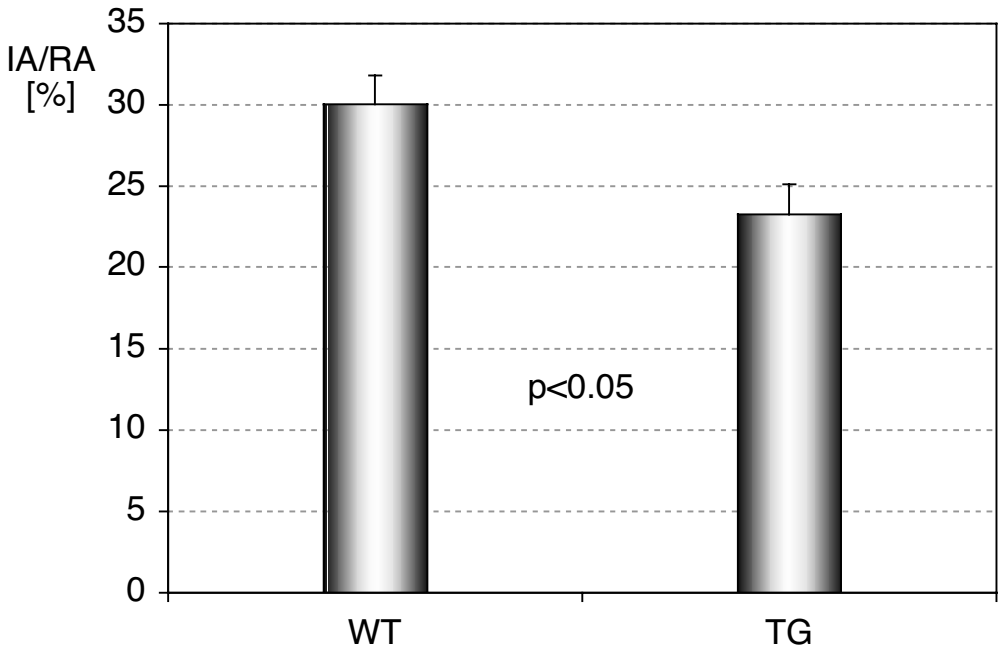

Fig. 6. *Quantification of the ratio IA/LV in WT (n=4) and TG (n=4) after 45-min CAO+3-day R.*

3.1.4. D-JNKI1 and infarct size

In order to investigate the role of SAPK/JNKs pathway activation in the induction of cardioprotection in MCP-1 TG mice, we injected the SAPK/JNK1/2 inhibitor D-JNKI1 before performing CAO. Injection of the inhibitor D-JNKI1 increased significantly infarct size in TG mice as compared with untreated TG mice (Fig. 7). In contrast, D-JNKI1-treated WT mice showed that infarct size did not increase significantly as compared with untreated WT animals (Fig. 7). Despide the fact that infarct size was still significantly different between D-JNKI1-treated TG and untreated WT mice, the infarct size of D-JNKI1-treated TG animals was not significantly different from treated WT mice (Fig. 7). Application of NaCl/DMSO without D-JNKI1 had no influence on infarct size in TG as well as in WT hearts (data not shown).

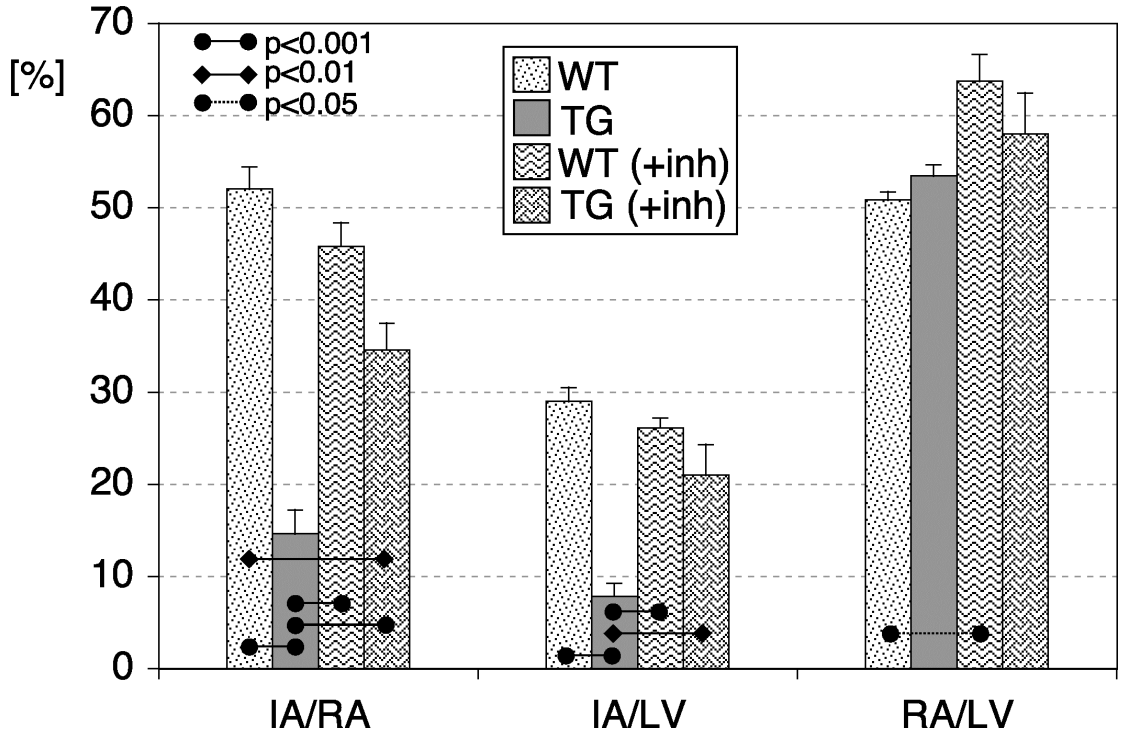

Fig.7. *Infarct size determination after injection of the SAPK/JNKs inhibitor D-JNKI1 in WT (n=4) and TG (n=5) mice. WT and TG treated mice (+inh in the figure) were compared one to each other and with WT and TG untreated mice. The statistical significances are reported.*

3.2. Western blot analysis

3.2.1. SAPK/JNK1/2

In order to check if the activation of SAPK/JNK1/2 showed different degree of expression in the hearts of TG and WT animals we performed Western blot analysis using an antibody against phosphorylated (activated) SAPK/JNK1/2. A significant (1.5-fold) increase in the phosphorylated-SAPK/JNK1/2 was found in the heart of TG animals as compared to the WT as shown in Fig. 8A,C. The activation was induced in both, p46 and p54 respectively isoforms, of SAPK/JNK1/2 in TG and WT mice. An antibody against JNK1 (inactivated SAPK/JNK1/2) was used to confirm the presence of SAPK/JNK1/2 proteins in the heart of TG and WT animals as well as equal amount of non-phosphrylated proteins (Fig. 8B).

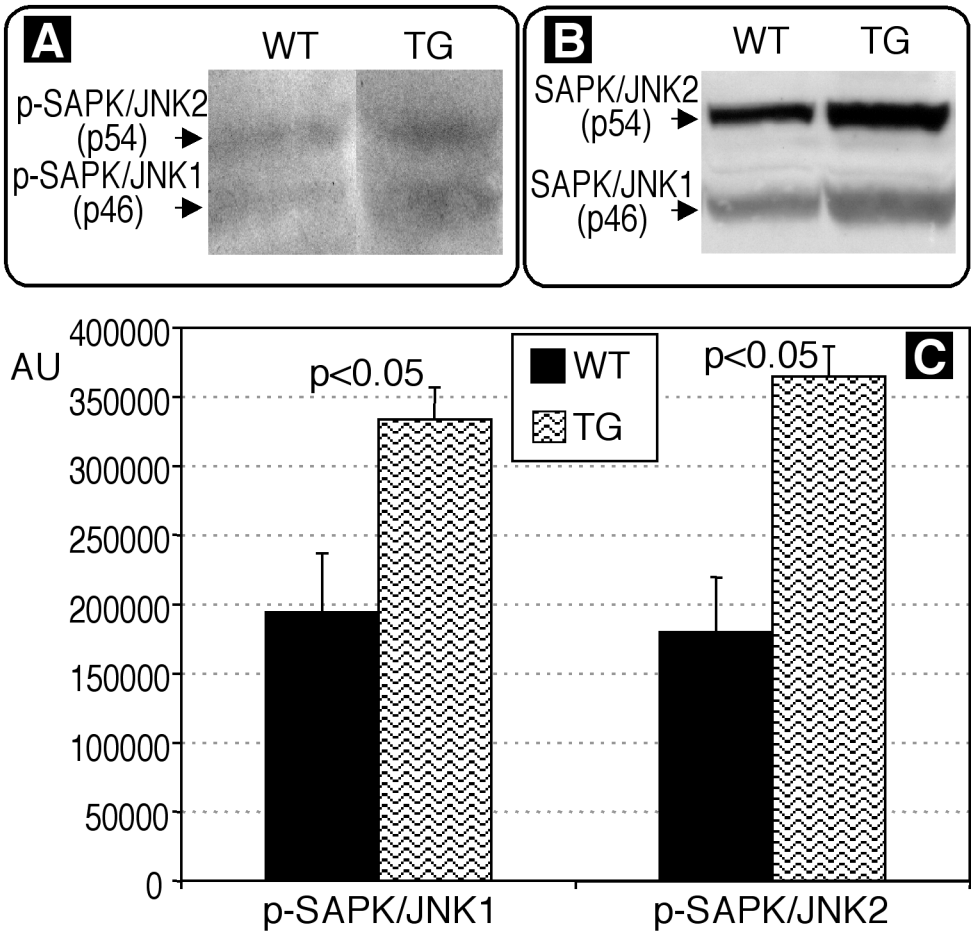

Fig. 8. *(A) Western blot assay of hearts from WT and TG animals using a polyclonal antibody against phosphorylated-SAPK/JNK1/2 (p-SAPK/JNK1/2 in the figure). The activation is induced in both, p46 and p54 respectively isoforms of SAPK/JNK1/2. (B) Western blot assay of the same WT and TG hearts used in A, using a polyclonal antibody against JNK1 (inactivated SAPK/JNK1/2). (C) Quantification of phosphorilated-SAPK/JNK1/2 proteins in WT (n=4) and TG (n=4) animals. The differences in the phosphorylated-SAPK/JNK1/2 levels between WT and TG mice are significant for both isoforms.*

3.2.2. ERK1/2

We performed Western blot analysis using an antibody against phosphorylated-ERK1/2 in order to check if other members of the MAPKs superfamily are activated in the MCP-1 TG animals. In hearts of TG and WT mice no differences were found in the protein levels of phosphorylated (activated) ERK1/2 (Fig. 9A). Western blot of ERK2 (inactivated form of ERK2) showed similar amounts of non-phosphorylated proteins in TG and WT hearts (Fig. 9B).

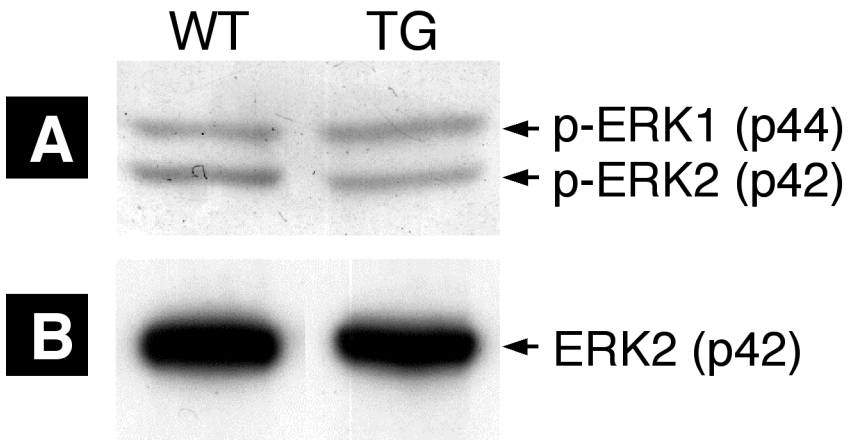

Fig. 9. *(A) Western blot analysis of hearts from WT and TG mice using a polyclonal antibody against phosphorilated-ERK1/2 (p44 and p42 isoforms, respectively). No statistical differences in the protein levels can be detected between WT (n=5) and TG (n=5) animals. (B) Western blot analysis of the same WT and TG hearts used in A using a polyclonal antibody against ERK2 (inactivated ERK2).*

3.2.3. p38

Finally, Western blot using an antibody against p38, the last big member of the MAPKs superfamily, was also performed. In hearts of TG and WT mice no differences were found in the protein levels of phosphorylated (activated) p38 (Fig. 10A). An antibody against the inactivated form of p38 was used and revealed equal amount of non-phosphorylated proteins in WT and TG hearts (Fig. 10B).

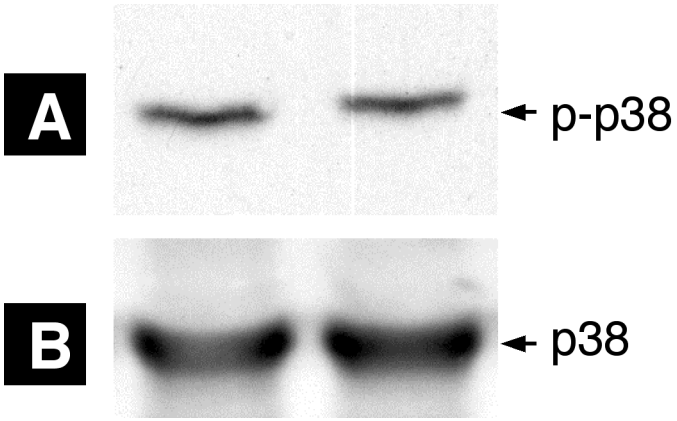

Fig. 10. *(A) Western blot analysis of hearts from WT (left) and TG (right) mice using a polyclonal antibody against phosphorilated-p38. No statistical differences in the protein levels can be detected between WT (n=5) and TG (n=5) animals. (B) Western blot analysis of the same WT (left) and TG (right) hearts used in A using a polyclonal antibody against p38 (inactivated p38).*

3.2.4. TNF-α

It is already known that activated leukocytes are the major source of TNF-α. In order to check if activated leukocytes, found in the hearts of TG animals (see *3.3.4.*), express TNF-α, we performed Western blot analysis using an antibody against TNF-α. TNF-α expression was significantly (1.8-fold) increased in the heart of TG mice when compared with WT (Fig.11).

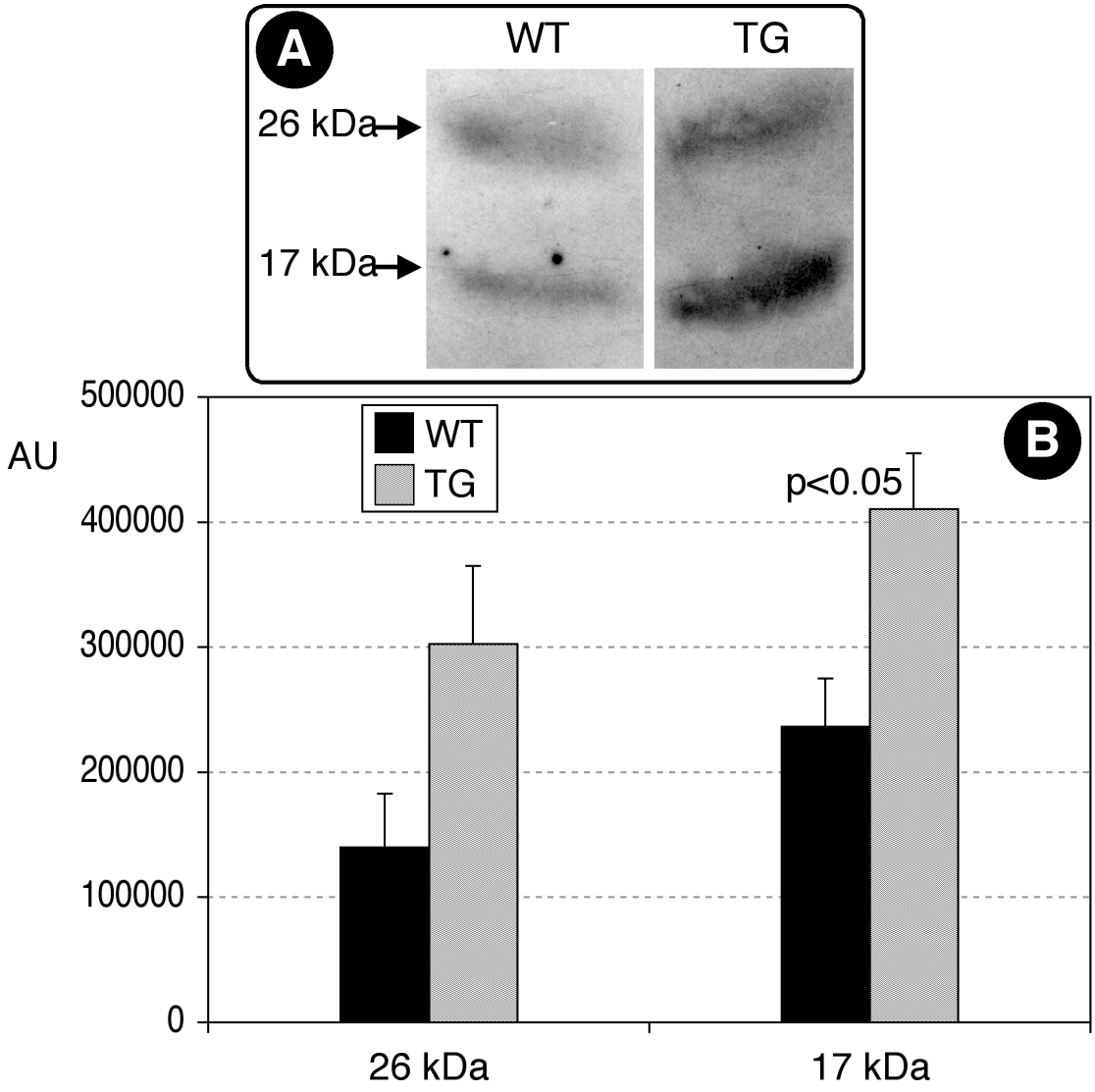

Fig. 11. *(A) Western blot analysis of hearts from WT and TG animals, using a polyclonal antibody against TNF-α. The band at 26 kDa level probably corresponds to a transmembrane precursor form of TNF-α, the band at 17 kDa corresponds to the active form of TNF-α. (B) Quantification of TNF-α protein in WT (n=4) and TG (n=4) hearts. The heart samples used for TNF-α protein level quantification were the same used for phosphorylated-SAPK/JNK1/2 protein level quantification by Western blot.*

3.3. Histomorphology, immunohistochemistry, and ultrastructure

We performed ultrastructural comparative study of neonatal, young, adult, and old TG mice in order to establish a temporal pattern of MCP-1 overexpression. In addition, histomorphological examination of hearts from TG and WT mice were performed to study morphological changes due to MCP-1 overexpression.

3.3.1. One day old TG mice

Using electron microscopic analysis we found that the myocytes of 1 day old TG mice were normal (Fig. 12A). Isolated monocytes/macrophages and fibroblasts in the myocardial interstitium were present (Fig. 12B).

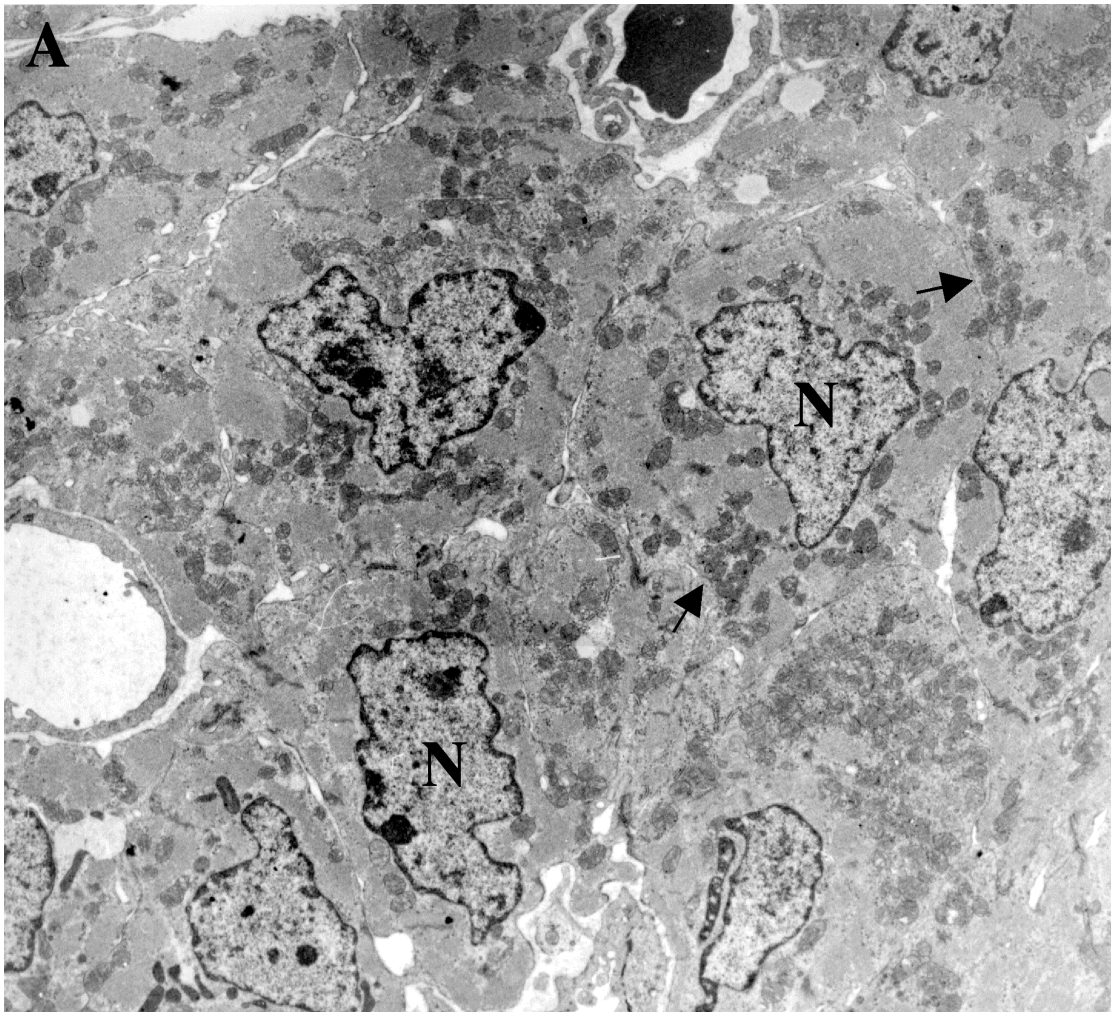

Fig. 12. *(A) Electronmicrograph of a heart from a 1 day old TG mouse. Cardiomyocytes show normal ultrastractural appearance of nuclei (N) as well as mitochondria (Arrows). x3000.*

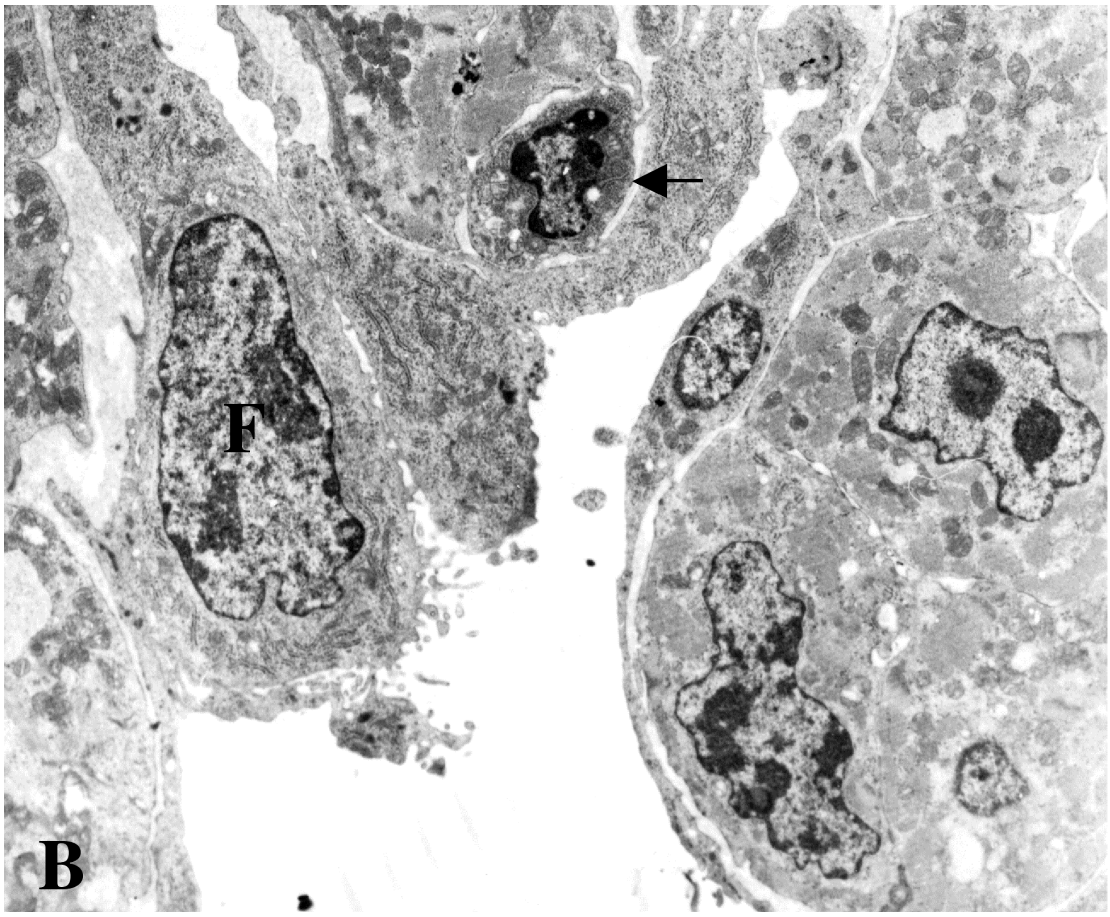

Fig. 12. *(B) Electronmicrograph of a heart from a 1 day old TG animal. TG myocardium. Isolated monocytes / macrophages (Arrow) and fibroblasts (F) were found. x3000.*

3.3.2. One month old TG mice

Electron microscopic analysis revealed the presence of normal myocytes in the heart of 1 month old TG mice (Fig. 13A,B). In addition, isolated fibroblasts (Fig. 13A), lymphocytes (Fig. 13B), and monocytes/macrophages (Fig. 13C) were found in the myocardial interstitium with increased number when compared with 1 day old TG mice.

3.3.3. Two months old TG mice

The myocardium of 2 months old TG mice exhibits a normal histological appearance (Fig. 14A). However, calcium accumulation were found in the cytoplasm of cardiomyocytes, and small accumulations of monocytes/macrophages, fibroblasts and lymphocytes in the ventricular interstitium were present (Fig. 14B,C).

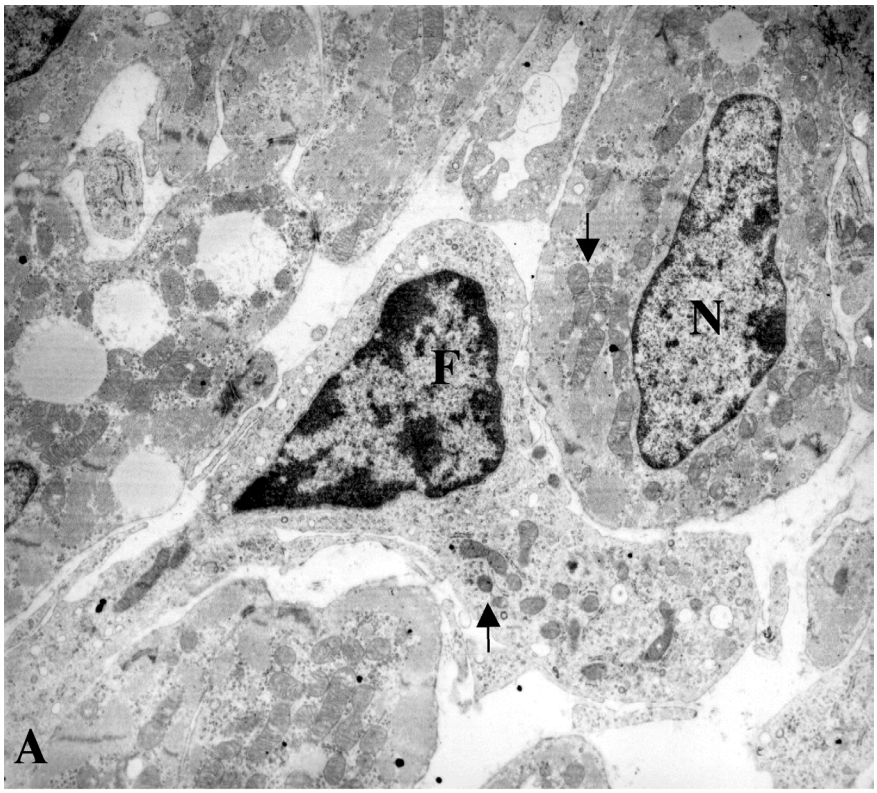

Fig. 13. *(A) Electronmicrograph of a heart from a 1 month old TG animal. TG cardiomyocyte nuclei (N) and mitochondria (Arrows) are of normal ultrastractural appearance. F: fibroblast. x4500.*

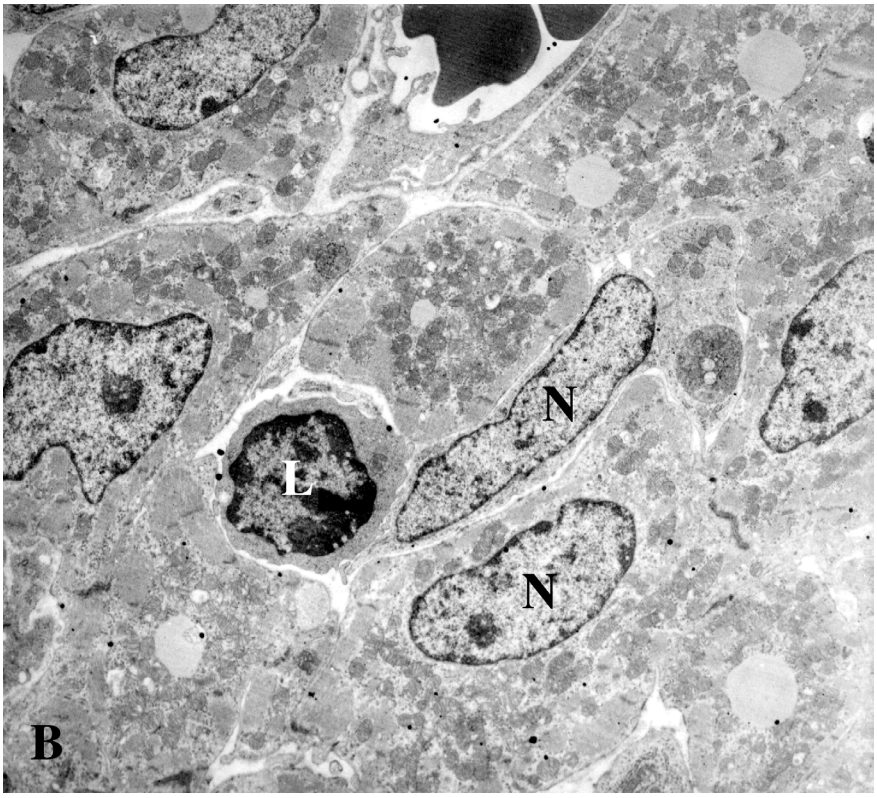

Fig. 13. *(B) Electronmicrograph of a heart from a 1 month old TG mouse. TG myocardium. N: cardiomyocyte nuclei. L: lymphocyte. x3000.*

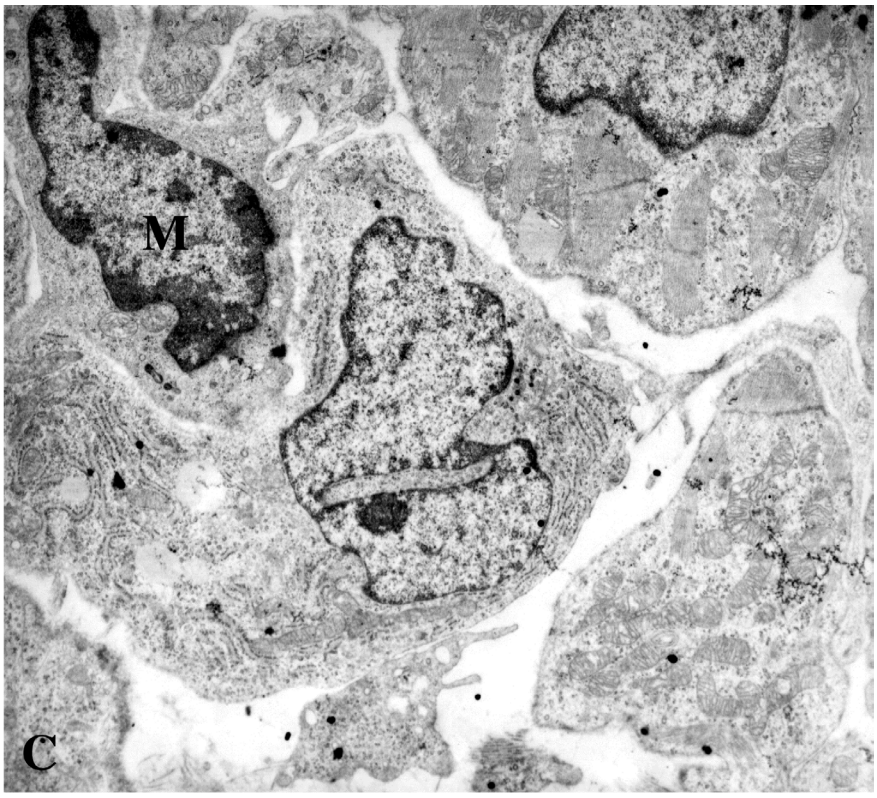

Fig. 13. *(C) Electronmicrograph of a heart from a 1 month old TG mouse. TG myocardium. M: monocyte / macrophage. x4500.*

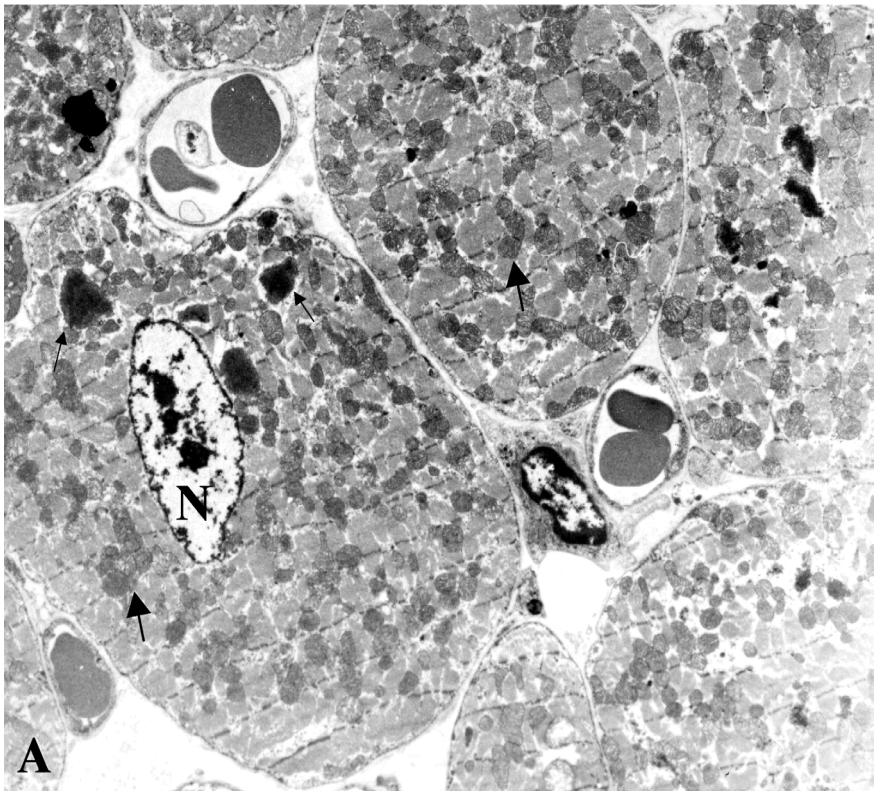

Fig. 14. *(A) Electronmicrograph of a heart from a 2 months old TG mouse. Cardiomyocytes show normal ultrastractural appearance of nuclei (N) as well as mitochondria (Big arrows). Small arrows: Intracellular calcium accumulations. x2000.*

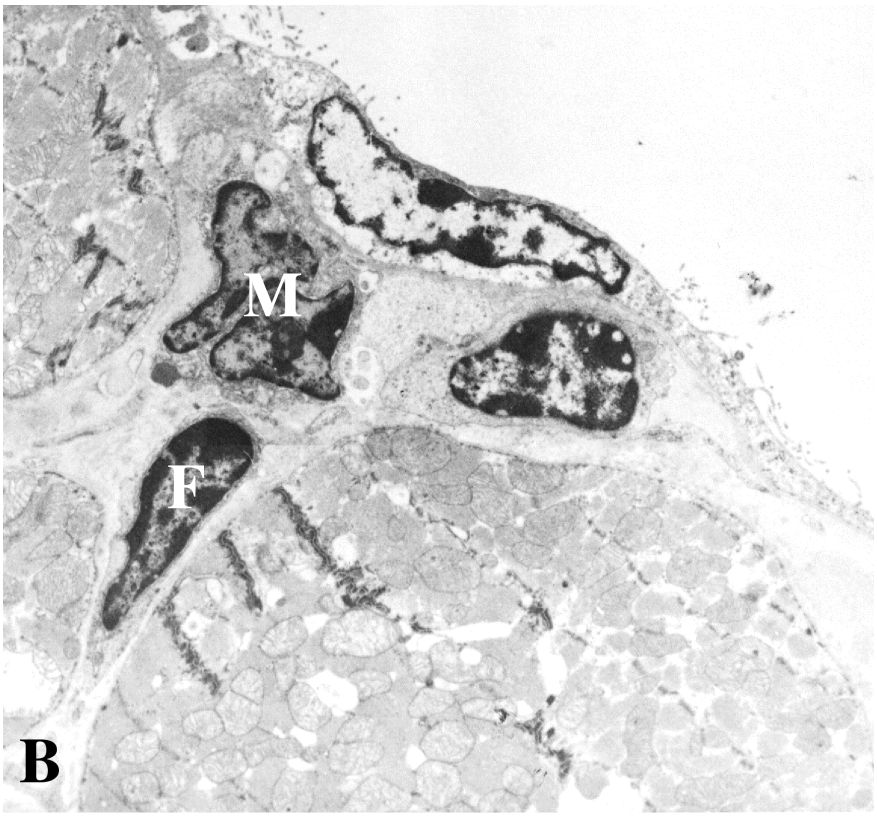

Fig. 14. *(B) Electronmicrograph of a 2 months old TG animal. Myocardial interstitium showing a small leukocyte accumulation composed of monocyte / macrophage (M) and fibroblast (F). x3000.*

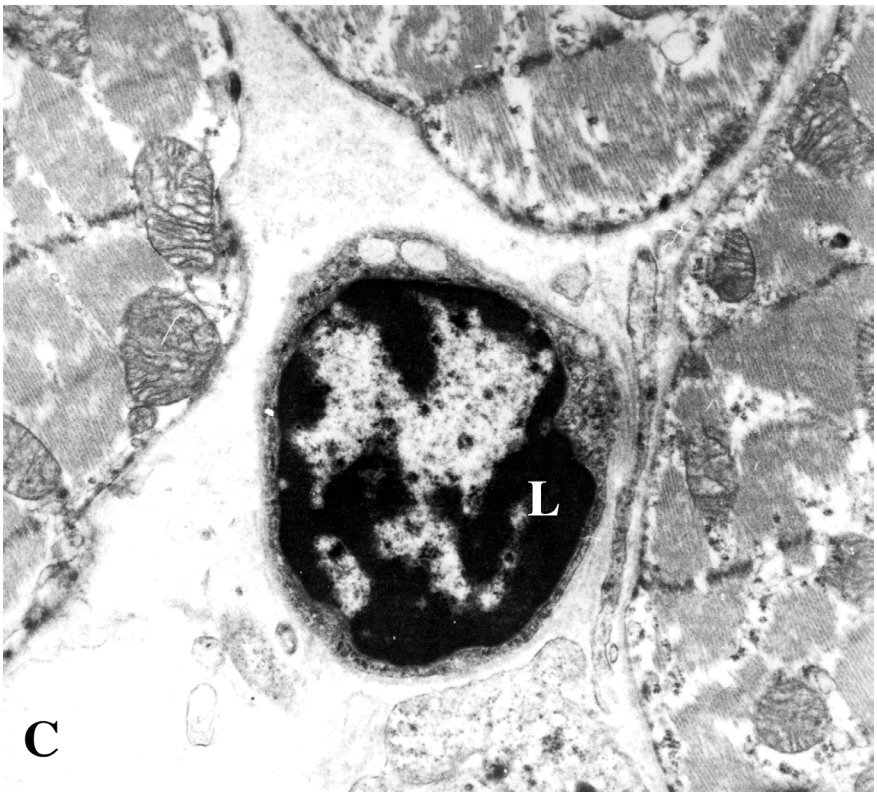

Fig. 14. *(C) Electronmicrograph of a heart from a 2 months old TG mouse. Myocardial interstitium. L: lymphocyte. x7000.*

3.3.4. Adult (3-5 months old) TG mice

The myocardium of WT mice was of normal histological appearance (Fig. 15A), whereas TG hearts showed large accumulations of infiltrating cells in the ventricular interstitium, surrounding the vessel walls of both, arteries and veins, and in the subepicardial space (Fig. 15B,C). Fig. 15B and C show ventricular sections of a 3-months and a 9-months old mouse, respectively. This picture clearly shows that the interstitial accumulations increased in number and size with the age of the animals. The interstitial cells were identified as monocytes/macrophages, lymphocytes, granulocytes, and fibroblasts (Fig. 15D). The atrial myocardium of TG mice showed also interstitial cell accumulations but the cells observed were mainly granulocytes (Fig. 15E).

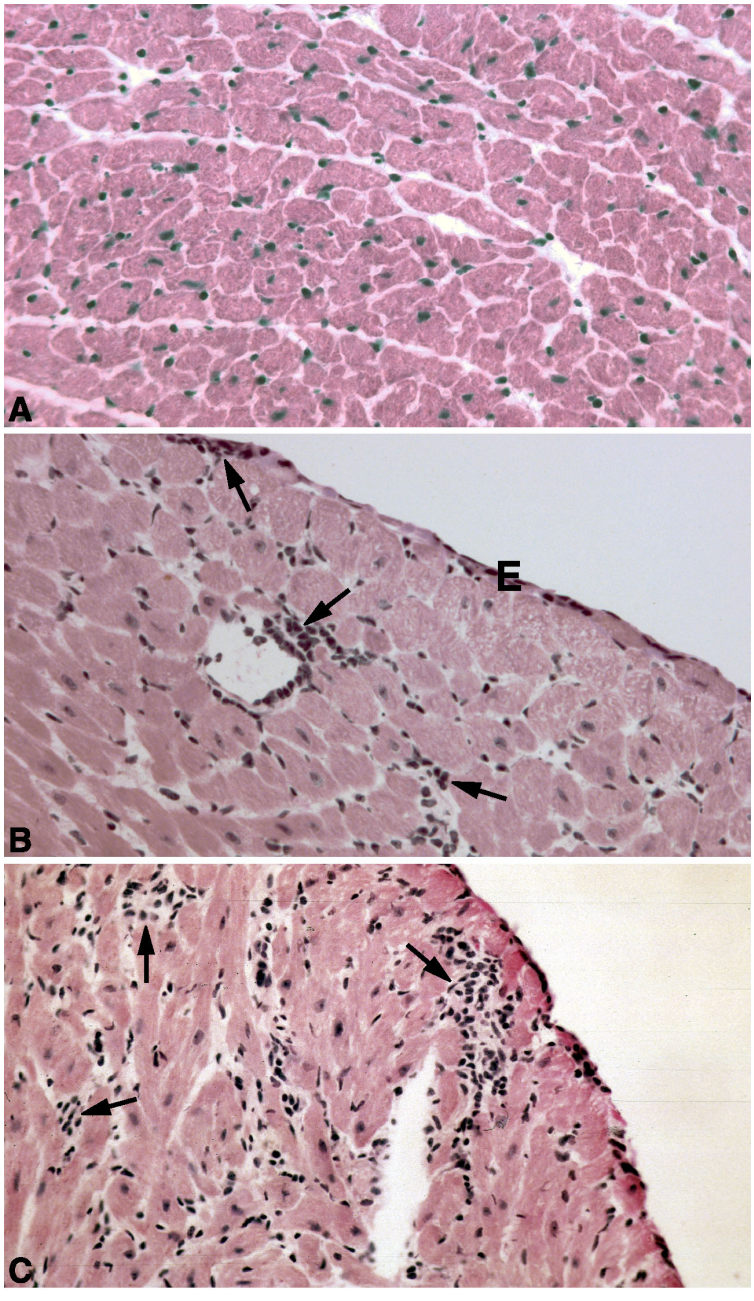

Fig. 15. *H&E staining. (A) Ventricular myocardium of a WT heart. x250. (B) Ventricular myocardium of a 3 months old TG mouse. Arrows:* foci *of interstitial cells. x250. (C) Ventricular myocardium of a 9 months old TG mouse. Arrows:* foci *of interstitial cells. x250.*

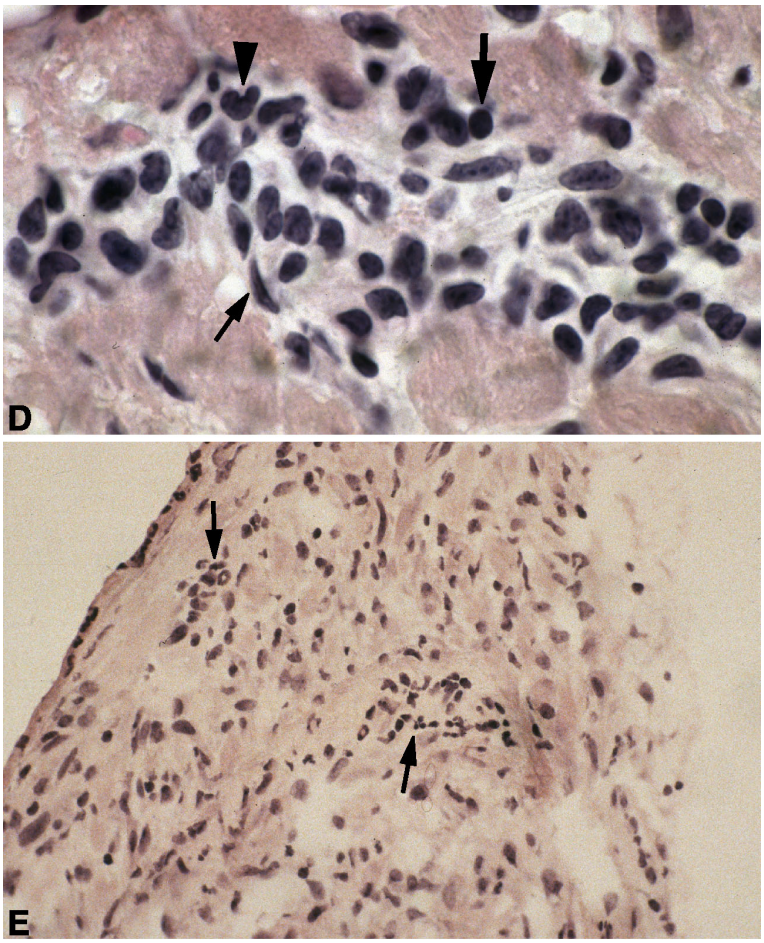

Fig. 15. *H&E staining. (D) High magnification of an interstitial cell accumulation. Big arrow: lymphocyte. Arrowhead: monocyte / macrophage. Small arrow: fibroblast. x630. (E) Atrial myocardium of a TG heart. Arrows:* foci *of granulocytes. x250.*

Electron microscopic analysis confirms the presence of monocytes/macrophages, lymphocytes, and fibroblasts in the myocardial interstitium of TG mice(Fig. 16A). In addition, electron microscopic analysis showed also the present of activated monocytes/macrophages as well as activated lymphocytes (Fig. 16B).

CD 71

In order to investigate the state of activation of the infiltrating cells present in the interstitial accumulations we used an antibody against the transferrin receptor (CD71), a known marker of activated lymphocytes (Knapp et al. 1989; Kishimoto et al. 1997). We found that TG animals showed a high proportion of activated lymphocytes (CD71-positive cells) in both the left and right ventricles, surrounding the vessel walls and in the subepicardial space (Fig. 17A). In WT mice, isolated positive interstitial cells were only sporadically detected (Fig. 17B).

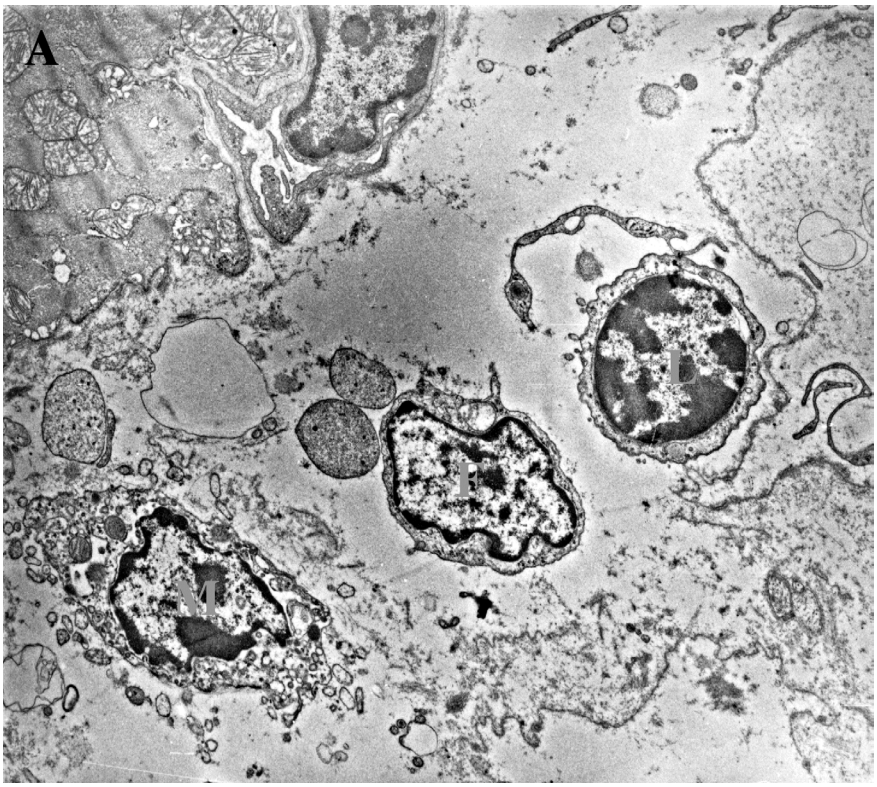

Fig. 16. *Electron microscopy. (A) TG heart. Myocardial interstitium. L: lymphocyte. M: monocyte / macrophage. F: fibroblast. x4500.*

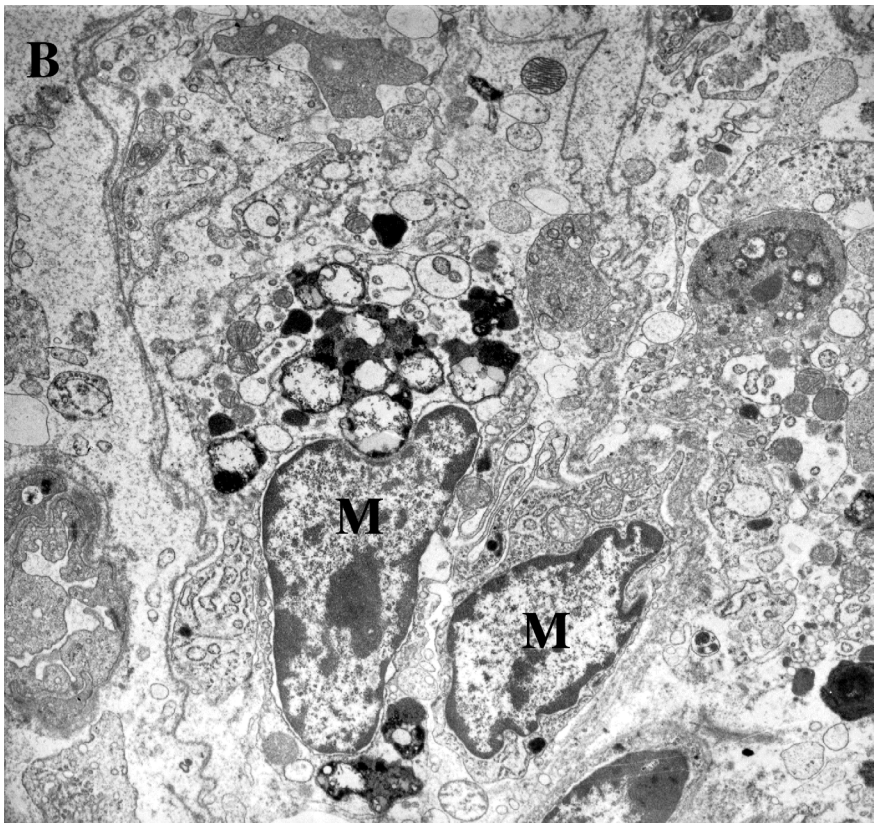

Fig. 16. *Electron microscopy. (B) TG heart. Myocardial interstitium. M: activated monocyte / macrophage. x7000.*

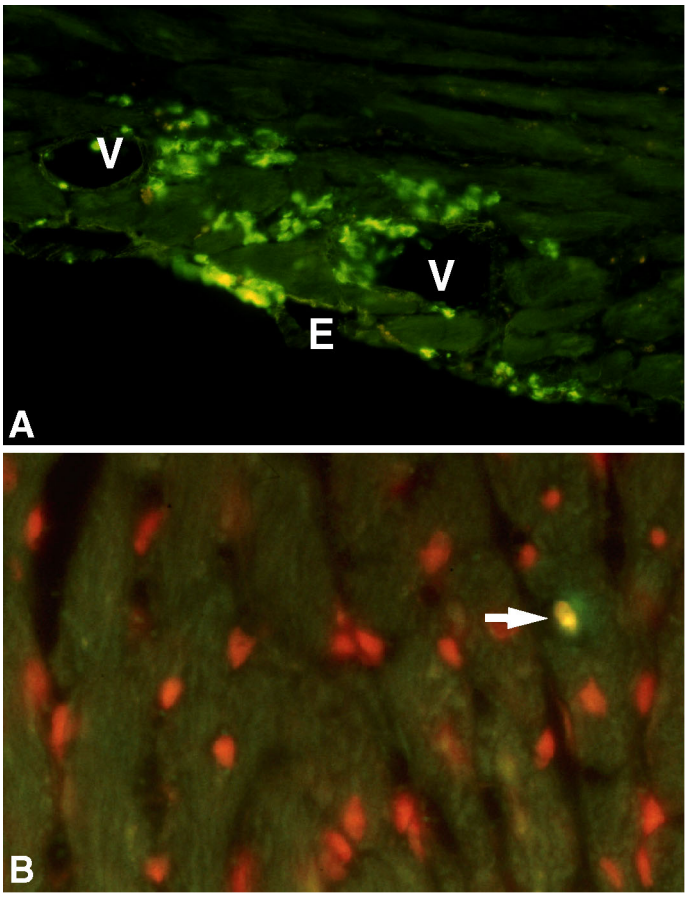

Fig. 17. *Immunofluorescence for CD 71 (green). (A) TG heart. The interstitial accumulations are composed by many CD 71-positive cells. E: epicardium. V: coronary vein. x400. (B) WT heart. Positive cells (arrow) are not frequent in WT mice. Nuclei are stained red with propidium iodide. x630.*

TNF-α

In order to check which kind of cells are expressing TNF-α in the heart of TG mice, we performed immunohistochemistry using an antibody against TNF-α. In TG hearts TNF–α immunostaining was detected in cells present in the myocardial interstitium and the subepicardial space (Fig. 18A). TNF-α immunostaining was also found weakly in the media of coronary vessels (not shown). TNF-α-positive interstitial cells were identified as monocytes/macrophages, lymphocytes and fibroblasts (Fig. 18B). In WT hearts, weak TNF-α immunostaining was also found in the media of coronary vessels, and in the myocardial interstitium (Fig. 18C).

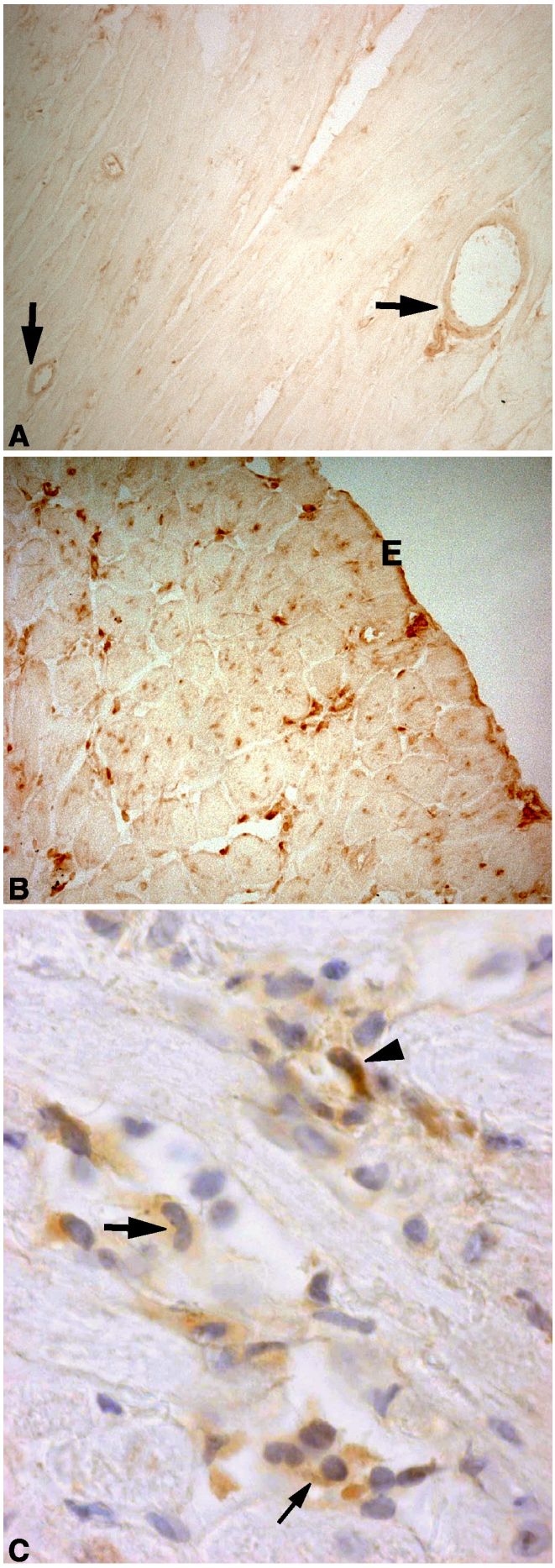

Fig. 18. *Immonuhistochemistry for TNF-α. (A) WT heart. TNF-α staining was found mainly in the media of coronary vessels (arrows) and weakly in the myocardial interstitium. x250. (B) TG heart. TNF-α staining is located in the myocardial interstitium and in the supepicardial space. E: epicardium. x250. (C) TG heart. High magnification of leukocyte accumulations showing TNF-α-positive interstitial cells. The nuclei were counterstained with hematoxilin. Big arrow: monocyte/macrophage, arrowhead: fibroblast, small arrow: lymphocyte. x630.*

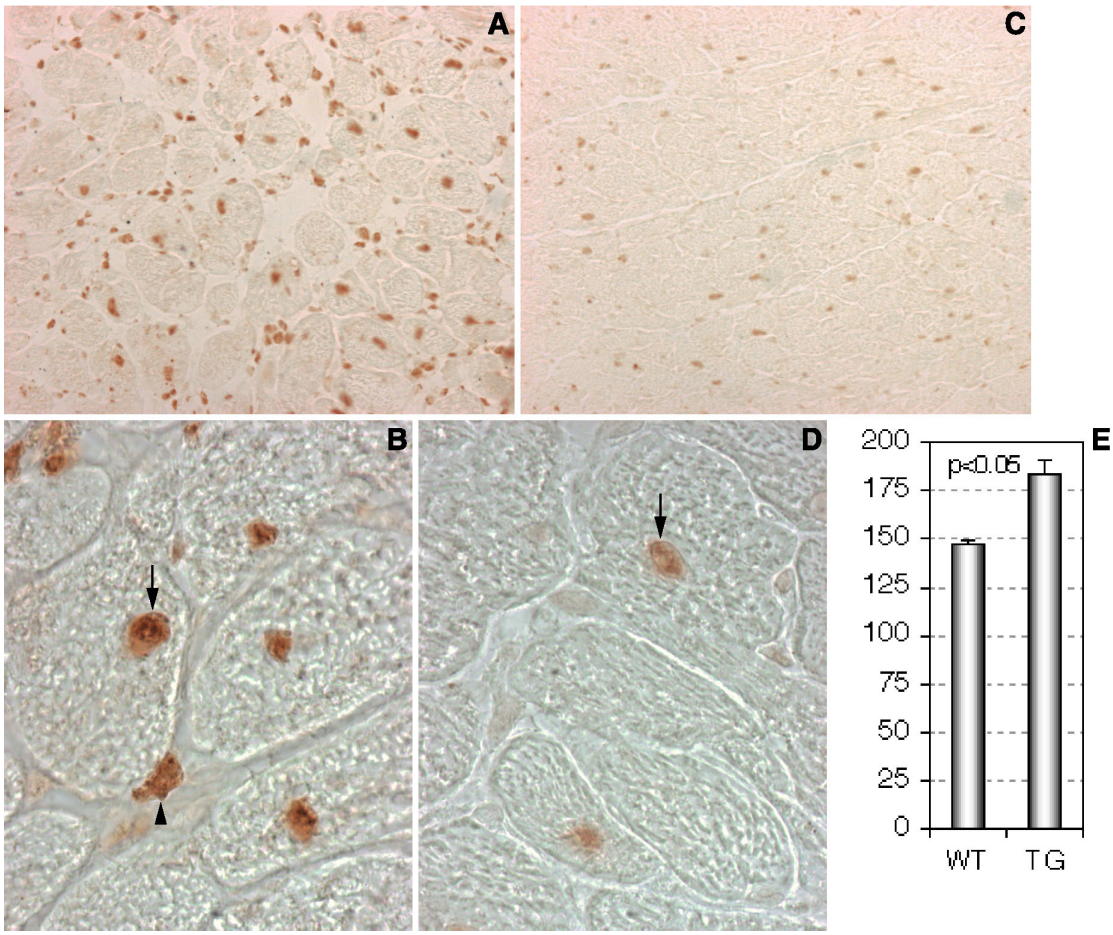

Fig. 19. *Immunohistochemistry for phosphorylated-SAPK/JNK1/2. (A) TG heart. Distribution of phosphorylated-SAPK/JNK1/2 in the left ventricle. x250. (B) TG heart. Phosphorylated-SAPK/JNK1/2 staining was found in the nuclei of myocytes (arrow) and leukocytes (arrowhead). x1000. (C) WT heart. Distribution of phosphorylated-SAPK/JNK1/2 in the left ventricular myocardium. x250 (D) WT heart. Phosphorylated-SAPK/JNK1/2 staining was detected in the nuclei of myocytes (arrow). x1000. (E) Quantification of the phosphorylated-SAPK/JNK1/2 intensity level in WT and TG mice. The sections used in this figure were stained and photographed using the same conditions. The difference in the optical density between TG and WT cardiomyocyte nuclei is evident.*

Phosphorylated-SAPK/JNKs

We performed immunohistochemistry using an anti-phosphorylated-SAPK/JNK1/2 antibody in order to identify the type of cells that express phosphorylated-SAPK/JNK1/2 in the heart of TG mice. We found phosphorylated (activated) SAPK/JNK1/2-positive cardiomyocytes as well as SAPK/JNK1/2-positive infiltrating cells in the hearts of TG mice (Fig. 19A). The phosphorylated-SAPK/JNK1/2 immunostaining was located around the nuclei as well in the cytoplasm of MCP-1 cardiomyocytes (Fig. 19A). The WT hearts showed also phosphorylated-SAPK/JNK1/2-positive cardiomyocytes (Fig. 19C). The phosphorylated-SAPK/JNK1/2 immunostaining was found in a perinuclear position but

not in the cytoplasm (Fig. 19C,D). To exclude the possibility that phosphorylated-SAPK/JNK1/2 positive leukocytes, detected in TG hearts, could be responsible for the increased levels of phosphorylated-SAPK/JNK1/2 found in TG animals by Western blot, we performed intensity quantification of phosphorylated-SAPK/JNK1/2-positive cardiomyocytes in TG and WT mice. The analysis revealed a significant increase in the optical density of the staining in TG cardiomyocytes as compared to WT (Fig. 19B,D,E).

3.3.5. Old (6-10 months old) TG mice

3.3.5.1. Cardiomyocyte injury

The hearts of adult and old TG mice showed damaged cardiomyocytes scattered throughout the myocardium increasing in number with increasing age of the animals (Fig. 20A). The damaged myocytes were surrounded by leukocytes as showed in Fig. 20A. Using electron microscopic analysis it was possible to demonstrate that the damaged cardiomyocytes containing damaged mitochondria appeared to be necrotic (Fig. 20B). In addition, the ultrastructural analysis showed that necrotic myocytes in the heart of TG mice were usually accompanied by leukocytes (Fig. 20B).

3.3.5.2. Cell death

In order to study the occurrence of different types of injury leading to cell death in MCP-1-overexpressing-cardiomyocytes, we investigated cardiomyocyte damage by ischemic injury using an antibody against complement 9, by autophagy using an antibody against ubiquitin, and antigen presentation using an antibody against MHC II.

Ubiquitin

In WT hearts ubiquitin-positive cardiomyocytes were only sporadically detected. Ubiquitin depositions were found in the nuclei of WT cardiomyocytes (Fig. 21A). The heart of TG mice showed accumulation of ubiquitin in the cytoplasm and in the nuclei in a high proportion of myocytes. In fact, 64‰ of the total number of myocytes were found ubiquitin-positive (Fig. 21B,C). In MCP-1 cardiomyocytes, ubiquitin exhibited different degrees of depositions (accumulations) including spots and very large and intense accumulations (Fig. 21B,C). For quantification we counted only perinuclear and cytoplasmic ubiquitin accumulations as shown in Fig. 21B,C excluding nuclear ubiquitin accumulations.

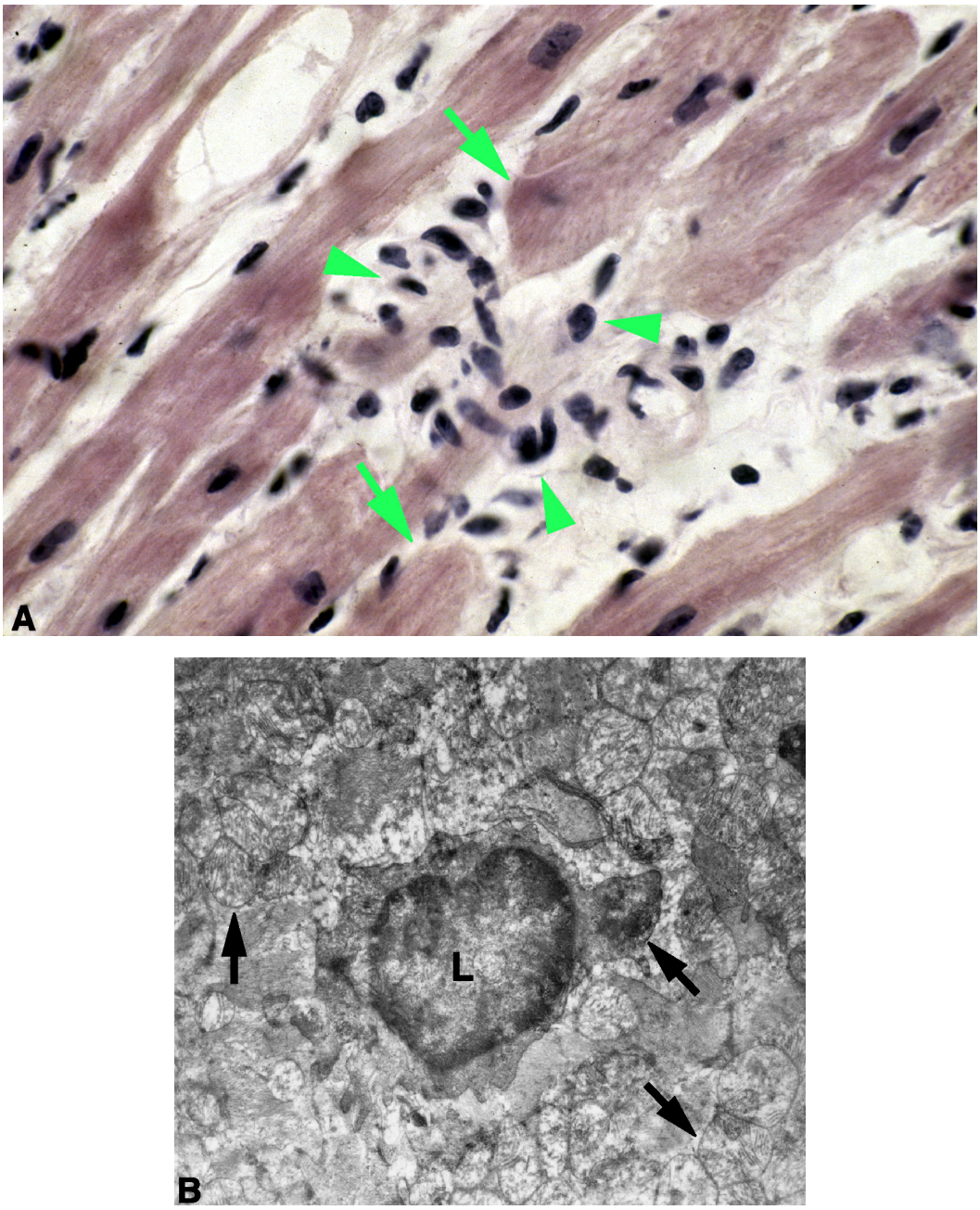

Fig. 20. *Nine months old TG mouse. (A) H&E staining. TG heart. Damaged cardiomyocytes (arrows) are surrounded by leukocytes (arrowheads). x630.*
(B) Electron microscopy. TG heart. A lymphocyte (L) appears located within a necrotic myocyte exhibiting damaged mitochondria (arrows). x1000.

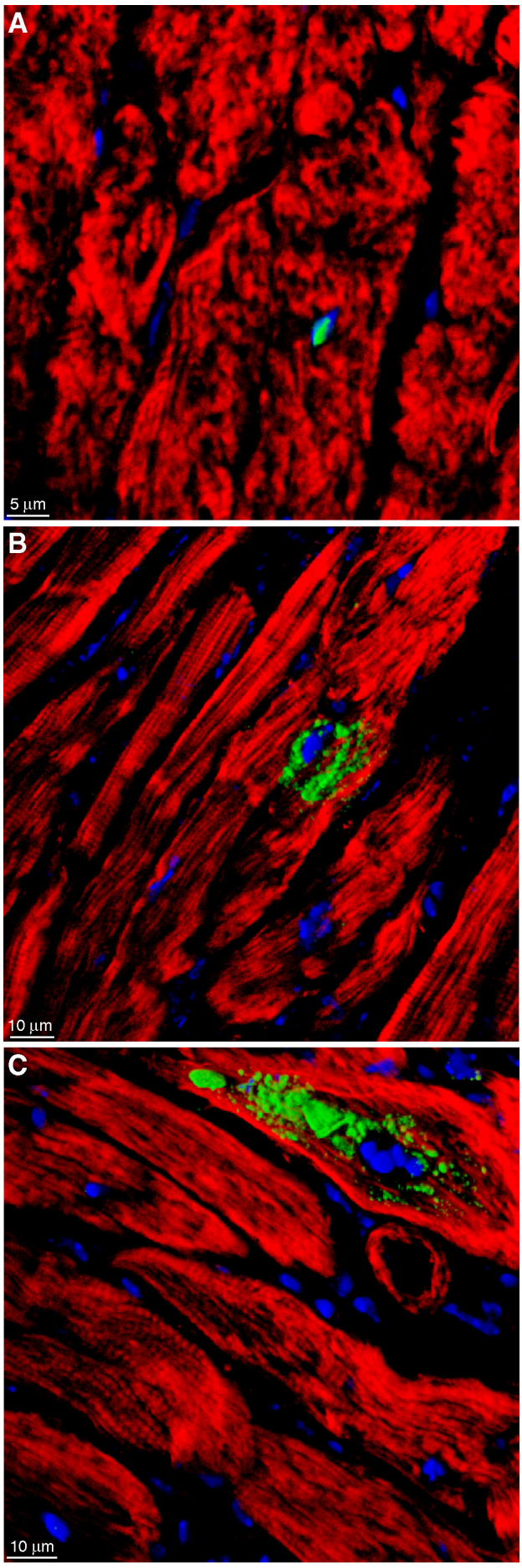

Fig. 21. *Immunofluorescence for ubiquitin antibody (green). Cardiomyocytes are stained in red with phalloidin. Nuclei are stained in blue with DAPI. (A) WT heart. Depositions of ubiquitin were accasionally detected only in the nuclei of myocytes. (B) and (C) TG hearts. Different degree of ubiquitin accumulations are present in TG cardiomyocytes.*

Complement 9

Using an antibody against complement 9, a known marker of acutely ischemic necrotic cells, it was possible to quantify the total amount of necrotic cardiomyocytes observed by conventional histology and ultrastructural analysis in TG animals (Fig.22A). We found that 3‰ of the total number of myocytes were complement 9-positive in TG hearts (Fig. 22A), while WT hearts showed no complement 9-positive-myocytes (Fig. 22B).

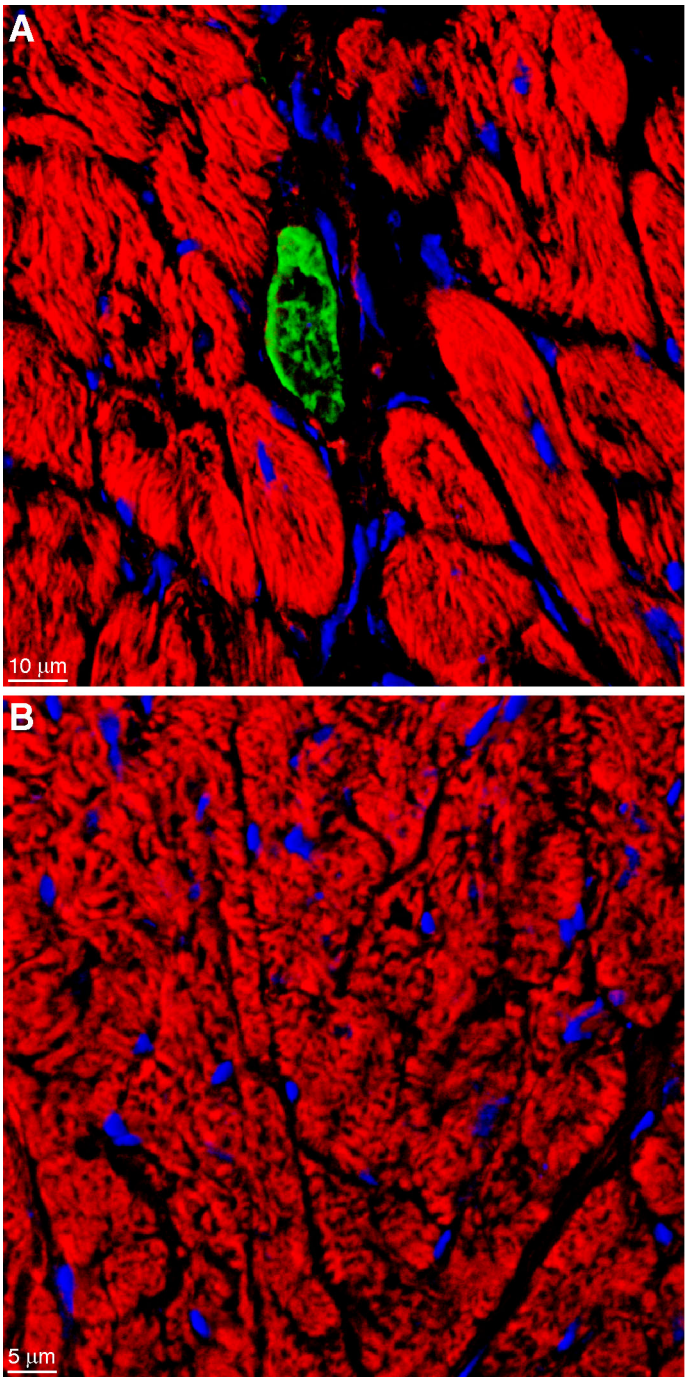

Fig. 22. *Immunfluorescence for complement 9 antibody (green). Cardiomyocytes are stained in red with phalloidin. Nuclei are stained in blue with DAPI. (A) TG heart. Complement 9-positive-cardiomyocyte (green). Note the lack of phalloidin staining in the complement 9-positive myocyte indicating irreversible necrosis. (B) WT heart. Complement 9-positive cells are not present.*

MHC II

Using an antibody against MHC II, no MHC II-positive- cardiomyocytes were found in the WT hearts (Fig. 23A) while TG cardiomyocyte were found positive for MHC II as shown in Fig. 22B. The MHC immunostaining was found in the myocyte cell membrane of 3‰ of total number of TG myocytes (Fig. 23B,C).

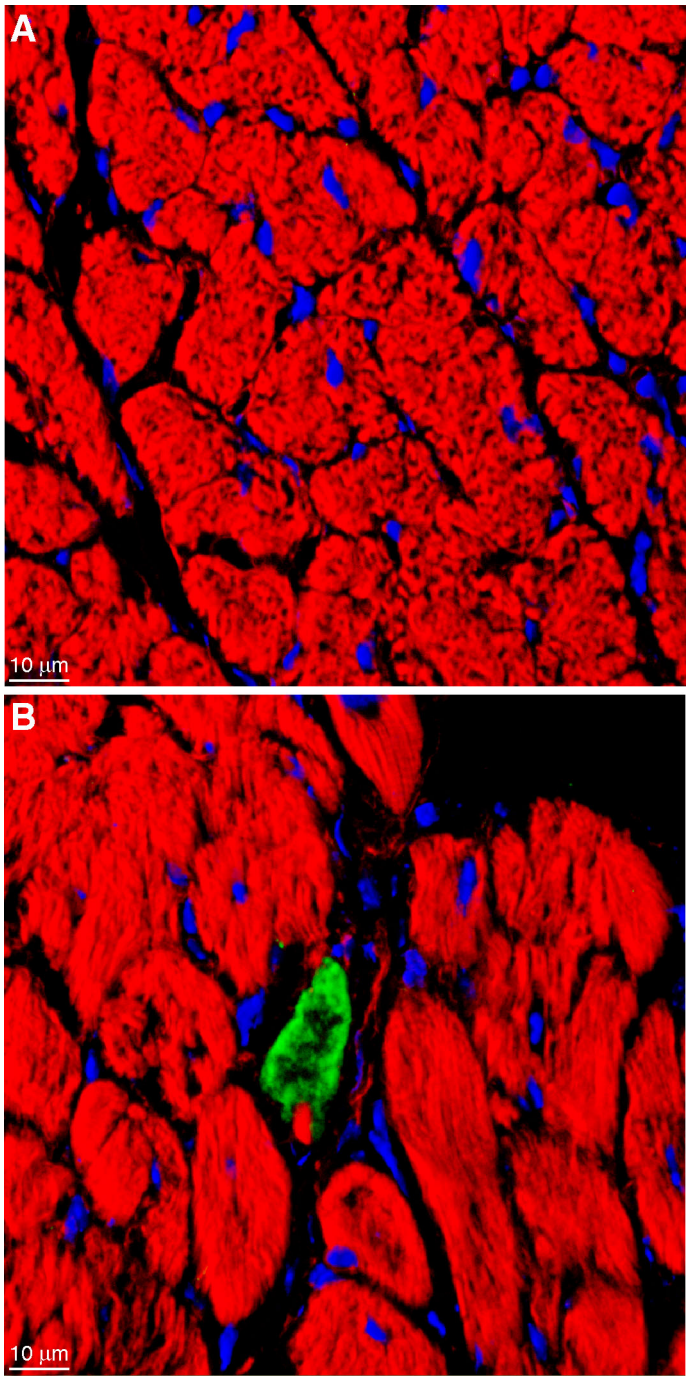

Fig. 23. *Immunofluorescence for MHC II antibody (green). Cardiomyocytes are stained in red with phalloidin. Nuclei are stained in blue with DAPI. (A) WT heart. MHC II-positive myocytes were not detected. (B) TG heart. MHC II-positive cardiomyocytes (green) were detected in TG ventricles. Note that this MHC II-positive cardiomyocyte is the same showed in Fig. 22, which is positive for complement 9.*

Apoptosis

The programmed cell death, apoptosis was checked in TG and WT animals in order to complete the study regarding MCP-1-overexpression-induced cell death. In addition, we wanted to investigate the possibility that activation of SAPK/JNK1/2 might cause apoptosis in the heart of TG mice. We used an apoptosis detection system. TUNEL-positive cardiomyocytes were detected neither in TG nor in WT animals (Fig. 24A,B). In the heart of TG mice, TUNEL-positive interstitial cells were found (Fig. 24B). In the duodenum, used as positive control, many TUNEL-positive cells were found (Fig. 24C).

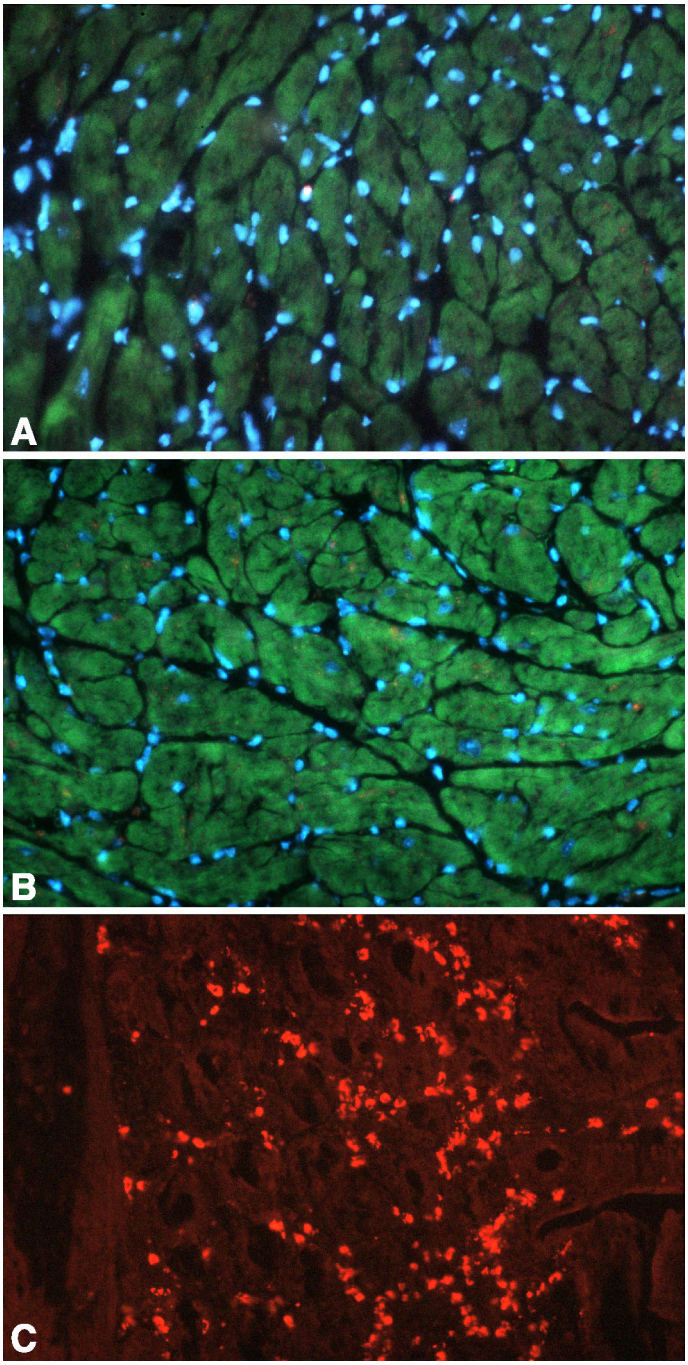

Fig. 24. *TUNEL staining (red). (A) TG heart. No TUNEL-positive myocytes were present. TUNEL-positive infiltrating cells (red) were occasionally detected. (B) WT heart. TUNEL-positive cardiomyocytes were not present. (C) Duodenum of pig used as positive control. Many TUNEL-positive cells (red) were present. Green: phalloidin. Blue: DAPI. Yellowspots:lipofuscin.*

4. Discussion

4.1. Cardioprotection

Our results indicate that MCP-1 overexpression exerts myocardial protection against short-term (45 min) vs longer term (3 days) of ischemia. In addition, these studies show that after short-term ischemia followed by 3 days of reperfusion the infarct size was still reduced in TG mice when compared with WT but it was increased when compared with 45-min of coronary artery occlusion demonstrating that some of the salvaged myocardium died during reperfusion but another part was rescued by reperfusion. This cardioprotection is not caused by angiogenic or arteriogenetic mechanisms because infarct size was similar in TG and WT mice after long-term (3 days) CAO. We can conclude that MCP-1 overexpression induces a permanent reduction in infarct size by a short-acting mechanism that mimics ischemic preconditioning.

The results presented here indicate an important role for the MAPK family in mediating the cardioprotective effect. Evidence for the activation of different MAKs subfamilies during ischemic preconditioning has been reported by different autors (Maulik, Watanabe et al. 1996; Weinbrenner, Liu et al. 1997; Barancik, Htun et al. 1999; Baines, Cohen et al. 1999; Nakano, Baines et al. 2000; Sato, Cordis et al. 2000; Saurin, Martin et al. 2000). Although many of these studied suggested MAPKs importance as signaling components in the protection of the myocardium achieved by ischemic preconditioning against ischemia (Maulik, Watanabe et al. 1996; Weinbrenner, Liu et al. 1997; Maulik, Yoshida et al. 1998; Yue, Ma et al. 1998; Barancik, Htun et al. 1999; Baines, Cohen et al. 1999; Nakano, Baines et al. 2000; Sato, Cordis et al. 2000; Strohm, Barancik et al. 2000), others supposed a deleterious effects of MAPKs activation during ischemic preconditioning (He, Li et al. 1999; Saurin, Martin et al. 2000). Moreover, in the last years, the available of specific inhibitors of MAPKs have permitted the study of the role of each MAPKs in ischemic preconditioning as well as the signaling transduction pathways downstream of each MAPK (Murray, Alessandro et al. 1998; Yue, Ma et al. 1998; Barancik, Htun et al. 1999; Strohm, Barancik et al. 2000; Sato, Cordis et al. 2000; Yue, Wang et al. 2000). However, the exact role of each member of the MAPKs superfamily is still controversial and under discussion. It has been demonstrated in different organs and animal models that the ERKs pathway is correlated with the induction of survival,

having a protection effect against apoptosis (and in general cell death) both *in vitro* and *in vivo* (Xia, Dickens et al. 1995; Gu, Jiang et al. 2000; Henaff, Hatem et al. 2000; Strohm, Barancik et al. 2000). *In vitro*, inhibition of the ERKs transduction pathway with specific inhibitors increased apoptosis in cultured cardiac myocytes in an ischemic-reoxygenation-induced apoptosis rat model (Yue, Wang et al. 2000), as well as exaggerated reperfusion injury in isolated perfused rat heart (Yue, Wang et al. 2000). *In vivo*, inhibition of the ERKs pathway results in an increased infarct size in a pig model of coronary artery occlusion (Strohm, Barancik et al. 2000). Although the activation of the ERKs pathway plays an important role in preventing stress-induced apoptosis in cardiac myocytes, in a cell culture model of seizure activity it has been reported that inhibition of the ERKs pathway protects hippocampal neurons suggesting a role of ERKs activation in the induction of cell death (Murray, Alessandro et al. 1998).

The activation of the p38 pathway is in general correlated with the induction of apoptosis and with stress responses (Yue, Wang et al. 2000; Bogoyevitch, Gillespie-Brown et al. 1996; Saurin, Martin et al. 2000; Kyriakis and Avruch 2001). Inhibition of the p38 cascade protects cultured myocytes against apoptosis in an ischemia/reoxygenation-induced apoptosis rat model (Yue, Wang et al. 2000) but also increases myocardial infarction in an isolated rat heart model of ischemic preconditioning followed by ischemia/reperfusion (Sato, Cordis et al. 2000), suggesting that activation of the p38 signal transduction pathway is an important (and maybe necessary) component in the ischemic preconditioning program (Nakano, Baines et al. 2000).

The activation of the SAPK/JNKs pathway was originally associated with the induction of cell death (He, Li et al. 1999; Lin, Weinberg et al. 2000; Yue, Wang et al. 2000). *In vitro*, activation of the SAPK/JNKs pathway occurs at the onset of apoptosis and inhibition of this pathway reduces hypoxia-induced apoptosis in cultured myocytes (He, Li et al. 1999; Lin, Weinberg et al. 2000; Baines, Cohen et al. 1999). *In vivo*, inhibition of SAPK/JNKs activation reduces reperfusion injury in perfused rabbit hearts (Yue, Ma et al. 1998) and protects hippocampal neurons against ischemia-induced apoptosis (Ozawa, Shioda et al. 1999). However, in marked contrast, recent studies have demonstrated that SAPK/JNKs activation exhibits a cytoprotective effect in nitric oxide-induced cardiac myocyte apoptosis (Andreka, Zang et al. 2001). Sato et al. (Sato, Cordis et al. 2000) reported that activation of the SAPK/JNKs pathway is obligatory for ischemic preconditioning in isolated rabbit hearts. In addition, stimulation of SAPK/JNK1/2 with okadaic acid and anisomycin reduced infarct size in a pig model of coronary artery occlusion (Barancik, Htun et al. 1999). Despite the confusion generated by the

controversial data regarding the role of MAPKs, it is now recognized that the functions of the MAPKs subfamilies can vary, and even oppose each other depending on the cell type, *stimuli*, and model system used. In addition, it is likely to suppose that in ischemic preconditioning and/or ischemia/reperfusion, the dynamic balance between MAPKs activity and the presence of specific signaling transduction-activating *co-stimuli* are crucial and critical in determining cardiomyocyte survival or death subsequent to injurious stimulation.

Our data show a permanent activation of the SAPK/JNK1/2 pathway in MCP-1 TG hearts. In addition, we reported that injection of the SAPK/JNKs inhibitor D-JNKI1 partially abrogates the cardioprotective effect of MCP-1 overexpression, leading to a significantly increased infarct size in treated TG mice *versus* untreated TG animals. In addition, the other two mayor MAPKs subfamilies, ERK1/2 and p38, are not activated in MCP-1 TG hearts, suggesting that, in our transgenic model, ERK1/2 and p38 signaling transduction pathways do not contribute to the cardioprotective effect observed in MCP-1 TG mice. We hypothesize that the permanent activation of SAPK/JNK1/2 in MCP-1 TG hearts could lead in TG cardiomyocytes to a preconditioned state that increases myocyte survival (with consequent infarct size reduction) after simulated ischemia. Our hypothesis is supported by data obtained with the use of the SAPK/JNKs inhibitor D-JNKI1. We can conclude that a permanent activation of the SAPK/JNKs signal tranduction pathway in MCP-1 TG mice is in part involved in the development of cardiac resistance against ischemia.

Very recently, Smith et al. (Smith, Lecour et al. 2002) have proposed innate immunity as an integral component of the “innate adaptive cardiac program” (ischemic preconditioning) that leads to cardiac protection after ischemic injury (Smith, Lecour et al. 2002). This cytoprotective program appears to be mediated by pro-inflammatory cytokines, in which TNF-α, an apical cytokine in the immune system, has been proposed to play an important and beneficial role. TNF-α is produced during viral myocarditis (Matsumori, Yamada et al. 1994) and ischemia/reperfusion (Gurevitch, Frolkis et al. 1996; Frangogiannis, Lindsey et al. 1998; Kupatt, Habazettl et al. 1999), both charaterized by inflammatory processes development. The production of TNF-αwas generally considered deleterious to the cardiovascular system. Administration of TNF-α aggravates myocarditis in a mouse model of myocarditis induced by encephalomyocarditis virus (Yamada, Matsumori et al. 1993). Neutralization of TNF-α with specific

antibodies has been reported to ameliorate viral and autoimmune myocarditis (Yamada, Matsumori et al. 1993; Smith and Allen 1992), and to improve myocardial recovery after ischemia/reperfusion (Gurevitch, Frolkis et al. 1997). Systemic administration of this cytokine results in myocardial depression and cardiomyopathy (Bozkurt, Kribbs et al. 1998; Hegewish, Weh et al. 1990; Kubota, McTiernan et al. 1997). In marked contrast, it is now being recognized that TNF-α production is not merely linked to detrimental effects but that it can have a protective action after both, viral myocarditis and myocardial infarction (Kurrelmeyer, Michael et al. 2000; Wada, Saito et al. 2001; Lecour, Minners et al. 1999; Smith, Suleman et al. 2002; Sack, Smith et al. 2000; Mann 2001). In fact, Wada et al. (Wada, Saito et al. 2001) demonstrated using mice lacking TNF-α that this cytokine plays a protective role during acute myocarditis because is necessary for adhesion molecule expression and leukocyte infiltration. A protective effect of TNF-α against apoptosis after coronary occlusion has been shown in mice lacking both TNFR1 and TNFR2 (Kurrelmeyer, Michael et al. 2000). Moreover, recently Smith RM et al. (Smith, Suleman et al. 2002) demonstrated that cardiac production of TNF-α is required for ischemic-preconditioning-induced cardioprotection.

TNF-α can activate the SAPK/JNKs signaling transduction pathway (Darnay and Aggarwal 1997), and the binding of TNF-α with its receptors can lead to an apoptotic or a survival signaling cascade, both through SAPK/JNKs activation (Darnay and Aggarwal 1997). In fact, new evidence demonstrated that TNFRs might be coupled to activation of different downstream transduction pathways, depending on the association of the receptors with adapter proteins. This is due to the ability of the TNFRs to associate with specific intracellular proteins: TRAFs and FADD/MORTs. These proteins can induce selective activation of SAPK/JNKs leading to the survival or death signaling cascade through MEKK1 or Caspase-8 activation, respectively (Darnay and Aggarwal 1997; see also Fig. 3). Roulston et al. (Roulston, Reinhard et al. 1998) showed that after the binding of TNF-αwith the receptors there is an early and transient activation of SAPK/JNKs, NF-kB, and p38 mediated through TRAF2, which is directly bound to TNFR2, and via TRADD to TNFR1. These authors propose that these signaling cascades may coordinate protective signals, preventing apoptosis and cell injury. If one of the protective signals is inhibited or specific pro-apoptotic *stimuli* are present, the apoptotic pathway will proceed, via FADD that is bound to TRADD, leading to the activation of a cascade of Caspases, which then result in late phase stress kinase activation and apoptosis (see Fig. 3). In MCP-1 TG hearts we found incresead level of TNF-α. Immunohistochemical analysis

revealed that leukocytes and fibroblasts accumulating in the myocardial interstitium of TG hearts are the source of TNF-α. We suggest that activated leukocytes secreting TNF-α serve as *stimulus* for the activation of the SAPK/JNK1/2 signaling transduction pathway in TG cardiomyocytes. In our model, the Caspase-8-independent pathway seems to be responsible for SAPK/JNKs activation leading to cell survival and not to apotosis. This is also confirmed by the fact that in TG hearts neither apoptotic TUNEL-positive cardiomyocytes nor activation of the pro-apoptotic factor p38 could be observed. It is likely that in our TG model the production of TNF-α plays a beneficial role in the induction of cardiac protection against short-term ischemia, possibly activating a signaling cascade down-stream of TNFR leading to cytoprotective and not to cell death programs. Our data may be further evidence for the beneficial link proposed by Smith et al. (Smith, Lecour et al. 2002) between the immune system and ischemic preconditioning. Moreover, we propose that the link existing between innate immunity and ischemic preconditioning could act trough SAPK/JNKs activation.

4.2. Cardiomyopathy

The MCP-1 TG mice have a very short life span (~10 months against ~18 months in WT mice) due to the development of inflammatory cardiomyopathy and pulmonary edema, which start approximately at 3-4 months after birth and increase until the death of the animal (Kolattukudy, Quach et al. 1998; Martire, Fernandez et al. 2001; Moldovan, Goldschmidt-Clermont et al. 2001). Kollatukudy et al. (Kolattukudy, Quach et al. 1998) described the development of cardiomyopathy in MCP-1 TG mice from neonatal to 2 months old. In TG hearts, MCP-1 overexpression starts before birth and increases until ~2 months of age where it remains constant until the death of the animal. At this early age the mice show infiltration of only monocytes/macrophages in the myocardial interstitium. They also show that the monocytes/macrophages were not activated.

Although it is established that *in vitro* MCP-1 is a chemoattractant for monocyte/macrophages and lymphocytes (Gunn, Nelken et al. 1997; Sekine, Yasufuku et al. 2000), the development of a variety of TG and deficient mice models indicate that MCP-1 has similar properties *in vivo*. In fact, TG mice overexpressing MCP-1 in a variety of organs showed a moderate number of monocytes/macrophages (and in the same animal model also lymphocytes) infiltrations and accumulations at the sites of MCP-1 overexpression (Gunn,

Nelken et al. 1997; Fuentes, Durham et al. 1995; Kolattukudy, Quach et al. 1998). Interestingly, other TG mice that also overexpress MCP-1 in a variety of organs showed any monocytes/macrophages infiltration at the sites of overexpression (Roulston, Reinhard et al. 1998).

In vitro, MCP-1 has been shown to activate monocytes as well as lymphocytes (Jiang, Beller et al. 1992; Lukacs, Chensue et al. 1997; Gavrilin, Deucher et al. 2000) but it is unclear whether MCP-1 *in vivo* may activate these cells (Jiang, Beller et al. 1992; Lukacs, Chensue et al. 1997; Gavrilin, Deucher et al. 2000). In fact, in all the transgenic model studies, MCP-1 alone does not induce activation of attracted cells but in some cases it can enhance the inflammatory response when treated with other pro-inflammatory *stimuli* (Gunn, Nelken et al. 1997). In general, the relatively benign appearance of monocytes/macrophages attracted *in vivo* in response to transgenically production of MCP-1 demonstrates that the degree (and organs) of overexpression maybe somewhat limited. The same applies to leukocyte activation. There is a variety of monocyte activation states, and maybe in these TG mice models MCP-1 does not activate monocytes to a degree sufficient to stimulate them to differentiate into macrophages and to express adhesion molecules and cytokines but enough to induce in same cases a moderate inflammatory process (Kolattukudy, Quach et al. 1998), as well as to enhance inflammatory responses when stimulated with other pro-inflammatory *stimuli* (Gunn, Nelken et al. 1997).

The MCP-1 TG mice described in the present study are young (1 day, 1 month, 2 months), adults (3-5 months old), and old (6-10 months old). Our results confirm the finding of Kollatukudy et al., which showed moderate infiltration of monocytes/macrophages leading to moderate autoimmune myocarditis in the heart of young TG mice. In addition, the present results indicate that, in adult TG mice, lymphocytes, together with monocytes/macrophages and fibroblasts, constitute the principal cell types in the ventricular interstitial cell accumulations. Moreover, we showed that an important fraction of the leukocytes were also activated in MCP-1 TG hearts. The present results provide *in vivo* evidence for the capacity of MCP-1 not only for attracting monocytes/macrophages and lymphcytes but also for its capacity to induce their activation. However, we cannot exclude that the moderate autoimmune myocarditis detected in MCP-1 TG mice may additionally induce attraction and activation of lymphocytes. In this case, chemotaxis and activation of lymphocytes would not be a direct effect of MCP-1 overexpression, but secondary to cardiomyocyte degeneration (see below).

Moldovan et al. (Moldovan, Goldschmidt-Clermont et al. 2001) showed necrotic cardiomyocytes in old MCP-1 TG mice. These authors described the presence of necrotic myocytes as a consequence of the occlusion of small arteries and arterioles found in TG hearts, which generates diffuse ischemic regions (Moldovan, Goldschmidt-Clermont et al. 2001). Occlusion of small arteries and arterioles should induce *foci* of necrotic myocytes at the site of coronary blood flow arrest. We found also necrotic cardiomyocytes in old MCP-1 TG mice (6-9 months old), but they were isolated. We suggest that the presence of necrotic cardiomyocytes is likely due to the general autoimmune inflammation described in TG hearts and not to ischemic injury. In fact, although we have not performed statistical analysis for colocalization of complement 9 and MHC II antibodies, we found complement 9- and MHC II-positive cardiomyocytes scattered through the myocardium in TG hearts (compare Fig. 22 with Fig. 23), suggesting that complement cascade activation is induded at least in part by the MHC II presentation in the cell membrane of cardiomyocytes (see belove). However, we cannot exclude that transgenic manipulation and prolonged protein overexpression could also induce cardiomyocyte degeneration (see below).

The ubiquitin system of intracellular proteolysis has been shown to be essential for cell viability (Weissman 2001). In fact, embryonic development and cell death, which are characterized by large intracellular reorganization and degradation of internal organelles and proteins, have been shown to activate the ubiquitin system, which leads to protein-ubiquitination of target proteins (Mayer, Arnold et al. 1991; Doherty, Laszio et al. 1989; Lowe and Mayer 1990). It has been described that in different pathological situations such as neurodegenerative, viral or other chronic degenerative diseases, ubiquitin is abnormally accumulated in nuclei or cytoplasm and functions are altered (Mayer, Arnold et al. 1991; Layfield, Alban et al. 2001). It has been proposed that ubiquitin deposits in degenerative and viral diseases are cellular responses to stress, damage and injury (Layfield, Alban et al. 2001). In neurodegenerative diseases, characterized by an abnormal production of damaged neuronal proteins or organelles, accumulation of ubiquitin has been proposed to have two opposite functions. Protein ubiquitination might cause neuron death as part of an irreversible pathological process or cytoprotection trying to rescue neurons as a part of reversible pathological processes (Mayer, Arnold et al. 1991). The other possibility proposed by the same authors is that protein ubiquitination starts as a cytoprotective process. When the process cannot combat neuronal degeneration or the degree of injury is too much, protein ubiquitination is involved in

destroying neuron proteins in the final stage of cell death (Mayer, Arnold et al. 1991). In fact, there are evidences suggesting that in neurodegeneration the cytoprotective role of protein ubiquitination in neurons probably occurs only at preterminal stages of diseases progression (Mayer, Arnold et al. 1991; Lowe and Mayer 1990).

In our model, the altered accumulations of ubiquitin found in the cytoplasm of TG cardiomyocytes could have also two opposite functions: cytoprotection or induction of cell death. In fact, cellular stress induced by transgenic production of MCP-1 by myocytes and its abnormal intracellular accumulation in cells could induce protein-ubiquitination for initiating the degradative process of uncontrolled MCP-1 protein accumulation. The degradative process could take place for both, rescue the myocytes from high and toxic levels of MCP-1 and when its production raises letal levels, for destroying irreversible damaged myocytes in the final stage of cell death. We propose that the degree of MCP-1 accumulation is important for establishing if the cell will be rescued or not. Our hypothesis is supported by the fact that immunofluorescence using an antibody against MCP-1 revealed that in MCP-1 TG hearts the degree of MCP-1 accumulated in the cells varies from myocyte to myocyte in the same animal (data not shown). MCP-1 accumulated in the myocytes could induce MCP-1-ubiquitination in order to rescue the myocytes from an abnormal and possibly toxic protein deposition in the cytoplasm. In our model could also occurs that when the cytoprotective program could not take place due to the large amount of MCP-1 accumulated in the cell, the MCP-1-ubiquitination could also induce the destruction and the death of TG cardiomyocytes. Immunohistochemical analysis performing colocalization of MCP-1 and ubiquitin antibodies could confirm our hypothesis.

Activation of the complement system has been shown to play an important role in the induction of humoral immune responses, in the elimination of immune complexes, in the protection against bacterial and viral infections, and in general in autoimmune and inflammatory responses. Two mechanisms are known to be capable of initiating activation of the classic complement pathway: an antibody-dependent mechanism in which specific immunoglobulins bind antigen and that complex leads to complement activation (Porter and Reid 1979), and an antibody-independent mechanism in which cytosolic particles leaking from necrotic cells react with different members of the complement system without requiring antibody-antigen complex formation, which then activates the complement system (Pinckard, Olson et al. 1975; Linder, Lehto et al. 1979). The antibody-

dependent mechanism is the best characterized. It has been demonstrated that in injuries, in which a rapid activation of the complement system occurs via antibody-dependent mechanisms, novel antigenic determinants are presented to pre-existing immunoglobulins, because the activation occurs too rapidly to permit the production of new immunoglobulins for an unknown antigen (Kelley, Olson et al. 1974).

The antibody-independent mechanism of complement system activation is still poorly understood (Giclas, Pinckard et al. 1979). However, complement 9 mRNA has been shown to be upregulated under stress characterized by an inflammatory reaction (Yasojima, Kilgore et al. 1998) and in general in models of muscle injury, including myocardial ischemia and ischemia/reperfusion injury (Giclas, Pinckard et al. 1979). In fact, in ischemia/reperfusion injury, mitochondrial proteins leaking from the injured cardiac cells could directly or indirectly cause the activation of complement without requiring antibody binding. In the present study, we showed necrotic complement 9-positive cardiomyocytes by immunofluorescence as well irreversible damaged cardiomyocytes by conventional microscopy. We suggest that the general autoimmune inflammation, detected in adult and old MCP-1 TG mice, induces cardiomyocyte damage and death. The loss of membrane integrity that occurs in irreversible damage could expose cytoplasmic molecules to the circulating members of the complement system, which in turn could induce the complement cascade activation. We suggest that the complement system attacks necrotic TG cardiomyocyte membranes with consequent activation of the complement cascade that leads to the removal of the irreversible damaged (necrotic) cardiomycytes.

The exposure of MHC II antigens in the cell surface induces tissue-specific immune destruction by lymphocytes (Klein 1990). Cells that expose MHC II molecules in the cell membrane are target cells for cytotoxic T lymphocytes-mediated lysis (Klein 1990). An important characteristic of the immune and autoimmune diseases is their association with MHC II antigens-presenting cells. It has been shown that cardiac myocytes do not express MHC II antigen on their surface under normal conditions (Klein 1990) but under pathological conditions, characterized by activation of the immune system, myocytes express MHC II antigen and they are susceptible to cytotoxic effects of the immune system (Caforio, Stewart et al. 1990; Sell, Tadros et al. 1988). In immune diseases cytotoxic activation of lymphocytes can be induced by viral infection or rejection after heart transplantation (Sell, Tadros et al. 1988), while autoimmune diseases

(chronic myocardial damage in the absence of competent infections) are characterized by the action of autoreactive lymphocytes (Caforio, Stewart et al. 1990). In both cases, activated cytotoxic lymphocytes induce MHC II expression in the cell membrane of target cells. In MCP-1 TG mice, competent infections capable of inducing cytotoxic activation of lymphocytes were detected neither in previously studies (Kolattukudy, Quach et al. 1998; Moldovan, Goldschmidt-Clermont et al. 2001) nor in the present study. The presence of MHC II antigens on the cell surface of MCP-1 cardiomyocytes may indicate an autoimmune inflammatory response of myocytes induced by autoreactive and cytotoxic activated lymphocytes already described in TG hearts. As mentioned above, autoimmune diseases are also characterized by complement system activation (Giclas, Pinckard et al. 1979). In TG hearts, we found colocalization of complement 9 and MHC II antibodies in cardiomyocytes. It is likely to suppose that the exposure of MHC II in the cell membrane of TG cardiomyocytes could induce the complement cascade activation for eliminating damage myocytes. This could be an evidence for a direct association between MHC II antigen presentation and complement activation in autoimmune inflammatory diseases. Further analysis performing quantification of complement 9 and MHC II colocalization could demonstrate if there is a significant association between them. Another possibility exists to explain the presence of MHC II in the cell membrane of TG cardiomyocytes. In fact, the expression of MHC II in cells has been shown to be regulated by elements in the upstream (promoter) region of each gene and in the first intron (Klein 1990; Johnson and Pober 1991; Jacob, Lewis et al. 1991). It has been shown that the genes for both human and mouse TNF-α and TNF-β are closely linked and located within the MHCs (Spies T et al. 1986), and they can regulate the MHCs expression (Spies, Morton et al. 1986). TNF-α increases the cell surface expression of MHC I (Collins, Lapierre et al. 1986). MHC II regulation by TNF-α is controversial, probably because of the different cell types in which it has been studies but in general an increase in the expression of MHC II antigens in the cell surface by TNF-α has been shown to occur with additional inflammatory stimuli (Johnson and Pober 1991). We cannot exclude that in our model the increased levels of TNF-α (and possibly others pro-inflammatory cytokines) found in the heart of TG mice could induce activation of SAPK/JNK1/2 pathway as well as induction of MHC II antigen presentation in TG cardiomyocytes. On the other hand, MHC II preferentially bind peptides from exogenous proteins (Klein 1990). For this reason it is likely to suppose that the overexpression of MCP-1 by myocytes can raise high and toxic levels to induce myocytes to express MHC II antigens (together with exogenous MCP-1 antigen) in their surface for eliminating damaged cardiomyocytes.

In summary, neonatal MCP-1 TG mice (1 day old) show moderate infiltration of monocytes/macrophages in the ventricular myocardial interstitium but neither accumulations nor activation of any type of leukocytes could be detected at this age. One month old MCP-1 TG mice exhibit increased number of monocytes/macrophages when compared with 1 day old animals. Small accumulations composed by monocytes/macrophages, lymphocytes, and fibroblasts were present in the myocardial interstitium. Two months old TG mice show monocyte/macrophage, lymphocyte, and fibroblast accumulations that are increased in size and number as compared with 1 month old mice.

In adult animals (3-6 months old), overexpression of MCP-1 by cardiomyocytes causes chronic infiltration and activation of leukocytes, resulting in permanent elevated TNF–αsecretion, which could induce activation of SAPK/JNK1/2 pathway. We also showed in adult TG mice that infarct size is permanently reduced after short-term coronary occlusion, suggesting a cardioprotective role of MCP-1 overexpression. The permanent activation of SAPK/JNKs pathway induced by TNF-αproduction in TG mice could be responsible for the preconditioning effect of MCP-1 overexpression, inducing activation of downstream protective (survival and not apoptotic) signaling transduction pathways in TG cardiomyocytes. The activation of survival pathways could induce resistance against infarction in TG mice, mimicking ischemic preconditioning. The data presented using adult TG mice strongly support the hypothesis proposed by Smith et al. (Smith, Lecour et al. 2002), that exists a beneficial link between ischemic preconditioning and innate immunity and that this could take place through (at least) SAPK/JNKs activation.

Old animals (6-10 months) show large accumulations of leukocytes leading to severe myocarditis, inflammatory cardiomyopathy, edema, and premature death. Recently, Mann (Mann 2001) has proposed the hypothesis that an appropriate and moderate production (or administration) of cytokines has beneficial effects whereas an excessive and prolonged synthesis (or administration) has detrimental actions. The data presented in the present study strongly support this hypothesis. In fact, the morphological comparative studies of neonatal, young, adult, and old mice support the hypothesis that a moderate and appropriate cytokine production (moderate inflammation observed in adult TG mice) has beneficial effects (cardioprotection in adult mice). In old animals the prolonged and excessive synthesis of cytokines (and MCP-1) has detrimental effects showing the old MCP-1 TG mice myocarditis, cardiomyopathy, different types of cardiomyocyte injury and death, and premature death.

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