Data Supplemental for "TNF Provokes Cardiac Myocyte Apoptosis and Cardiac Remodeling Through Activation of Multiple Cell Death Pathways"

Blood Vessel Formation

To determine whether blood vessel loss may have been responsible for the adverse cardiac remodeling in the MHCsTNF mice, we examined blood vessel formation in12 week old littermate control (LM), MHCsTNF, MHCsTNF/Bcl-2 and Bcl-2 mice by staining tissue with griffonia lectin, which specifically stains blood vessels. Briefly, perfusion fixed, deparaffinized tissue sections were incubated with 3% hydrogen peroxide (Sigma Aldrich) followed by trypsin solution (Digest-All kit; Zymed). After washing and blocking in 2% horse serum, sections were stained with biotinylated griffonia simplicifolia lectin I (Vector Laboratories). Staining was visualized by avidin/HRP incubation (Vactastain ABC kit, DAB kit; both from Vector Laboratories) resulting in a brown color for positively stained areas. Images were scanned using a microscope equipped with a digital camera (AxioCam HRc, Carl Zeiss MicroImaging Inc.) and the number of positively stained vessels were counted using Zeiss IMAGE analysis software.

Representative images are seen in the supplemental Figure S1A (200X). Quantitative analysis showed that the amount of blood vessels in myocardial tissue was not different between groups (LM: 1249±87/mm^2, MHCsTNF: 1237±25/mm^2, MHCsTNF/Bcl-2: 1312±117/mm^2, Bcl-2: 1337±248/mm^2; p=0.8, n= 3/group).

Myocyte Proliferation

To determine whether differences in the prevalence of proliferating myocytes may have been responsible for the adverse cardiac remodeling in the MHCsTNF mice, we examined the

prevalence of Ki67 positive myocytes in 12 week old littermate control (LM), MHCsTNF, MHCsTNF/Bcl-2 and Bcl-2 mice, which is a cell cycle related nuclear protein expressed in proliferating cells during the cell cycle. Briefly, sections were prepared as above, then incubated with rabbit anti-mouse Ki67 (Novus Biologicals) followed by a secondary Alexa Fluor 594 antibody (abs/em: λ= 590/617; Molecular Probes). Sections were counterstained with DAPI (Santa Cruz Biotechnology) to visualize all cell nuclei. Staining was detected using a fluorescence microscope (Delta Vision SpectrisTM, Applied Precision) that was equipped with the appropriate filters and the numbers of positively stained nuclei were counted.

Representative images are seen in the supplemental Figure S1B (400X; top row = red Ki67 stain, bottom row and enlarged image = merged image of red Ki67 and blue DAPI stain resulting in pink nuclei). Positively stained nuclei are indicated by arrows. Quantitative analysis showed that the prevalence of Ki67 positive nuclei did not differ between the LM ($170\pm12/10^4$ nuclei), MHCsTNF ($180\pm9/10^4$ nuclei), MHCsTNF/Bcl-2 ($167\pm2/10^4$ nuclei) and Bcl-2 ($172\pm10/10^4$ nuclei) groups ($p=0.8$, $n= 3$/group). Qualitative analysis indicated that proliferation occurred primarily in interstitial cells (~97% of all positively stained cells), but did not appear in significant amounts in cardiac myocytes.

Supplemental Figure S1