Effects of selective TNFR1 inhibition or TNFR2 stimulation, compared to non-selective TNF inhibition, on (neuro)inflammation and behavior after myocardial infarction in male mice

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ABSTRACT

Background: Myocardial infarction (MI) coinciding with depression worsens prognosis. Although Tumor Necrosis Factor alpha (TNF) is recognized to play a role in both conditions, the therapeutic potential of TNF inhibition is disappointing. TNF activates two receptors, TNFR1 and TNFR2, associated with opposite effects. Therefore, anti-inflammatory treatment with specific TNF receptor interference was compared to non-specific TNF inhibition regarding effects on heart, (neuro)inflammation, brain and behavior in mice with MI.

Methods: Male C57BL/6 mice were subjected to MI or sham surgery. One hour later, MI mice were randomized to either non-specific TNF inhibition by Enbrel, specific TNFR1 antagonist-, or specific TNFR2 agonist treatment until the end of the protocol. Control sham and MI mice received saline. Behavioral evaluation was obtained day 10–14 after surgery. Eighteen days post-surgery, cardiac function was measured and mice were sacrificed. Blood and tissue samples were collected for analyses of (neuro)inflammation.

Results: MI mice displayed left ventricular dysfunction, without heart failure, (neuro) inflammation or depressive-like behavior. Both receptor-specific interventions, but not Enbrel, doubled early post-MI mortality. TNFR2 agonist treatment improved left ventricular function and caused hyper-ramification of microglia, with no effect on depressive-like behavior. In contrast, TNFR1 antagonist treatment was associated with enhanced (neuro) inflammation: more plasma eosinophils and monocytes; increased plasma Lcn2 and hippocampal microglia and astrocyte activation. Moreover, increased baseline heart rate, with reduced beta-adrenergic responsiveness indicated sympathetic activation, and coincided with reduced exploratory behavior in the open field. Enbrel did not affect neuroinflammation nor behavior.

Conclusion: Early receptor interventions, but not non-specific TNF inhibition, increased mortality. Apart from this undesired effect, the general beneficial profile after TNFR2 stimulation, rather than the unfavourable effects of TNFR1 inhibition, would render TNFR2 stimulation preferable over non-specific TNF inhibition in MI with co-morbid depression. However, follow-up studies regarding optimal timing and dosing are needed.

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1. Introduction

Cardiovascular disease and depression are amongst the major causes of morbidity and mortality in the western world. These two conditions seem interrelated as prognosis declines with severity of co-morbid depression (Barefoot & Schroll, 1996). Major depression was observed in 15–22% of patients with a myocardial infarction (MI) (Frasure-Smith & Lesperance, 2003), while even 65% reported symptoms of depression (Carney et al., 2004). The other way around, being depressed also increased the risk of developing myocardial infarction (Pereira et al., 2013; Rugulies, 2002) up to 2–3 times (Liu et al., 2013). However, optimal cardiovascular post-MI treatment does not reduce depression,
and anti-depressant therapy does not improve cardiovascular prognosis, reviewed by Thombs (Thombs et al., 2008). Hence, a common mechanism rather than a causal relationship may underlie this heart-brain interaction.

A detailed inflammatory state may provide a good candidate for a common underlying mechanism (Quan, 2014). Acute MI evokes activation of the innate immune system in order to heal the damaged cardiovascular tissue, as seen by increased cardiac and circulating levels of pro-inflammatory cytokines. Depression and heart failure share the increased production of pro-inflammatory mediators, such as IL-1beta, IL-6 and TNF-alpha (Johansson et al., 2011; Pasic et al., 2003), with a further increase in the case of co-morbid heart failure with depression (Shang et al., 2014). In our previous review (Liu et al., 2013), we emphasized the potential role of Tumor Necrosis Factor-alpha (TNF) in the MI-depression interaction. Despite acknowledgement of this role (Levine et al., 1990), therapeutic potential of TNF inhibition appeared rather poor (Kotyla, 2018; Padfield et al., 2013).

TNF mediated processes are complex. TNF occurs as transmembrane protein and as soluble molecule that is released from the membrane-bound form, and is regarded as a pro-inflammatory cytokine. However, the two TNF forms can bind to two different receptors that can elicit opposite effects (Medler & Wajant, 2019). Soluble TNF preferably activates TNF receptor 1 (TNFR1) causing pro-inflammatory effects and cell death. Membrane TNF binds and activates both TNF receptors, with TNF receptor 2 (TNFR2) activation being associated with cell survival and cytoprotection (Medler & Wajant, 2019). Accordingly, TNF was shown toxic via TNFR1 and protective via TNFR2 in a mouse model of MI (Monden & Monden, 2007); yin and yang in myocardial infarction (Schulz & Heusch, 2009). These dichotomous TNF-specific effects could provide an explanation for the failure of anti-TNF treatment in clinical trials (Hamid et al., 2009), as the overall balance between the opposing receptor-specific responses may determine the ultimate impact of TNF inhibition.

In hearts of mice with MI, TNFR1 and TNFR2 both increased in concert to increased TNF levels (Hamid et al., 2009). In a recent study, we showed in brains of mice with MI elevated expression of TNF precursor protein, but at a more than doubled TNFRI expression and almost 70% decline in TNFR2 expression, suggesting persistent neuro-inflammation (Gouweleeuw et al., 2017b). Thus, in addition to the opposite effects of TNFR1-versus TNFR2 receptor stimulation, an organ-specific shift in receptor expression after MI may contribute to the lack of therapeutic effects of non-specific TNF inhibition. Despite the overall indication of altered TNF signaling in the brain after MI, subsequent staining on TNF did not reveal significant changes in relevant areas, such as hippocampus, hypothalamus (paraventricular nucleus) and prefrontal cortex, although in the latter area the number of TNF-positive cells was about twice as much in MI versus sham mice (Gouweleeuw et al., 2017b). Analyses of gene expression by RNA sequencing in prefrontal cortex and hippocampus revealed alterations in genes related to inflammation (Frey et al., 2014). In our previous study (Gouweleeuw et al., 2017b), the altered brain TNF signalling was accompanied by microglia activation in a specific area of the hippocampus, the dentate gyrus, but not in areas involved in depressive-like behavior and cardiovascular regulation; the amygdala and paraventricular nucleus of the hypothalamus, respectively.

We hypothesize that the anticipated anti-inflammatory effects of selective TNFR1 inhibition as well as the cytoprotective effects of selective TNFR2 stimulation will be more effective than non-selective TNF inhibition in the heart-inflammation-brain triangle. Therefore, in the present study selective TNFRI inhibition and selective TNFR2 stimulation will be compared to non-selective TNF inhibition with Etanercept (Enbrel) in mice with MI, with regard to effects on the heart, inflammation, brain and behavior.

2. Materials and methods

2.1. Animals

Male 12-weeks old C57BL/6Jrj mice were obtained from Janvier (France). A total of 140 mice were included in the study. Because of complementary expertise at different Institutes in the Netherlands, part of the study was performed in Groningen (immunohistochemistry and behavior; 76 mice) and part in Maastricht (cardiac function and behavior; 64 mice). Behavioral studies in both institutes were performed by the same investigator using the same set of equipment. Mice were kept in individual cages for the duration of the experiment and were habituated to the housing conditions at least 2 weeks before experiments started. Housing occurred in climate rooms (temperature 22 ± 1 °C, humidity 50 ± 10% and a reversed light/dark (LD) schedule of 12 h light (±50 lx) and 12 h dark, normal tap water and food (standard rodent chow: RMHB/2180, Arie Block BV, Woerden, NL) ad libitum. Daily, all mice were weighed to get used to handling, and checked for health/activity/food/water and abnormal behavior. All procedures were in accordance with the regulation of the ethical committee for the use of experimental animals of the University of Groningen, and the University of Maastricht, The Netherlands (DEC 6145C).

2.2. Experimental protocol

Mice were subjected to permanent coronary artery ligation or sham surgery. One hour after surgery, infarcted mice were randomized over the 4 experimental groups (Fig. 1) and received their first treatment. Treatment was continued throughout the protocol. Behavioral tests were performed day 10–14 after surgery. Mice were sacrificed 18 days after surgery. Effects on cardiac function were measured by echocardiography and left ventricular pressure development. Blood samples were collected, and heart and brain tissues were dissected and processed for later molecular analyses and (immuno)histochemistry.

2.3. Surgery

Mice underwent either sham surgery or permanent ligation of the left coronary artery to induce myocardial infarction (MI) as described previously (Daskalopoulos et al., 2019). Briefly, mice were anesthetized with 2.5% isoflurane and placed on a heating pad. Mice were intubated and the left anterior descending coronary artery was ligated to induce permanent myocardial infarction. Sham mice underwent the same procedure without the actual ligation of the coronary artery. Whereas according to local regulations, analgesia in Groningen was provided by one time 1 ug buprenorphine at the start of surgery to limit post-operative pain, in Maastricht regulations required 1 ug buprenorphine 30 min before surgery, as well as twice a day for 2 days after surgery.

2.4. Treatment

Mice were treated with TNF interfering agents starting one hour after MI induction, and lasting for 18 days after MI, according to the schedule in Fig. 1. Enbrel (Etanercept) was purchased from Pfizer (Capelle aan den IJssel, the Netherlands). The TNFR1 antagonist was the commercially available monoclonal antibody (HM1097; mAB 55R-170, Hyvult Biotech, Uden, the Netherlands), specifically targeting the TNFR1 receptor (Sheehan et al., 1995). The TNFR2 agonist (TNC-sc-mTNF(221 N/223R)) consisted of a fusion protein of the chicken tenasin-C tri merization domain and a single chain-encoded TNF trimer domain composed of three soluble TNF trimers (aa 91–235 of murine TNF) containing point mutations conferring specificity for TNFR2 (Chopra et al., 2016). Treatment was administered by intraperitoneal (ip) injections of 200 µl. Dosing and treatment schedule for Enbrel, 100 µg 3xper week was based on the study of Yi et al. (Yi et al., 2014), as being effective in a mouse model for arthritis. Antibody dose (100 µg) and
schedule for TNFR1 antagonist treatment was based on effects in the Multiple Sclerosis study of Williams et al. (Williams et al., 2014) and the mechanistic study of Sheehan et al. (Sheehan et al., 1995). Studies implicated anti-inflammatory effects in conditions of activated TNF signaling. For TNFR2 agonist treatment, the study of Chopra et al., (Chopra et al., 2016) was used as basis for the 75 $\mu$g per mouse on days 0, 2, 4, 7, 9, given in 200 $\mu$l. The TNFR2 agonist did not affect body weight, nor serum or tissue levels of cytokines, over a 2 weeks treatment course in control mice (Chopra et al., 2016). Treatment was given as a fixed dose per mouse of 100 $\mu$g per administration for Enbrel and the TNFR1 antagonist, and 75 $\mu$g for the TNFR2 agonist, resulting in approximately 3.6- and 2.7 mg/kg, respectively. Control sham and MI mice received 200 $\mu$l saline.

2.5. Behavioral tests

Except for the sucrose preference test, behavioral tests were performed in the dark (=active) phase of the mice, excluding the first and last 2 h. All tests, except the forced swim test, were performed in a square arena ($50 \times 50$ cm).

2.5.1.Sucrose preference test

The sucrose preference test was performed on days 4, 5, 6, 12 and 13 to measure anhedonic behavior. Day 4 comprised habituation to the test for 24 h using 2 pipets with normal drinking water placed on the left and right side of the cage. The actual 48 h test was performed on days 5 and 6, using 2 pipets, 1 containing drinking water and 1 containing a 1% sucrose solution. After 24 h the position of the pipets was reversed, to prevent potential effects of side preference. On days 12 and 13 the test was repeated following the same procedure as on days 5 and 6. Sucrose preference was measured by calculating the percentage of sucrose solution intake relative to the total liquid intake (mean values of days 5-6 and 12-13), with reduced sucrose preference as an indicator for anhedonic behavior.

2.5.2. Open field test

The open field test was performed on days 10 and 11 to measure exploration of a new environment and anxiety. Mice were placed in an empty square arena and then behavior was recorded for 5 min. This was repeated the next day. Time spent in corner, border or center areas, distance and speed were measured with Ethovision software (Noldus, Wageningen, the Netherlands). Time spent in the corners was regarded as a measure for anxiety, while distance may reflect exploration, combined with physical condition. Furthermore, the percentage of time spent on the behaviors rearing, sniffing, walking, sniffing while walking, exploration of the wall and grooming were analyzed with in-house produced software (e-line). The first five behaviors composed total exploration time (%) as an outcome measure for interest in environment.

2.5.3. Social interaction test

The social interaction test was performed on days 10 and 11 to measure the interest for interaction with other mice. Mice were placed in a square arena with in 2 opposite corners a small container; one empty and one containing a (strain, gender and age matched, but unfamiliar) mouse. Behavior was recorded for 5 min and this was repeated the next day in opposite position with a different stimulus mouse. Absolute time spent at the mouse container and preference of being at the mouse rather than the empty container (mouse time/(mouse time + empty time)) were used as outcome measures for social interest.

2.5.4. Novel object and novel location recognition test

The novel object (NOR) and novel location (NLR) recognition tests were performed on day 13, to measure object and spatial memory. Before the actual tests, mice were habituated to the test arena. Then, mice performed a baseline test with two identical objects (either 2 drinking glasses or 2 candle holders) and behavior was recorded for 3 min. Subsequently, in random order all mice performed a NOR as well as the NLR test for 3 min with 1 min in between. For the NOR test, one of the objects from the baseline test was replaced by a novel object, while for the NLR test one of the objects from the baseline test was relocated to the opposite corner. Time spent at the unchanged, novel or relocated object was measured with in-house produced software (e-line). Preference for the novel or relocated object was calculated as time spent on the
novel/relocated object divided by the time spent on both objects were used as outcome measures for object or spatial memory, respectively.

2.5.5. Forced swim test
The forced swim test was performed on day 14 to measure depressive-like behavior, especially helplessness. Mice were placed in a beaker (20 cm diameter), filled with 15 cm deep 26 °C water. Behavior was recorded for 6 min. Time spent struggling, swimming and floating was measured with in-house produced software (e-line). Time spent on floating was regarded as giving up, representing depressive like/helplessness behavior, while struggling was seen as a measure for active coping.

2.6. Sacrifice and tissue preparation
Eighteen days after surgery, echocardiography and cardiac function measurements (see 2.7) were performed (Maastricht), and subsequently mice were sacrificed. For that, mice were anaesthetized with a high dose of pentobarbital (80 mg/kg). Immediately, a blood sample was obtained by cardiac puncture and collected in EDTA coated tubes and kept on ice before further processing. Then, mice were perfused with a saline solution with heparin for 1 min, followed by perfusion with 4% PFA in saline (Groningen). The heart, lungs, liver, kidney, and spleen were removed and weighed. An increased heart weight body weight ratio was considered as measure for cardiac hypertrophy, while increased lung weight body weight ratio was used to indicate pulmonary congestion and heart failure.

The brain was excised and processed for later (immuno)histochemistry (Groningen). For that, brains were fixed in 4% PFA solution, cryoprotected with 30% sucrose solution, frozen in liquid nitrogen and stored at –80 °C, until being cut for immunohistochemistry. A similar procedure was followed for a transverse mid ventricular section of the heart for later staining to visualize cardiac collagen. Alternatively, the hippocampus and prefrontal cortex were dissected, frozen in liquid nitrogen and stored at –80 °C for later molecular analyses (Maastricht).

2.7. Cardiac measurements

2.7.1. Echocardiography
Echocardiography was performed 18 days after MI, according to previously published methods (van den Borne et al., 2009). Parameters measured were end diastolic volume (EDV), end systolic volume (ESV), stroke volume (SV) and ejection fraction (EF) of the left ventricle.

2.7.2. Cardiac function
At the time of sacrifice, cardiac function measurements were performed as previously published (van den Borne et al., 2009). Briefly, mice were anaesthetized with 2.5% isoflurane, intubated and ventilated and kept on a 37 °C body temperature. A Millar pressure tip catheter was inserted in the left ventricle through the carotid artery. Maximal negative and positive pressure development and heart rate were recorded. Subsequently, responses to increasing dosages (20–120 ng/milligram) of i.v. Dobutamine were obtained. Responses to 60 ng/milligram were regarded most relevant to represent beta-adrenergic responsiveness.

2.7.3. Cardiac collagen
Cardiac collagen was measured as estimate for wound healing and scar formation in subgroups of mice. For that purpose, transverse midventricular slices were stained for collagen with Sirius red, as previously described (Gouweleeuw et al., 2016). Macroscopic (3x magnification) pictures are taken to measure infarct size as percentage of left ventricular circumference (2–3 sections per mouse). Infarct healing was measured as percentage of collagen in the center of the infarcted area.

2.8. Peripheral inflammation

2.8.1. Hematology
To obtain a measure for peripheral inflammation, a blood cell differentiation was performed. For that, 250 μl whole blood samples, collected in EDTA tubes at the time of sacrifice were transported to the department of Hematology at the University Medical Center Groningen or University Medical Center Maastricht for blood cell differentiation, platelets, and hemoglobin. In a subsection of MI mice, (Maastricht), further differentiation into the different white blood cells was performed.

2.8.2. Lipocalin 2
Since in our previous study plasma cytokine levels were not found increased 2 weeks after MI in mice (Gouweleeuw et al., 2017a, 2017b), Lipocalin 2 levels were measured as sensitive marker for inflammation (Gouweleeuw et al., 2016, 2017a) and potentially linking depression and cardiovascular disease (Gouweleeuw et al., 2015, 2016; Naudé et al., 2014). For that, the mouse NGAL quantikine ELISA kit (R&D systems; detection limit 78.1 pg/ml) was used according to manufacturers’ instructions. Samples were diluted 100x and measured in duplicate.

2.9. Neuroinflammation

2.9.1. Microglia
Immunohistochemical staining was performed to visualize microglia (ionized calcium-binding adaptor protein; IBA-1, Wako, Neuss, Germany. Dilution 1:2500) as previously described (Hovens et al., 2014). From sections (20 μm thickness) photographs were taken (200 × magnification) of the CA1, CA3, dentate gyrus (DG), hilus of the dorsal hippocampus (bregma: −1.34_-2.30 mm), the prefrontal cortex (bregma: +2.34_-1.18 mm), the piriform cortex (bregma: −1.34_-2.30 mm) and the paraventricular nucleus of the hypothalamus (PVN) (bregma: −1.06_-1.34 mm), for image analyses. For overall hippocampal microglia parameters, values for CA1, CA3, dentate gyrus (DG) and hilus areas were averaged.

2.9.2. Astrocytes
Immunohistochemical staining to visualize activated astrocytes was performed by staining against the astrocyte specific Glial Fibrillary Acidic Protein (GFAP). Sections (20 μm thickness) were pretreated for 30 min. with 0.3% H2O2 in 0.1 M TBS pH 7.4. Then sections were incubated for 24 h with 1:10000 mouse anti-GFAP (G3893, Sigma) in 3% bovine serum albumin (BSA) and 0.1% triton X-100 at 4 °C. Next, the sections were incubated for 1 h with a goat-anti-mouse labeled secondary antibody (1:500 dilution, Jackson, Wet Grove, USA) at room temperature. The sections were incubated for 2 h with avidin-biotin peroxidase complex (1:500) (Vectastain ABC kit, Vector, Burlingame, USA) at room temperature. The labeling was visualized with 0.075 mg/ml diaminobenzidine (DAB) solution, activated with 0.1% H2O2. All dilutions used in the staining steps were made in 0.01 M TBS pH 7.4 and in between staining steps sections were washed 5 times. Images were taken (200x magnification) from the hippocampus CA1, CA3 and DG regions for later image analyses.

2.9.3. Neurogenesis/neuroplasticity
To investigate the hippocampal neurogenesis, and piriform cortex neuroplasticity (Klempin et al., 2011), sections were stained for double cortin (DCX; Santa Cruz, California, USA, Dilution 1:5000), as described before (Gouweleeuw et al., 2017a, 2017b). Pictures were taken (50x magnification) of the dentate gyrus of the hippocampus and the piriform cortex, for later image analyses.

2.9.4. Image analysis
Images of sections of Iba-1, GFAP and DCX staining were taken using
quantitative imaging software (Leica QWin, Leica Microsystems), at 50x magnification for the DCX staining and 200x magnification for the IBA-1 and GFAP staining. The obtained images were analyzed by using image analysis software (Image-Pro Plus 6.0.0.26, Media Cybernetics, Inc. Rockville, USA). Values represent the average of 3–4 sections per area per mouse. Image analysis was performed blinded for experimental procedures.

For the IBA-1 staining all regions were analyzed in the same way, following our previously described method (Hovens et al., 2014). Number of cells (density) and total cell surface (coverage), as well as area of cell bodies were measured. Microglia activity was expressed by the calculated cell body size to cell size ratio, as previously described (Hovens et al., 2014).

In order to obtain a more detailed analysis of microglia morphology, a Sholl analysis was performed. For that photographs of single randomly selected microglia were preprocessed by binarization and removal of single scattered positive pixels in the background. Morphological analysis was performed using the Sholl-analysis plugin from the Fiji plug-in software for Image J. Concentric circles, increasing 1 pixel in radius every step, were used to analyze the number of intersections of the processes with every circle. Values were averaged for 20 microglia.

From the GFAP images, astrocyte density as well as coverage were obtained. Higher number of stained astrocytes or increased coverage were regarded as astrocyte activation.

For the DCX pictures, the area of the DCX positive cells was measured using intensity threshold histogram, as well as the length of the dentate gyrus or piriform cortex. The surface of DCX positive cells per dentate gyrus length was used as measure for neurogenesis, while the same parameter in the piriform cortex was used as measure for neuroplasticity.

2.9.5. Brain TNF expression

Expression of TNF-alpha and its receptors, as well as Lipocalin 2 (Lcn2), in brain areas were measured using western blot, as described in detail previously (Gouweleeuw et al., 2017a, 2017b), but using different antibodies. Briefly, antibodies against TNF (1:1000, ab66579), TNFR1 (1:1000, ab19139), TNFR2 (1:200, ab15563) and lipocalin-2 (1:1000, ab63929) were all purchased from Abcam (Cambridge, UK). Specific bands were observed for TNF (25Kda), TNFR1 (55Kda), and TNFR2 (75Kda) and lipocalin-2 (23Kda). Actin served as an equal loading control and was used at 1:1000 000 dilution (mouse anti-actin, ImmunO, MP Bio Medicals Inc.). An enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA) was used to visualize labelling, which was subsequently analysed (chemiluminescence image analysis; Bio-Rad Laboratories, Hercules, CA, USA).

2.10. TNFR2 agonist dose response

The outcomes of the TNFR2 agonist treated group showed the highest mortality and survivors seemed to have larger infarcts. This may be attributed to a disturbed healing process as indicated by the glassy appearance of the infarct area, as was also observed for another receptor of the TNF receptor superfamily (Pacheli et al., 2013). Furthermore, the observed increase in platelet count could potentially increase blood clotting. On the other hand, cardiac function parameters indicated prevention of left ventricular dysfunction. Although the dose used in the present study did not have effects in healthy mice (Chopra et al., 2016), in our model of (cardiac)damage the dose may have been too high. For that, an extra dose–effect relation experiment was performed. MI mice were randomized for treatment with different dosages of the TNFR2 agonist according to schedule in Table 1 (the “high” dose being the same as in the main study) and sacrificed at day 4, as most of the extra mortality in TNFR2 treated mice occurred between day 3 and 7 post-MI.

Since in the main study no clear behavioral differences were observed for this treatment, examination was limited to survival, echocardiographic analysis of cardiac function, measurement of collagen in the infarct zone, and blood cell analyses, as described in detail before.

2.11. Data analysis

All measurements and analyses were performed by persons blinded for the experimental procedures of the mice or samples. Statistical analyses were performed using IBM SPSS Statistics 20 (IBM, Armonk, New York, USA). Only mice that survived the full protocol were included in the analysis, unless indicated otherwise. Roses of mice that exceeded mean ± twice the standard deviation of their group were regarded as outliers and omitted (resulted in maximally 1 mouse left out per group).

Effects of MI were analysed by a student-t-test for independent samples for results of saline treated sham mice versus saline treated MI mice. Differences between the five treatment groups were analysed using one-way ANOVA followed by LSD post-hoc test. Subsequently, results of only actively treated groups were compared with one-way ANOVA followed by a Dunnett post-hoc test with the Enbrel treated group as reference, in order to test preference of selective TNF receptor intervention. A probability of p < 0.05 was considered statistically significant. All graphs were made with GraphPad Prism version 5.00 for Windows (Graphpad software, San Diego, California, USA) and results are expressed as mean ± S.E.M.

3. Results

3.1. General

From the total of 140 mice, 29 mice died during MI surgery. Another 36 mice died spontaneously, or had to be sacrificed prematurely because of greater than 15% loss of body weight. This resulted in 17 sham mice and 58 MI mice that finished the protocol (12–17 mice per experimental group). Fig. 2 shows the survival in the different experimental groups. Mortality mainly occurred in the first week after MI, after that mice survived the complete protocol. Although differences between the different MI groups did not reach statistical significance (p = 0.10), curves suggest that whereas Enbrel did not affect mortality compared to saline treated MI mice, selective interference with the TNF receptors further increased post-MI mortality (mortality MI + saline: 24%; MI + Enbrel: 25%; MI + TNFR1 antagonist: 44%; MI + TNFR2 agonist: 52%).

![Survival](image_url)

**Fig. 2.** Survival curves after MI or sham surgery and the effects of different TNF interventions. MI = myocardial infarction; TNFR1 = TNF receptor 1; TNFR2 = TNF receptor 2.

| Table 1 | Schedule for dose response relationship for the TNFR2 agonist. MI = myocardial infarction; TNFR2 = TNF receptor 2. |
|---------|--------------------------------------------------|
| 1. MI + high dose TNFR2 agonist (75 μg/mouse: ±2.68 mg/kg), (n = 12) |
| 2. MI + mediate dose TNFR2 agonist (25 μg/mouse: ±0.89 mg/kg), (n = 11) |
| 3. MI + low dose TNFR2 agonist (7.5 μg/mouse: ±0.27 mg/kg), (n = 9) |
| 4. MI + saline (n = 9) |
| 5. Sham + saline (n = 6) |
Results were comparable for both institutes.

For mice that finished the protocol, body weight at start (28.2 ± 0.3 g) did not differ from body weight at the end (27.9 ± 0.3 g), nor differences between experimental groups were observed. Table 2 presents organ weights of these mice. Mice with MI had higher heart weight compared to sham mice (heart weight body weight ratio sham 0.62 ± 0.03 versus MI 0.74 ± 0.04; p < 0.05), without increased lung weight (lung weight body weight ratio sham 0.86 ± 0.09 versus MI 0.91 ± 0.11) suggesting no congestive heart failure. TNFR2 agonist treated mice displayed significantly increased heart weights compared to all experimental groups, without affecting lung weight (lung weight body weight 0.874 ± 0.105) or other organ weights.

Mice that had to be sacrificed prematurely because of losing too much weight (1 from saline treated MI; 1 from Enbrel treated MI; 2 from TNFR1 antagonist treated MI and 7 from TNFR2 agonist treated MI) had average heart weights of 0.27 ± 0.02 g, but strongly increased lung weights; 0.39 ± 0.05 g (p < 0.05 versus survivors), indicating development of pulmonary congestion and heart failure.

Notably, mice treated with TNFR2 agonist, seem to show macroscopic signs of an altered infarct healing, as infarcts appear more vesicular and edematous. Apart from this observation at sacrifice, no side effects on general behavior and appearance were seen.

3.2. Effects on the heart

Left ventricular dysfunction after MI was evidenced by significantly increased left ventricular volumes and reduced ejection fraction (Fig. 3 and Table 3). MI mice showed increased cardiac weight, without increased lung weight, suggesting cardiac remodeling without heart failure (Table 3). Accordingly, with increased left ventricular volumes without increased left ventricular pressures, stroke volume was preserved at lower ejection fraction (Fig. 3 and Table 3). Infarct collagen content was not affected by treatment (1 ± 1% in sham + saline, n = 6; 54 ± 8% in MI + saline, n = 5; 52 ± 5% in MI + Enbrel, n = 7; 45 ± 9% in MI + R1agonist, n = 4 and 54 ± 7% in MI + R2agonist, n = 6), neither was absolute infarct length (263 ± 37 in MI + saline; 298 ± 16 in MI + Enbrel; 288 ± 24 in MI + R1 antagonist and 300 ± 31 pixels in MI + R2 agonist), indicating that effects on cardiac dimensions and function could be attributed to the non-infarcted myocardium, rather than the infarct itself.

Except for significantly improved left ventricular relaxation, Enbrel did not significantly alter left ventricular volumes and functional parameters compared to saline treated MI mice. However, parameters were also not different from sham anymore.

Although effects of the TNFR1 antagonist did not reach statistical significance, they may indicate physiological relevance. The TNFR1 antagonist further dilated the non-infarcted myocardium, increasing left ventricular volumes, resulting in lower infarct sizes (as % of left ventricular circumference). The lower EF and SV may then be compensated for by the increased heart rate, in order to preserve cardiac output. Notably, the significantly increased baseline heart rate coincided with significantly impaired beta-adrenergic responsiveness (Fig. 3).

The opposite was observed for the TNFR2 agonist; less dilatation as seen by reduced left ventricular volumes, resulting in increased EF and preserved SV. The increased infarct size measured as percentage of left ventricular circumferene may then be attributed to the reduced dilatation of the non-infarcted myocardium (Table 3 and Fig. 3), since absolute length of the scar did not differ from saline MI mice.

3.3. Peripheral inflammation

3.3.1. Lipocalin 2

Plasma lipocalin 2 (Lcn2) was measured as a sensitive marker for inflammation. MI did not increase plasma levels of Lcn2 (Fig. 4). However, the TNFR1 antagonist significantly increased Lcn2 levels. Moreover, the plasma Lcn2 levels of mice that had to be sacrificed prematurely were more than 10 times higher (1839 ± 457 ng/ml; n = 9, p < 0.05) than those of the mice that survived the complete protocol.

3.3.2. Hematology

To investigate effects on peripheral inflammation, blood cells were analyzed. MI reduced white- (p = 0.058) and red blood cells counts, as well as platelet counts, reaching statistical significance for red blood cells (Fig. 5). Accordingly, hemoglobin levels were also significantly reduced (6.7 ± 0.5 versus 9.5 ± 0.6 mmol/L). Enbrel more than doubled the number of white blood cells, which was reflected in the number of lymphocytes. The TNFR2 agonist significantly increased the number of platelets.

Further differentiation of white blood cells counts in subgroups of MI mice revealed increases of basophils (ns), eosinophils (p < 0.05) and monocytes (p < 0.05), but not neutrophils in TNFR1 antagonist treated mice, while Enbrel and the TNFR2 agonist did not affect these cell counts.

3.4. Neuroinflammation

3.4.1. Microglia activity

Neuroinflammation was evaluated by analysis of morphological changes of microglia. Activated microglia display de-ramification, evident from an increased cell body to cell size ratio. Microglia parameters obtained in the hippocampus, the prefrontal cortex and piriform cortex showed no difference between de groups. Within the different hippocampal areas, no differences were observed for the CA1 and dentate gyrus. However, TNFR1 antagonist treatment significantly increased microglia activation in the hilus (4.2 ± 0.4 vs 2.3 ± 0.5 in saline treated MI mice).

In contrast, microglia morphology in the Paraventricular Nucleus of the hypothalamus (PVN) changed significantly (Fig. 6). Altered microglia density is compensated by the size of the individual microglia, as total coverage is similar in all groups. Surprisingly, microglia activity, measured as cell body to total cell size ratio declined in all MI groups. Since microglia cell body area is similar in all groups, increased microglia cell size after MI can be completely attributed to increased area of processes. The significantly declined microglia activity measure in all MI groups compared to sham was exaggerated in TNFR2 agonist treated mice and attributed to a significantly increased processes area.

Since an increased area covered by processes could be attributed to either an increased number of processes, thicker processes, or both, this aspect is evaluated by a Sholl analysis of individual microglia profiles (Fig. 7). The curves for saline treated sham and MI overlap, indicating that the higher processes area in MI mice may represent thicker, rather than more processes. All treated groups differ significantly from saline treated MI. For the treated groups, at least part of the increased

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Table 2

| Organ       | Lung   | Liver  | Kidney | Spleen | Brain | Heart |
|-------------|--------|--------|--------|--------|-------|-------|
| Sham + saline (n = 17) | 0.24 ± 0.03 | 1.30 ± 0.06 | 0.22 ± 0.01 | 0.10 ± 0.09 | 0.47 ± 0.08 | 0.18 ± 0.02 |
| MI + saline (n = 15) | 0.25 ± 0.03 | 1.18 ± 0.06 | 0.21 ± 0.01 | 0.08 ± 0.07 | 0.47 ± 0.00 | 0.18 ± 0.01 |
| MI + Enbrel (n = 15) | 0.21 ± 0.03 | 1.30 ± 0.06 | 0.21 ± 0.01 | 0.11 ± 0.07 | 0.46 ± 0.02 | 0.22 ± 0.00 |
| MI + R1 agonist (n = 14) | 0.23 ± 0.03 | 1.26 ± 0.05 | 0.20 ± 0.01 | 0.10 ± 0.09 | 0.46 ± 0.02 | 0.22 ± 0.00 |
| MI + R2 agonist (n = 13) | 0.23 ± 0.03 | 1.20 ± 0.06 | 0.20 ± 0.01 | 0.10 ± 0.09 | 0.46 ± 0.02 | 0.27 ± 0.00 |

Additional reading:

- L. Gouweleeuw et al., Brain Behavior and Immunity 93 (2021) 156–171
- Brain Behavior and Immunity 93 (2021) 156–171
processes area was due to a higher number of processes.

3.4.2. Astrocyte activity

Astrocyte activity was measured by density as well as coverage of astrocytes in the hippocampus. Results are presented in Table 4. No astrocyte activation was observed after MI; in the CA1 even a significantly lower coverage was found. TNFR1 antagonist treatment increased coverage in all areas, which became statistically significant for the CA1 area.

3.4.3. Brain TNF and TNF receptors

Expression of TNF and its receptors as well as Lcn2 were measured in homogenates from the hippocampus and prefrontal cortex (Table 5). Absence of data indicates absence of bands in the western blot for these groups. No significant signs of neuroinflammation by increased TNF expression were observed, nor did expression of the TNF receptors change in saline treated MI versus sham mice. In the hippocampus, a consistent, though not statistically significant, reduction in TNFR2 receptor expression was observed in all MI groups, being most pronounced after TNFR2 agonist treatment. TNFR1 antagonist treatment significantly reduced TNFR1 expression in the hippocampus. In contrast, in the prefrontal cortex, TNF receptor interference, but not Enbrel, significantly increased TNF expression. Lcn2 expression almost doubled in both brain areas in saline treated MI versus sham mice. Enbrel significantly further increased the Lcn2 expression in the prefrontal cortex.

Regarding expression in the supernatant fraction versus pellet fraction, shifts from one fraction to the other, rather than absolute expression patterns, seemed consistent for the interventions. Similar and
Table 3

Left ventricular volume parameters obtained by echocardiography in the different experimental groups, and infarct size in subgroups (Groningen) of mice: EDV = end-diastolic volume; ESV = end-systolic volume; SV = stroke volume; EF = ejection fraction; MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2. *: significantly different from sham. #: significantly different from saline treated MI. $: significantly different compared to general TNF inhibition by Enbrel.

| Group                        | EDV (mL) | ESV (mL) | SV (mL) | EF (%) | % Infarct size (%) |
|-----------------------------|----------|----------|----------|--------|-------------------|
| Sham + saline (n = 9)       | 0.08 ± 0.03 | 0.035 ± 0.003 | 53.0 ± 0 | 4 (4) |
| MI + saline (n = 8)         | 0.10 ± 0.07 | 0.031 ± 0.004 | 35.0 ± 28 ± 8 (4)* | 7* |
| MI + Enbrel (n = 6)         | 0.11 ± 0.07 | 0.035 ± 0.004 | 39.0 ± 29 ± 5 (6)* | 6* |
| MI + R1 antagonist (n = 7)  | 0.02 ± 0.02 | 0.003 ± 0.004 | 8.0 ± 21 ± 3 (3)* | 6* |
| MI + R2 agonist (n = 4)     | 0.08 ± 0.05 | 0.037 ± 0.005 | 44.0 ± 44 ± 3 (6) | 6* |

Fig. 4. Plasma levels of lipocalin 2 (Lcn2) in the different experimental groups (n = 11–13) 18 days after MI/sham surgery. MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2. *: significantly different from sham. #: significantly different from saline treated MI. $: significantly different compared to general TNF inhibition by Enbrel.

Fig. 5. Blood cell counts of the different experimental groups (n = 6–7 per group). A: White blood cells; B: Lymphocytes; C: Red blood cells; D: Platelets. MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2. *: significantly different from sham. #: significantly different from saline treated MI. $: significantly different compared to general TNF inhibition by Enbrel.

3.4.4. Neurogenesis/neuroplasticity

Although no significant differences between groups were seen, hippocampal DCX seemed lower in all MI groups compared to sham. Except for Enbrel treated mice, this is also seen in DCX values in the piriform cortex (Table 6).

3.5. Effects on behavior

Table 7 shows an overview of the performances in the behavioral tests. Apart from a significant lower resting behavior in the Open Field, no significant differences were observed between saline treated MI and sham mice. Enbrel did not significantly affect any parameter. Mice treated with the TNFR1 antagonist walked significantly less distance, showed lower exploratory behavior and more resting in the Open Field than saline treated MI mice. TNFR2 agonist treated MI mice displayed more grooming in the Open Field, and declined NOR in the cognitive test, though still significantly better than random (p=50%). Tests that further examines depression/anxiety-like behaviors; location preference in the Open Field, social preference and the forced swim test, were not affected by either treatment. Accordingly, overall sucrose preference increased from day 5-6 to day 12–13 (from 81.7 ± 1.8 to 88.3 ± 1.6%), but was not different between groups.

3.6. TNFR2 agonist dose-effect

As the above results indicate mixed results for the TNFR2 agonist treatment, an extra dose-effect relationship experiment was performed. The main results are presented in Fig. 8, while the other data are summarized in Table 8. Effects were measured after 4 days of treatment since mortality mainly occurred early after MI. If anything, the 2 higher consistent effects of Enbrel and the TNFR1 antagonist could be observed. In the hippocampus, Enbrel and TNFRI antagonist treatment were associated with a shift from pellet to supernatant for TNF and shifts in the opposite direction for TNFR1 and Lcn2, whereas in the prefrontal cortex a similar effect on TNFR1 expression was accompanied by opposite shifts for TNFR2 and Lcn2 expression. For the TNFR2 agonist treatment only in the prefrontal cortex shifts may be seen; from supernatant to pellet for TNF and the other way around for Lcn2.
dosages may increase mortality (30% and 33% for the mid and high dose, respectively, compared to 11% for the low dose). Left ventricular dysfunction was evidenced by increased left ventricular volumes, reduced stroke volume at decreased ejection fraction. Notably stroke volumes at 4 days were substantially lower than at 18 days (Table 3). None of these parameters were significantly affected by treatment at any dose. Similarly, infarct healing, obtained from infarct collagen was not affected by any dose of the TNFR2 agonist. The different blood cells and platelets were not changed by MI, and neither a dose-dependent effect of R2 agonist was observed.

4. Discussion

TNF signalling is increased after MI, in the heart, the circulation and the brain (Francis et al., 2004) and is associated with decreased cardiovascular prognosis and higher incidence of depression (Liu et al., 2013). However, therapeutic effects of TNF inhibition so far have been disappointing. This may be explained by TNF activating receptors with opposing effects, as TNF was shown toxic via TNFR1 and protective via TNFR2 in MI mice (Monden & Monden, 2007). Therefore, in the present study effects of treatment with a specific TNFR1 antagonist or TNFR2 agonist were investigated, compared to non-specific TNF inhibition in MI mice, regarding the triangle: heart, inflammation and brain (neuro-inflammation and depressive like behavior). We hypothesized that inhibition of TNFR1 and stimulation of TNFR2 will show therapeutic benefit over non-specific TNF inhibition by Enbrel.

Main findings were an almost doubled mortality in receptor-specific interventions compared to general TNF inhibition. However, whereas TNFR2 agonist treatment improved cardiac outcome and microglia activity in the brain, but did not affect inflammation nor behavior, TNFR1 antagonist treatment did not affect cardiac outcome, surprisingly increased (neuro)inflammation and sympathetic activity, and negatively affected behavior. In general, effects of Enbrel treatment appeared in between.
Hence, the general profile after TNFR2 stimulation, rather than that after TNFR1 inhibition, may suggest beneficial effects of selective TNFR2 intervention over non-specific TNF inhibition. However, the concerning increased mortality after this early TNFR2 agonist treatment requires additional studies regarding timing and dosing.

4.1. Effects on the heart

4.1.1. Mortality

In accordance with literature in this strain of mice (van den Borne et al., 2009), mortality was about 25% in saline treated MI mice. Mortality was not affected by Enbrel treatment, but approximately doubled by both TNFR1 antagonist and TNFR2 agonist treatment. TNF receptors affecting cardiovascular mortality was previously indicated by ablation of TNFR2 receptor increasing mortality and ablation of TNFR1 receptor inhibiting mortality (Higuchi et al., 2004). This is in accordance with TNFR1 receptors being toxic and TNFR2 receptors being protective in a mouse model of MI (Monden & Monden, 2007). Although TNF is reported to interfere with reactive fibrosis associated with cardiac rupture (Monden & Monden, 2007) and was positively correlated with cardiac arrhythmias after MI (Xiao et al., 2008), mice that died spontaneously or had to be sacrificed prematurely showed increased lung weights and pronouncedly increased circulating Leu2 levels, indicating development of fatal congestive heart failure, rather than cardiac rupture or fatal arrhythmias.

Since the infarcted area looked more oedematous after TNFR2 agonist treatment, infarct healing could have been affected, as has been described for TWEAK, modulating TNF signalling in a similar way as TNFR2 (Pachel et al., 2013). However, in contrast to the present study, TWEAK was also associated with impaired left ventricular function. Moreover, since infarct parameters (length and collagen content) were described for TWEAK, modulating TNF signalling in a similar way as TWEAK was also associated with impaired left ventricular function. As TNF uncouples the beta receptors from adenylyl cyclase. In our previous study in MI mice, we did not observe increased TNF plasma levels (Gouweleeuw et al., 2017a, 2017b). As discussed above this may suggest TNFR2-rather than TNFR1 signalling, hence limiting the effects of TNFR1 inhibition.

4.1.2. Cardiac function

Coronary artery ligation in mice resulted in 30% infarction of the left ventricle. An increased left ventricular volume to preserve stroke volume at reduced ejection fraction confirmed post-MI left ventricular dysfunction, but without overt heart failure as lung weights had not increased. TNFR2 agonist treatment, but not TNFR1 antagonist treatment, significantly improved left ventricular function. Effects of Enbrel treatment appeared in between. This would be consistent with an anticipated myocyte protection by stimulation of TNFR2 receptors (Defer et al., 2007; Monden & Monden, 2007), but not with the anticipated positive effect of inhibition of the toxic effects of the TNFR1 receptor. TNFR1 antagonism significantly increased baseline heart rate, associated with a blunted heart rate response on beta adrenergic stimulation; signs of increased sympathetic activity (van den Borne et al., 2009). A significant contribution of the interaction between TNF signalling, sympathetic nervous system activation and cardiovascular regulation, is supported by the effects of soluble TNF inhibition in a model of spinal cord injury (Mironets et al., 2018). A shift in autonomic balance may indeed have played a role. Vagal nerve stimulation after MI was associated with increased TNF expression, accompanied by higher TNFR2 but lower TNFR1 expression, which was interpreted as protection (Katate et al., 2010). Moreover, TNF decreased contractile function in isolated rat cardiomyocytes (Feldman et al., 2000), as TNF uncouples the beta receptors from adenylyl cyclase.

4.2. Effects on inflammation

As in our previous study no increased levels of proinflammatory cytokines were observed in MI mice (Gouweleeuw et al., 2017a, 2017b), peripheral inflammation was assessed by the number of circulating white blood cells and plasma levels of the inflammation marker lipocalin 2.

4.2.1. Blood cells

Cell count after MI appeared lower for all blood cells, suggesting general hemodilution because of fluid retention after MI, which would be consistent with the increased left ventricular volumes. However, effects of treatment appeared rather distinct. White blood cell count was partly restored by selective TNF receptor modulation, but completely normalized with Enbrel, suggesting receptor modulation working in concert. However, eosinophils, monocytes and basophils seemed to interfere with remodelling of the spared myocardium after MI through both TNFR1 and TNFR2 signalling (Monden & Monden, 2007). Surviving mice still developed substantial infarcts (30% of the left ventricle), suggesting activation of the innate immune system (TNF) for healing, providing a relevant model to investigate effects of the different TNF interferences in the present study.

Table 4

Density and coverage of GFAP positive astrocytes in the hippocampal areas; CA1, CA3 and Dentate Gyrus (DG). MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2. *: significantly different from sham. #: significantly different from saline treated MI. S: significantly different compared to general TNF inhibition by Enbrel.

| Group                        | CA1 density (#/AOI) | CA1 coverage (pixel.104/AOI) | CA3 density (#/AOI) | CA3 coverage (pixel.104/AOI) | CA3 coverage (pixel.104/AOI) | DG density (#/AOI) | DG coverage (pixel.104/AOI) |
|------------------------------|---------------------|-------------------------------|---------------------|-------------------------------|-------------------------------|---------------------|-----------------------------|
| Sham + saline (n = 3–5)      | 57 ± 4              | 20.6 ± 0.9                    | 50 ± 10             | 22.4 ± 2.3                    | 54 ± 5                       | 19.9 ± 2.7          | 23.4 ± 2.7                  |
| MI + saline (n = 4)          | 60 ± 2              | 15.9 ± 0.8                    | 52 ± 3              | 19.3 ± 1.2                    | 54 ± 4                       | 19.9 ± 1.7          | 21.9 ± 0.8                  |
| MI + Enbrel (n = 5)          | 53 ± 5              | 17.3 ± 1.3                    | 49 ± 5              | 20.3 ± 1.5                    | 57 ± 5                       | 21.9 ± 0.8          | 23.3 ± 1.7                  |
| MI + R1 antagonist (n = 2–3) | 58 ± 1              | 21.2 ± 1.0 (#)                | 58 ± 16             | 23.9 ± 2.4                    | 70 ± 8                       | 23.3 ± 1.7          | 19.0 ± 2.4                  |
| MI + R2 agonist (n = 4)      | 59 ± 4              | 17.7 ± 2.0 (#)                | 45 ± 4              | 19.0 ± 1.4                    | 54 ± 9                       | 19.0 ± 2.4          | 19.0 ± 2.4                  |

Fig. 7. Sholl analysis of microglia profiles. Inlay shows schematic presentation of Sholl analysis for each experimental group (n = 20 per group). No differences were observed between saline sham and saline MI groups. All actively treated groups differed significantly from these sham and the MI saline control groups (p < 0.001). MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2.
above indicates that TNFR1 antagonist-, in contrast to Enbrel- or TNFR2 agonist treatment. Literature specifically increased after TNFR1 antagonist treatment. Literature identifies different roles for the TNF receptors via T-cells (Yang et al., 2019). However, as the present study was not focused on a more detailed discrimination of the different inflammatory cells, unfortunately no further conclusions could be drawn here. Nevertheless, the above indicates that TNFR1 antagonist-, in contrast to Enbrel- or TNFR2 agonist treatment, may even increase peripheral inflammation.

Striking finding was the significant increase in platelet count after TNFR2 agonist treatment. In addition to their well-established role in thrombosis, platelets have been recognized as inflammatory and immune cells (von Hundelshausen & Weber, 2007). In an in vivo model for arteriolar thrombosis, a prothrombotic effect of TNF seemed amplified by the absence of TNFR1 and requires TNFR2 (Pircher et al., 2012). However, both TNFR1- and R2 deficient mice have normal thrombogenesis (Cambien et al., 2003). TNF reduced platelet count, which entirely depended on TNFR1, but without actual presence of TNFR1 or TNFR2 receptors on platelets (Tacchini-Cottier et al., 1998), suggesting that TNF works on host cells rather than on the platelets themselves. Platelets play an important role in leukocyte adhesion (von Hundelshausen & Weber, 2007), which mainly take place during the early healing phase. The sample collection at 18 days post MI may have passed beyond this phase, but the dose–effect experiment for the TNFR2 agonist 4 days after MI, did not show increased platelet number at any dose.

**Table 5**
Inflammatory markers measured in homogenates of the dorsal hippocampus and prefrontal cortex. Expression was relative to expression of actin. Upper panel presents examples of western blots in hippocampal tissue for the different experimental groups. Sham values were set to 1 and expression per groups was expressed relative to sham. Number of included mice between brackets. -: none or only one of the samples showed a measurable band; MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor2; Lcn2 = lipocalin 2. *: significantly different compared to general TNF inhibition by Enbrel.

|            | Hippocampus | Prefrontal Cortex |
|------------|-------------|-------------------|
| MI + Enbrel| 1.00 ± 0.08 | 1.00 ± 0.20       |
| TNFR1 pellet| 1.00 ± 0.11 | 1.00 ± 0.13       |
| TNF supernatant | 1.00 ± 0.11 | 1.00 ± 0.13       |
| Lcn2 pellet | 1.00 ± 0.13 | 1.00 ± 0.04       |
| Enbrel MI | 1.00 ± 0.08 | 1.00 ± 0.20       |
| TNFR1 | 1.00 ± 0.11 | 1.00 ± 0.13       |
| TNF | 1.00 ± 0.11 | 1.00 ± 0.13       |
| Lcn2 | 1.00 ± 0.13 | 1.00 ± 0.04       |

**Table 6**
Double cortin (DCX) positive cells in the dentate gyrus of the hippocampus (neurogenesis) and the piriform cortex (neuroplasticity) measured as DCX positive area per length. MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor2. *: significantly different from sham. #: significantly different from saline treated MI. $: significantly different compared to general TNF inhibition by Enbrel.

| Group          | Hippocampus DCX | Prefrontal Cortex DCX |
|----------------|-----------------|-----------------------|
| MI + Enbrel    | 1.00 ± 0.08 (10)| 1.00 ± 0.20 (7)       |
| TNFR1 pellet   | 1.00 ± 0.11 (3) | 1.00 ± 0.13 (8)       |
| TNF supernatant| 1.00 ± 0.11 (3) | 1.00 ± 0.13 (8)       |
| Lcn2 pellet    | 1.00 ± 0.13 (3) | 1.00 ± 0.04 (3)       |
| Enbrel MI      | 1.00 ± 0.08 (10)| 1.00 ± 0.20 (7)       |
| TNFR1 | 1.00 ± 0.11 (3) | 1.00 ± 0.13 (8)       |
| TNF | 1.00 ± 0.11 (3) | 1.00 ± 0.13 (8)       |
| Lcn2 | 1.00 ± 0.13 (3) | 1.00 ± 0.04 (3)       |

4.2.2. Lipocalin 2 (Lcn2)
Plasma levels of Lcn2 were measured as general and sensitive marker for inflammation (Gouweleeuw et al., 2017a, 2017b). In agreement with left ventricular dysfunction without overt heart failure, Lcn2 levels in MI mice had not increased. Surprisingly, anti TNFR1 treatment significantly increased Lcn2 levels, as lower levels of Lcn2 were anticipated since TNFR1 receptor stimulation is associated with strong pro-inflammatory effects. However, the tendencies for higher left ventricular volumes and
lower stroke volume and ejection fraction values compared to the other MI groups may represent a lower cardiac function, which could be in line with the higher Lcn2 levels. Parameters of peripheral inflammation were only measured at the time of sacrifice. In hindsight, measurement of early cytokine responses after TNFR1 antagonist treatment may have given us a better insight in the pro/anti-inflammatory effects of this treatment.

Thus, whereas Enbrel and TNFR2 agonist treatment seemed to have no effect on peripheral inflammation, surprisingly, TNFR1 antagonist treatment was associated with an increased rather than decreased peripheral inflammatory state in MI mice.

4.3. Effects on the brain

4.3.1. Neuroinflammation

Neuroinflammation was measured as expression of TNF and its receptors, as well as microglia and astrocyte activity in the brain. Also, expression of Lcn2 in the hippocampus and prefrontal cortex were obtained, as a close interaction between TNF signalling and Lcn2 has been described (Naude et al., 2012). Lcn2 is produced after activation of TNFR1 receptors, as well as microglia and astrocyte activity in the brain. Also, expression of lcn2 in the hippocampus and prefrontal cortex were described (Naude et al., 2012). Lcn2 is produced after activation of TNFR1 receptors, as well as microglia and astrocyte activity in the brain. Also, expression of lcn2 in the hippocampus and prefrontal cortex were described (Naude et al., 2012).

Table 7

Summary of behavioral test outcomes. NOR = Novel Object Recognition; NLR = Novel Location recognition; MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2. *: significantly different from sham. #: significantly different from saline treated MI. $: significantly different compared to general TNF inhibition by Enbrel.

| group | sham + saline | MI + saline | MI + Enbrel | MI + R1 antagonist | MI + R2 agonist |
|-------|--------------|------------|-------------|-------------------|---------------|
|       | 12–17        | 10–16      | 10–15       | 10–14             | 10–13         |
| Open field |             |            |             |                   |               |
| Distance (cm) | 2675 ± 100   | 2768 ± 148 | 2293 ± 175  | 2230 ± 273#       | 2589 ± 181    |
| Corner (%) | 46 ± 2       | 45 ± 2     | 47 ± 3      | 42 ± 3            | 41 ± 2        |
| Grooming (%) | 13 ± 1       | 13 ± 1     | 11 ± 2      | 12 ± 2            | 15 ± 1        |
| Exploration (%) | 95 ± 1       | 97 ± 1     | 93 ± 2      | 86 ± 5*#          | 94 ± 2        |
| Resting (%) | 0.42 ± 0.10  | 0.60 ± 0.14| 0.67 ± 0.14 | 0.80 ± 0.19       | 1.09 ± 0.30*  |
| Social interaction | 4.6 ± 0.9   | 1.9 ± 0.9* | 8.2 ± 2.4 | 13.5 ± 5.5*#      | 5.4 ± 1.4     |
| Preference (%) | 34 ± 4       | 30 ± 4     | 42 ± 5      | 39 ± 7            | 45 ± 7        |
| Cognition | 67 ± 4       | 75 ± 4     | 69 ± 5      | 77 ± 3            | 64 ± 4#       |
| Struggle (%) | 33.2 ± 2.3   | 36.0 ± 2.9 | 36.7 ± 3.4 | 35.9 ± 3.8        | 32.8 ± 3.5    |
| Swim (%) | 38.4 ± 2.9   | 38.0 ± 4.1 | 32.8 ± 2.4 | 41.9 ± 5.0        | 39.8 ± 4.7    |
| Float (%) | 26.7 ± 3.0   | 23.1 ± 3.6 | 28.3 ± 3.5 | 18.7 ± 4.1        | 27.5 ± 4.1    |
| Anhedonia |             |            |             |                   |               |
| Sucrose preference (%) | 89.4 ± 1.3 | 86.2 ± 2.5 | 87.0 ± 2.4 | 86.5 ± 2.3        | 85.7 ± 2.3    |

Fig. 8. Main results of the dose-effect study of the TNFR2 agonist 4 days after MI. A: Survival curves; B: Left ventricular ejection fraction as measure for cardiac function; C: Collagen in the infarct area as measure for infarct healing; D: Number of platelets in blood. MI = myocardial infarction; R1 = TNF receptor1; R2 = TNF receptor 2. *: significantly different from sham. #: significantly different from saline treated MI.
soluble proteins. Both TNFR1 antagonist- and TNFR2 agonist treatment, but not Enbrel, increased pellet expression of TNF in the prefrontal cortex, but not in the hippocampus, hence indicating an increased membrane bound TNF (precursor) expression in the prefrontal cortex. Altered expression between the two fractions might then indicate a shift; translocation. These shifts, rather than absolute expression patterns, seemed consistent for the interventions. A reduction in the supernatant, accompanied by increased expression in the pellet fraction for the TNFR1 receptor in TNFR1 inhibition (TNFR1 antagonist and Enbrel), but not for TNFR2 stimulation, in both the hippocampus and prefrontal cortex. Opposite shifts for Lcn2 expression in the hippocampus and prefrontal cortex would further support brain-area specific effects, as was seen for TNF expression.

Microglia are the immune cells of the brain. Upon stimulation they can become activated and thereby change their morphology. Classical activation consists of retraction of processes (dendrites); de-ramification, increased cell body and changes towards an amoebic and/or phagocytotic phenotype (Beynon, Walker, 2012). This microglia activation has been reported in rats with MI (Dworak et al., 2012), but not so much in mice (Gouweleeuw et al., 2017a, 2017b), and would be associated with injury and inflammation related signals. Alternative activated microglia show opposite morphological changes, as they become hyper-ramified as response to non-pathological stimuli, such as stress (Beynon & Walker, 2012), and may represent a state of prolonged altenrness (Tashbighou et al., 2018) and tissue protection. However, microglia hyper-ramification in the hippocampal DG was associated with depression (Hellwig et al., 2016). In the present study, classic microglia activation was observed only after TNFR1 antagonist treatment and only in the area of the hippocampus enclosed by the DG, the hilus. Alternative activated hyper-ramified microglia, noted as reduced activity, were found in the PVN in all MI groups but most pronounced in the TNFR2 agonist treated group. Subsequent Sholl analysis suggests that the higher dendrite area in saline treated MI mice was due to thickening rather than increased number of dendrites, whereas in the treated MI groups at least part of the increased area could be explained by increased number of dendrites. The PVN is a very important area in coupling cardiovascular regulation and higher brain functions (Badner, 2010a, 2010b). In rats with MI, this area showed prolonged inflammatory responses, indicated by increased expression of TNF and IL1beta (Kang et al., 2008), and classical microglia activation (Dworak et al., 2012). Why mice with MI respond different from rats in this regard would be a subject for further investigation. However, our findings are supported by the study of Frey et al. (Frey et al., 2014) showing no major effects on neuroinflammation in other brain areas in MI mice. In the present study, the observed hyper-ramification of microglia in the PVN may then represent a state of increased alertness rather than reflecting neuroinflammation.

Astrocytes are suggested to be involved in the heart-brain inflammation network after myocardial infarction (Bascunana et al., 2020). Whereas astrocyte density was not affected by MI nor by treatment, coverage was lower after MI, reaching statistical significance for the CA1 area. This is in accordance with literature data (Isegawa et al., 2014). Treatment with the TNFR1 antagonist increased astrocyte coverage in all hippocampal areas, statistically significant in the CA1 area. In general, microglia activation and astrocyte activation seem to change in parallel after myocardial infarction (Rinaldi et al., 2015). In the present study, actual (classical) activation of microglia and astrocytes were not observed after MI, but a paralleled lower activity may indeed have been observed. TNFR1 antagonist treatment seemed to enhance activity of both cell types in the hippocampus, again indicating a pro- rather than anti-inflammatory effect.

Thus, none of the measured parameters indicated overt neuroinflammation 18 days after MI. Overall, effects of the different TNF interventions revealed minor localized proinflammatory effects of TNFR1 antagonist treatment, and none or tissue protective (microglia) effects of TNFR2 agonist treatment, while effects of Enbrel appeared in between.

### 4.3.2. Behavior

In patients with myocardial infarction the risk of developing mental problems, such as depression and cognitive decline is increased. In our previous studies (Schoemaker & Smits, 1994) as well as studies by others (Grippo et al., 2003), behavioral changes that can be interpreted as depressive-like behavior were observed in rats with MI, and appeared sensitive to inhibition of TNF by Enbrel (Grippo et al., 2003). In later studies however, these behavioral effects did not present as clear anymore (Gouweleeuw et al., 2016). In an extensive behavioral study in mice 6 weeks after MI (Frey et al., 2014), several behavioral changes were reported that were interpreted as depressive-like symptoms or cognitive impairment. In contrast, in the present study, no behavioral effects of MI were found. The timing after MI, 6 weeks versus the 18 days after MI in the present study, may have played a role. However, comparing the exact same parameters in the present study to those in the study of Frey and co-workers, time in the center of the open field and sucrose preference at 2–3 weeks, similar results were obtained. The lack of behavioral consequences of MI in the present study may also relate to the observation that our MI mice displayed left ventricular dysfunction rather than heart failure, which may indicate a milder pathological condition. Accordingly, the lack of peripheral- and neuroinflammation 18 days after MI suggests completion of the healing process and hence cessation of the inflammatory process, although substantial infarcts implicate transient activation of the innate immune system for healing.

Whereas Enbrel and the TNFR2 agonist had no effect on behavior, the TNFR1 antagonist resulted in impaired exploration in the open field. This lower exploration may then represent a change toward a more pro-inflammatory status in mice treated with the TNFR1 antagonist; increased plasma Lcn2, hippocampal microglia and astrocyte activation, and increased TNF expression in the prefrontal cortex. Moreover, TNFR1 treatment was associated with sympathetic activation. The interrelation between the sympathetic nervous system and inflammation is rather complex as it may integrate signals from different pathways. Sympathetic outflow to the cardiovascular system is mainly regulated in the
PVN. TNF-α in the PVN contributes to sympathoexcitation in heart failure (Kang et al., 2010). The increase in PVN noradrenaline levels as well as the increased sympathetic nerve activity after myocardial infarction in rats could be completely prevented by the cytokine synthesis inhibitor pentoxifyllin or by etanercept (Embrel). Alternatively, inflammation is associated with increased sympathetic tone in cardiovascular disease (Yu et al., 2010). Accordingly, in the present study the pro-inflammatory effect of the TNFR1 antagonist treatment was associated with increased sympathetic activity. As autonomic balance also plays an important role in balancing pro- and anti-inflammatory influences, a shift towards higher pro-inflammatory sympathetic nervous system activity may have contributed to inflammation as well as depressive-like behavior (Raison et al., 2006), seen in our TNFR1 antagonist treated mice.

4.4. Pharmacology of TNF signalling

Although our research question was rather straightforward; whether selective TNF receptor interventions would be preferable over general TNF inhibition in mice with MI, the complexity of TNF signalling renders interpretation challenging. The endogenous ligand TNF presents itself as transmembrane protein with its membrane-bound and soluble molecule. Soluble TNF preferably activates TNFR1, resulting in pro-inflammatory effects and cell death, while membrane TNF can activate both TNF receptors, with TNFR2 stimulation being associated with cell survival and cytotoxicction (Medler & Wajant, 2019). Accordingly, in mice with MI TNF was shown toxic via TNFR1 and protective via TNFR2 (Monden & Mondon, 2007). In the present study, it was speculated that expression of TNF and its receptors in the pellet and the supernatant of brain homogenates represented the membrane-bound and soluble fractions, respectively. Shifts from one phase to the other, rather than absolute expression patterns, seemed consistent for the interventions. A reduction in the supernatant, accompanied by increased expression in the pellet fraction for the TNFR1 receptor in TNFR1 inhibition (TNFR1 antagonist and Embrel) would be in agreement with general receptor dynamics; receptor binding and subsequent inactivation by internalisation evokes increased expression of new receptors on the membrane. Lcn2 is produced after activation of TNFR1 receptors, and subsequently inhibits TNFR2 mediated cell survival (Naude et al., 2012). Opposite shifts for Lcn2 expression in the hippocampus and prefrontal cortex suggest brain-area specific effects. Besides the increased TNF expression in prefrontal cortex pellets, TNFR2 agonist treatment did not affect absolute TNF receptor expressions nor seemed to induce shifts between pellet and supernatant receptor expression.

Most of the studies regarding the function of the TNF receptors are performed in receptor knock-out mice, disregarding the complex interplay between the two TNF receptors in general (Naude et al., 2011) and in cardiomyocytes specifically (Defer et al., 2007). Accordingly, an older study in cell cultures as well as in vivo mouse models (Sheehan et al., 1995) using mouse antibodies against TNFR1 (p55) and TNFR2 (p75), indicated that the two antibodies appear selective for p55 or p75 in cell cultures, while in vivo the p55, but not p75 antibodies protected against lethal endotoxin shock, and both antibodies blocked skin necrosis after TNF treatment. To add to this complexity, expression of the 2 receptors may change differently and inconsistently after MI, as either more TNFR2 at similar TNFR1 expression (Monden & Mondon, 2007), or the opposite, more TNFR1 than TNFR2 (Hamid et al., 2009) have been reported in the heart. In the brain, we recently showed doubled TNFR1 and strongly declined TNFR2 in the face of increased TNF precursor expression (Gouweleeuw et al., 2017a, 2017b). Apart from receptor subtype, the action of TNF also depends on concentration and duration of exposure, (Schulz & Heusch, 2009). The differences in dissociation constants between TNFR1 and TNFR2 would indicate that high TNF levels would interact with both TNFR1 and TNFR2, while low levels may only stimulate TNFR2 pathways (Monden & Mondon, 2007). This may provide an explanation of our unexpected finding of pro-inflammatory effects of TNFR1 antagonist treatment; partial agonism. According to general pharmacodynamics, when a partial agonist would be introduced at high ligand levels, it indeed will act as an antagonist, but with low ligand levels it may even act as agonist. This suggestion would be supported by the study of Sheehan et al (Sheehan et al., 1995), showing that the 55R-170 antibody is not capable of completely inhibiting the binding of labeled TNF. Moreover, the 55R-170 antibody could inhibit, but not completely prevent the effect of TNF on the L292 killing. Our previous study showed no increased circulating TNF levels after MI (Gouweleeuw et al., 2017a, 2017b). Accordingly, no MI-induced increase in TNF expression in the brain was observed. Hence, theoretically it could be that the lack of endogenous TNF stimulation may have turned the TNFR1 antagonist HM1097 55R-170, into a partial agonist. On the other hand, potential partial agonistic effects of HM1097 55R-170 would by minor, and become only visible at higher HM1097 55R-170 concentrations, which both seem not evident in the present study. Hence, the ambivalent effect of TNF, organ specific changes and receptor dynamics would make prediction of effects of specific receptor interference in a pathological condition with changing TNF levels at least challenging.

5. Limitations

In the present study extensive data have been collected in order to gather further insight in the role of inflammation, reflected by TNF signalling, in heart-brain interactions after MI.

Coronary artery ligation in mice resulted in 30% infarction of the left ventricle. Post-MI left ventricular dysfunction was confirmed, however without overt heart failure, suggesting mild cardiovascular pathology. This was reported before in MI mice (Frey et al., 2014) and has recently been extensively reviewed (Daskalopoulos et al., 2019) as a concern for wider use of this model. In accordance with the absence of heart failure, no signs of (neuro) inflammation nor depressive-like behavior were observed. Nevertheless, our mice developed substantial infarcts that would have evoked activation of the innate immune system (TNF) for healing. Therefore, the MI mice in the present study are providing a relevant model to investigate effects of the different TNF system interferences.

Most of the knowledge regarding the function of the two TNF receptors is derived from studies with genetically modified mice, lacking one of the receptors. Mice experience the absence of that receptor their whole life, with all opportunities to compensate for the function. Though these models may give a good insight in the role of that specific receptor, it ignores the contribution of TNF receptor cross-talk (Naude et al., 2011). For that, results from those studies may be incomparable with the pharmacological interference used in the present study; the latter may potentially be more relevant for therapeutic goals.

Mortality and premature sacrifice were highest in the TNFR2 agonist treated group. Survivors could therefore represent a selective group of “strong” animals, rather than general TNFR2 agonist-treated MI mice. Although we cannot fully exclude this aspect, measurements of infarct size (absolute length as well as relative to the left ventricular circumference) and collagen content of the infarct in this group do not indicate a selection of mice based on smaller infarcts and consequently better cardiac function.

In addition to the obvious reason of interference of the estrus cycle in female mice and better comparison with literature mainly obtained in male mice, our choice for only using male mice was based on our own experience in female rats, not developing heart failure nor depressive behavior after MI, and hence differing from female humans (non-published data). Still, we are well aware of sex-related differences of MI, regarding inflammatory responses and behavioral consequences in humans (Mommersteeg et al., 2019).

In the present study, mice were studied at one time point after induction of MI and for the main study at one dose. We realize that this is a time point chosen in a series of processes activated after MI, each with its
own time course. Hence, the findings in the present study may appear different when treatment would have been started after the healing period, or evaluation at different time points, as well as using different dosages. Especially the increased mortality for both receptor-specific interferences in the early phase could suggest preference for a delayed start of treatment, as the early inflammatory response may be necessary for healing.

Finally, two research institutes were involved because of their specific expertise. Although serious attempts were made to ensure comparability by the same investigator and equipment, we cannot exclude that this aspect may have increased variation. At the least it limited the group size for some measurements, which may have hampered strong conclusions. For these specific aspects, follow-up studies need to be performed.

6. Conclusion

In the present study, effects of specific modulation of TNFR1 and TNFR2 activity aimed at inhibition of inflammation, were studied in a mouse model of MI, and compared to non-specific TNF-alpha inhibition. Apart from undesired increased mortality, the general beneficial profile after the TNFR2 stimulation, rather than the unfavourable effects of TNFR1 inhibition, would render TNFR2 stimulation referable over general TNF inhibition in MI with comorbid depression. However, follow-up studies regarding optimal timing and dosing are needed.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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