Th1/M1 conversion to Th2/M2 responses in models of inflammation lacking cell death stimulates maturation of monocyte precursors to fibroblasts

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INTRODUCTION

The immune system uses similar tools whether responding to infections or sterile tissue injury. The same steps of an inflammatory response followed by resolution and healing take place under both circumstances (1). For example, the liberation of heat shock proteins (2), mitochondrial DNA (3), HMGB1 (high-mobility group box 1) (4, 5), and a host of other internal components from dead cells [alarmins (6)] can trigger inflammation in the absence of infection. Others, such as adenosine (7), apoptotic cell membranes (8, 9), or self DNA (10), can at the same time initiate the anti-inflammatory response that will bring the organism back to homeostatic balance. Both types of influences may act via chemokines that attract different subsets of immunocytes or may skew the subsequent maturation of the cells (11). In contrast to that schema, this report describes and defines similar responses, both proinflammatory and anti-inflammatory, in the absence of cell death and its associated signaling.

Our murine model of intermittent cardiac ischemia and reperfusion (I/R) in vivo, despite the absence of cell death, still results in an immune response. This treatment, which leads to cardiomyopathy, is the daily repetitive brief (15 min) I/R of a coronary artery, in which the major chemokine induced is monocyte chemotactant protein-1 (MCP-1) and histology reveals interstitial fibrosis but no necrosis (12). We have shown that the fibrosis results from an influx of CD34+CD45+ monocytes that become fibroblasts making collagen (13). As in many models of fibrosis, these cells bear the markers of M2 macrophages while also assuming the shape and markers of fibroblasts (14). Because this model does not result in the death of cardiomyocytes, the mediators inducing the monocyte influx and transition into fibroblasts are limited in comparison to those accompanying a myocardial infarct, including both proinflammatory and anti-inflammatory or profibrotic influences (12). Neither the influx nor the fibrosis occurs in animals when MCP-1 is genetically deleted, indicating the necessity of this chemokine for initiation of the immune response leading to fibrosis (15, 16).

To simulate these events using human cells in vitro, we designed a transendothelial migration (TEM) assay using human cardiac microvascular endothelial cells (HCMEC) growing on a model basement membrane over an insert with 8 µm pores. Human mononuclear cells are attracted to migrate through the endothelium and the pores in response to MCP-1 below the insert (17). Some of the transmigrating monocytes become M1 macrophages, whereas others become M2 macrophages that mature into fibroblasts, and the numbers of the latter vary with different stimuli. We have used this TEM model to investigate the response of
monocytes to Fc receptor engagement (17), angiotensin-II (18), and ROCK-1 knockdown (19), with parallel results to the corresponding in vivo mouse models. As with the in vivo models, MCP-1 is necessary for the migration of the monocytes through the endothelium (17). Classically, MCP-1 is regarded as a stimulus for M1 development, but MCP-1 may be accompanied by other chemokines to which a subset of monocytes capable of maturation into M2 macrophages may migrate (20). A major inducer of the M2 phenotype is the Th2 cell product, IL-13 (11), and addition or production of IL-13 is required for the formation of monocyte-derived fibroblasts in our in vitro assay (21). This suggests that it may be pertinent to human disease states.

Since IL-13 has been associated with fibrosis in the aging heart and in other organs (21–23), we elected to investigate its presence and kinetics in our cardiomyopathy model as well as in our in vitro model. Although IL-13 can be made by many kinds of cells, the source of it in an inflammatory infiltrate is likely to be predominantly from T cells, and so we verified the presence of T lymphocytes. Our data suggest that the presence of a Th2 response is integral to the fibrosis in these models of immune activation, in which there is an initial induction of the Th1 and M1 responses that then evolve into a Th2/M2 response in the absence of cell death. The Th2 response is obligate for the formation of myeloid fibroblasts and the development of a fibrotic cardiomyopathy.

RESULTS

MURINE IN VIVO MODEL

To quantify the number of M2 macrophages and T lymphocytes in heart tissue from shams or animals treated with the I/R protocol, we digested the heart to isolate cells and performed flow cytometry to measure the expression of the markers CD45 (cells of hematopoietic origin), CD301 (M2 macrophages), and CD3 (T cells). These markers were combined with fluorescence from calcein, which identifies viable cells. Mechanical disruption and digestion of the heart tissue as optimized in our protocol breaks cardiomyocytes apart, so the live cells are all non-myocytes, representing endothelial cells, smooth muscle cells, fibroblasts, and any hematopoietic cells that may have entered the heart. In Figure 1, it can be seen that the number of CD45+CD301+ M2 macrophages increased after I/R, as did the number that were CD45+CD301+ collagen type I+ (and 38 ± 7% of the CD45+CD301+ were positive for collagen type I), consistent with our previous histological results that these cells are in the heart and are making collagen (13, 14). In addition, it can be seen that there were also T lymphocytes, identified by their CD45 and CD3 markers. Of note, in human heart tissue from cases of cardiomyopathy, we also found CD45+ cells in a perivascular location expressing either a myofibroblast marker (α-smooth muscle actin) or the T cell marker CD3, as well as CD301+ α-smooth muscle actin+ cells (Figure 2).

FIGURE 1 | Flow cytometry for the indicated markers on cells harvested from mouse hearts after 5 days of I/R or from sham-treated animals. (A) Through (G) are representative histograms for sham-treated animals. (D) Through (F) are representative histograms for I/R animals. (G) Through (I) are summary data graphs. (A,D,G) Represent the percent of all viable cells (calcein positive) that were positive for both CD45 and CD301. (B,E,H) are the percent of all cells positive for CD45 that were also positive for both CD301 and collagen type I. (C,F,I) are the percent of all viable cells that were positive for both CD45 and CD3 (n = 3, *p < 0.05).
MURINE CYTOKINES
In our mouse model, we were interested in the temporal gene expression of MCP-1 and IL-13. Our results showed high levels of MCP-1 mRNA early during the I/R response that waned thereafter (Figure 3). The Th2 product, IL-13, showed a later peak, but was present throughout the 2-week period of I/R. Neither of these responses took place in mice deficient in MCP-1 expression (genetic knockout, KO). IL-33, another mediator of interest because of its ability to induce IL-13, was increased with a similar pattern to that of IL-13. Unlike IL-13, IL-33 was induced in both the wild-type (WT) and the KO mice, and its expression was maintained at 14 days in the KO but not the WT mice.

We also measured specific cytokines that would characterize the T cells in the mouse heart as Th1 or Th2 by performing protein arrays on sham and I/R hearts after 3 days of treatment (Table 1).

The Th2-related IL-13 protein was the most elevated protein in I/R over sham levels. The Th2 cytokine IL-4, by contrast, was not as increased, and neither was the major Th1 cytokine, IFN-γ.

HUMAN IN VITRO MODEL
In our human in vitro TEM assay, we stained macrophages for M1 versus M2 markers after two different times of migration,

| Cytokine | Fold change from sham | Accession number |
|----------|-----------------------|-----------------|
| G-CSF    | 0.6 ± 0.2 | NP_034101.1 |
| GM-CSF   | 0.9 ± 0.1 | NP_034099.2 |
| IFN-γ    | 1.8 ± 0.4 | NP_032363.1 |
| IL2      | 0.9 ± 0.1 | NP_032392.1 |
| IL3      | 1.1 ± 0.2 | NP_034688.2 |
| IL4      | 1.9 ± 0.6 | NP_067258.1 |
| IL5      | 1.7 ± 0.3 | NP_034688.1 |
| IL6      | 1.4 ± 0.3 | NP_112445.1 |
| IL9      | 0.7 ± 0.1 | NP_032399.1 |
| IL10     | 1.0 ± 0.2 | NP_034678.1 |
| IL12p40p70 | 1.1 ± 0.1 | NP_032378.1 (p40) |
| IL12p70  | 1.3 ± 0.1 | NP_001152896.1 (p35) |
| IL13     | 3.7 ± 0.9 | NP_032381.1 |
| IL17     | 2.4 ± 0.8 | NP_034682.1 |
| MCP-1    | 1.5 ± 0.2 | NP_035463.1 |
| MCP-5    | 0.8 ± 0.1 | NP_035461.2 |
| RANTES   | 1.1 ± 0.1 | NP_038681.2 |
| SCF      | 1.7 ± 0.2 | NP_038626.1 |
| sTNFR1   | 2.2 ± 0.5 | NP_035739.2 |
| TNF      | 2.0 ± 0.7 | NP_038721.1 |
| Thrombopoietin | 3.1 ± 0.9 | NP_033405.1 |
| VEGF     | 1.9 ± 0.6 | NP_001020421.2 |

*Cardiac protein densities were obtained from three sham and three I/R animals normalized to the array positive controls. Densities from I/R animals were divided by the mean densities of the shams and averaged. Only proteins that gave a positive signal on the array are included.
To characterize further the kinetics of macrophage M2 development, we performed quantitative PCR on in vitro samples. The cells were stained for the external markers CD86, CD206, and CD301, and after fixation and permeabilization, for the internal markers NOS2 and prolylhydroxylase (labeled prolylhydroxylase in the figure). DAPI was used to stain cell nuclei blue. The insets are cells at either higher magnification (CD301) or cells with external staining and an internal marker (CD86 plus NOS2 and CD206 plus prolylhydroxylase) with white arrows extending from the label. The white arrow within the center panel indicates a CD86 positive cell in the low magnification field, and the inset at top right shows a cell that is double positive for CD86 and CD206. White bars represent 20 µm.

DISCUSSION

Under homeostatic regulation, inflammation is followed by its resolution (1). In infections, removal of the responsible agent leads to the quieting of the immune response. In injury, cell death by various modes leads to active anti-inflammatory signaling. In our in vivo and in vitro models, in which cell death is avoided, it is clear that the same transition from proinflammatory to anti-inflammatory responses can take place, as well as a subsequent profibrotic environment. In both simplified in vivo and in vitro models, the initiating factor is MCP-1, considered a proinflammatory cytokine and as we have found in other studies (27), followed by a Th2 response (IL-13) and downregulation of Th1 gene expression.

IL-13 can also be upregulated by IL-33, which is a member of the IL-1 family (28, 29). We measured the gene expression of IL-33 in our human model, and found that it was highly expressed both early and late during TEM (Figure 6).

HUMAN CELL IDENTIFICATION

To characterize further the kinetics of macrophage M2 development, we performed quantitative PCR on in vitro human transmigrated cells and isolated monocytes from blood. We found that the shift from inflammation to fibrosis may be mediated at least partially by IL-13, which is well known for this effect (30, 31).

Indeed, the targets of IL-13, M2 macrophages, were present in the human model, and found that it was highly expressed both early and late during TEM (Figure 6).
mediators. The source of the IL-13 may be the CD3+ T cells that transmigrated in response to MCP-1, because IL-13 was lacking in the MCP-1 knockout. We also demonstrate that T cells were found in the human heart with cardiomyopathy; this indicates that our in vivo model may be relevant to human disease. Another stimulus for the upregulation of IL-13 in WT animals may be increased IL-33, expected to be made by infiltrating immune cells. However, IL-33 gene expression occurred even in the absence of MCP-1. IL-33 can be secreted from cells under mechanical strain (32), is constitutively expressed in endothelial cells (33), so these may be sources of IL-33 that are independent of an inflammatory infiltrate. In the MCP-1 KO animals, IL-33 gene expression did not decline at the 14-day time point, indicating the possibility that MCP-1 could be responsible for the eventual downregulation of IL-33 mRNA.

As in the murine in vivo model, the presence of MCP-1 in the human in vitro model induced first a Th1 (IFN-γ) and M1 (CD86, NOS2) response that within a day shifted to a Th2 (IL-13) and M2 (CD206, KLF4, and SOCS1) plus fibrotic (prolylhydroxylase) response without the addition of any further mediators. Therefore, the proinflammatory response uncomplicated by known external stimuli for its resolution was down regulated and evolved into a fibrotic response. A resolution to this conundrum may be found in further investigation of the action of MCP-1.

The view of MCP-1 as being responsible only for inflammation has been challenged by findings in other models; effects were noted either directly on myeloid cells or indirectly via T cell production of cytokines. Human monocytes in vitro and in tumor environments can be induced by MCP-1 to undergo M2 polarization (34). T cells treated with MCP-1 elaborate IL-4 and IL-13 (Th2 cytokines) (35, 36) and MCP-1-deficient mice cannot mount Th2 responses (37). Some of the influence of MCP-1 on the promotion of Th2 responses may be mediated through MCP-1-induced protein-1 (MCP1P1), a down regulator of inflammatory responses that degrades cytokine mRNAs and deubiquitinates TNF receptor-associated factor family proteins (38, 39), thus allowing an unfettered Th2 response. MCP-1 may induce both inflammation and anti-inflammation, perhaps at different times or in different environments, and the cessation of its proinflammatory effects may operate through the induction of anti-inflammatory gene products.
In previous studies of myocardial infarction injury (with extensive cell death), the presence of two monocyte subsets, one inflammatory and the other reparative, was noted in mouse models (40) and in human patients (41). In myocardial infarction, the proinflammatory macrophages are thought to be required for terminal digestion of tissue and removal of necrotic debris, which would not be necessary in our models. Later after infarction, reparative macrophages (M2) dominate. Thus, there is the generation of a temporal pattern such that the influx of inflammatory macrophages (phenotypically and functionally identical to M1 macrophages) early after injury is followed by reparative macrophages (identified as M2) (11, 42). In our models, despite the absence of cardiac injury sufficient to cause cell death, there was a reprise of this temporal pattern, including a transient M1 response followed by a robust M2 response, despite there being nothing to “repair.” In fact, our previous studies demonstrated a transition to a state of unstrained fibrosis resulting from dysregulation of immune function in M2 macrophages (14, 21). One cause may be that IL-13 can drive further production of MCP-1, which may set up a profibrotic feed forward loop (43). Thus the fibrotic response may represent dysregulation of a potentially protective immunological evolution (21).

Members of the adaptive immune system recognize foreign or altered self molecules via their multichain immunoreceptors, and innate immune cells recognize conserved pathogen-associated molecules through their pattern recognition receptors. In the absence of infection or cognate antigen presentation, however, there can be cytokine-induced cytokine production by both conventional and innate lymphoid cells (44). For example, the IL-1 family member IL-33 (an alarmin) causes antigen-independent IL-13 production (28, 29). IL-33 gene expression was present in both our models, although we do not know if this was an ancillary or primary stimulus for IL-13 production. The lack of IL-13 and presence of IL-33 in the MCP-1 knockout animals may be interpreted as the production of IL-33 in resident cells, which thereafter cannot induce the production of IL-13 from an absent inflammatory infiltrate. In either case (antigen recognition or cytokine-induced), we have demonstrated that MCP-1 alone can initiate an immune response that moves rapidly through a Th1/M1 inflammatory stage and into a Th2/M2 anti-inflammatory and profibrotic environment. Thereafter, the establishment of a chronically fibrotic state may depend on dysregulation of the immune response, as in diseases such as systemic sclerosis (45). Although MCP-1 and its receptor CCR2 historically have been targeted to reduce inflammation [reviewed in (46)], the possibility that it may initiate chronic fibrosis without chronic inflammation has not been considered. Our simplified models may allow an examination of this issue, as we propose that MCP-1 may initiate both an M1 and an M2 response leading to fibrosis, or promote M1 to M2 conversion.

The data from a murine model with daily episodes of brief ischemia not inducing infarction and an in vitro model of mononuclear cell TEM driven by MCP-1 both demonstrate the presence of a sequential inflammatory reaction occurring without evidence of tissue injury or infection and leading to interstitial fibrosis. The reaction is initiated by MCP-1-induced TEM of mononuclear cells (monocytes and T lymphocytes) and does not occur in the absence of MCP-1. Initially, the mononuclear cells are predominantly in an inflammatory phenotype (M1, Th1) and secrete cytokines and lymphokines appropriate to that phenotype (Figure 7).

As the reaction progresses, mononuclear cells of an “anti-inflammatory” phenotype begin to appear and subsequently dominate the cell population. This is followed by the appearance of a population of fibroblasts expressing collagen while still bearing the markers of their myeloid origin (CD45) and alternative polarization (CD206, CD301). The data suggest that the signaling cascade also contains intrinsic “brake mechanisms” that limit the intensity and duration of the acute inflammatory response. From the data presented, we suggest potential signaling molecules that may orchestrate this “braking” response as well as those initiating the transition from M1 to M2 phenotypes (Figure 7). We also suggest that our previous findings of CD34+CD45+ cells infiltrating the mouse heart during injuries may provide evidence for a relatively primitive monocyte precursor for either or both the M1 and M2 macrophages, although the finding of collagen in these cells suggests that they may be destined preferentially for the M2 phenotype (13, 16).

As described above, IL-33 is a powerful inducer of IL-13 and the Th2 phenotype without requiring an antigen response. Our data suggest that it also may be an important factor in the initiation of a Th2 response in an area of inflammation without tissue necrosis. In addition, MCPIP1 is secreted from activated M1 macrophages and has been observed to block M1 signaling (47), so this modulator may also aid in the transition from a Th1/M1 to a Th2/M2 response. Our previous and current data suggest that the chronic
presence of M2 macrophages is associated with some of them undergoing maturation into secretory myofibroblasts. The mechanism and signaling associated with this maturation has not been well characterized although the phenomenon has been described in vivo and in vitro (13, 17, 48, 49). When the stimulus continues, as it does in our in vitro model, even the basic response may change its nature to that of a chronic pathology (fibrosis) very different from the initial inflammation. Having thus identified the sequential immune responses and the initial signals involved, we will investigate further pathways and additional responses involved in chronic fibrosis in future studies.

**MATERIALS AND METHODS**

**ANIMALS AND ISCHEMIA REPERFUSION PROTOCOL**

Ten to twelve week old B6.129S4-Cd21^tm1Rol/J (MCP-1-KO) and C57BL/6j WT mice (both from Jackson Laboratory) were subjected to closed chest surgery as previously described (12). Briefly, 1 week after suture implantation, 15-min occlusions of the left anterior descending artery were performed for the number of indicated days, allowing a 24-h reperfusion period in between. Mice were euthanized 5 h after the last ischemic episode. All mice were fed standard mouse chow and water ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All animals were treated in accordance with the guidelines of the Baylor College of Medicine Animal Care and Research Advisory Committee.

**HUMAN HEART TISSUE**

Tissue pieces from human hearts with cardiomyopathy were obtained under an Institutional Review Board-approved protocol (50). The formalin-fixed tissue was paraffin embedded and sectioned.

**MOUSE CELL IDENTIFICATION**

After 5 days of I/R, mice were anesthetized with 2% isoflurane followed by cervical dislocation. Hearts were isolated and non-necrotic pieces were isolated by enzymatic digestion using 0.1 mg/ml Liberase TH Research Grade (Roche Applied Science), as described previously (17, 18). For flow cytometric analysis, 1–3 × 10^6 freshly isolated cells were incubated with 0.5 μg biotin-conjugated anti-CD45 antibody (BD Biosciences) followed by PE/Cy-5-conjugated streptavidin (BD Biosciences), together with either 0.5 μg CD45 antibody (BD Biosciences) followed by PE/Cy-5-conjugated streptavidin (BD Biosciences), or PE-conjugated anti-CD3 (Jackson ImmunoResearch), or PE-conjugated anti-rat secondary antibody (Jackson ImmunoResearch). For a TEM assay, HCMEC (Lonza, passages 4–9) were seeded on collagen type I antibody (Rockland), followed by FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). Fluorescence intensities were measured on a Beckman Coulter Epics XL.MCL using EXPO32 software.

**HUMAN CELL IDENTIFICATION**

Human heart sections were stained with H&E according to standard procedures (12). Before immunofluorescence staining, tissue sections were autoclaved in citrate buffer pH 6 for antigen retrieval and then permeabilized in 1% Triton X-100; after staining, sections were exposed to 0.3% Sudan Black in 70% alcohol to quench autofluorescence. Tissue sections were stained as described previously (17, 18) using anti-CD45 (BD Biosciences), -α-SMA (Sigma), -CD3ε (Beckman Coulter), and -CD301 (Dendritics).

Cells that transmigrated through an endothelial barrier were allowed to attach to poly-lysine-coated coverslips (Neuvitro) at the bottom of the lower well. The cells were fixed and stained with anti-CD86 (BioLegend), -CD301 (Dendritics), or appropriate isotype controls. For the internal antigens, the cells were also permeabilized (BD Pharmingen) and stained with anti-prolylhydroxylase (Millipore), -CD206 (Epitomics), or -NOS2 (Abcam Inc). These were followed by affinity-purified F(ab')2 IgG specific fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch) and the coverslips were mounted with the cell nucleus stain DAPI. Microscopy was performed on an Olympus AX70 using a QImaging Retiga 2000R camera. Colors were assigned and merged using ImageJ software (version 1.46r, NIH).

For cytometry, mononuclear leukocytes were stained fresh or after a 4-day transmigration with anti-CD3 plus either anti-CD4 or -CD8 all from R&D Systems). Cells were analyzed on a Cell Lab Quanta SC flow cytometer (Beckman Coulter) using the Quanta Analysis software.

**PROTEIN ARRAY**

After 3 days of I/R, hearts were harvested and protein was isolated using Cell Lysis Buffer (RayBiotech) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Protein (250 μg) was loaded onto standard mouse cytokine antibody array membranes (cytokine array C1, RayBiotech). Membranes were processed according to the manufacturer’s instructions, images on film were scanned, and densitometry was assessed by ImageJ software.

**TRANSENDOTHELIAL MIGRATION**

As detailed earlier (17, 18), normal blood was obtained from volunteers under a protocol approved by the Institutional Review Board of Baylor College of Medicine. Anticoagulated blood was fractionated by Ficol-Hypaque gradient centrifugation (Histopaque-1077; Sigma) to collect mononuclear cells (PBMC). For a TEM assay, HCMEC (Lonza, passages 4–9) were seeded on insert membranes with 8 μm pores. Human PBMC [25 × 10^4 in RPMI 1640 with no serum but with ITS-X (Sigma) and antibiotic-antimycotic (Invitrogen)] were then added to each insert and the same medium including 650 ng/ml MCP-1 (R&D Systems) for chemoattraction was added to the well below. PBMC were allowed to migrate for 16 h, and then the inserts were moved into new medium-containing wells (without additional MCP-1) for another 80 h (96 h total migration time, but separate from the 0–16 h time frame for migration). At the end of each migration period, the migrated cells were expanded in medium plus 5% FBS (HyClone) plus PMA plus calcium ionomycin (both from Sigma) for 3 days and then harvested for RNA.

**RNA ISOLATION AND TRANSCRIPTIONAL EXPRESSION**

Cells were lysed and total RNA was isolated with TRIzol reagent (Life Technologies) and treated with RNase-free DNasel (Qiagen).
Complimentary DNA was synthesized from 1 µg of RNA with a Verso cDNA synthesis kit (Thermo Scientific) using random hexamer and oligo-dT primers (3:1). Real-time PCR amplification reactions were performed with SsoAdvanced™ SYBR Green Supermix (Bio-Rad) in triplicate using a CFX96 thermal cycler (Bio-Rad). Cycling conditions (annealing temperature/time) were optimized for each primer pair. Gene expression was measured by the ΔΔCT method and was normalized to HPRT or 18S ribosomal RNA levels. Primers were designed using Primer 3, checked for homologies using BLAST, and for secondary structures using the mfold server. All primer pairs were verified to adhere to the MIQE guidelines using a temperature gradient and serial dilutions.

### Human:

- **KLF4**: ACTCGCCTTGCTGATTGCT and CCCCCAGT-CAACGGAGAG
- **SOCS1**: CGAGCATCAGGCTACCTT and GCAGCTGCAA-GGGAGTGCTGAA
- **IL-13**: CAATGGCAGCATGGTATGG and AGAATCCGCTCAG-CAACGAAGAGA
- **KLF4**: ACTCGCCTTGCTGATTGCT and CCCCCAGT-CAACGGAGAG
- **HPRT-1**: GCCCCAAAATGGTTAAGGTT and TTGCGCT-HPRT-1
- **18sRNA**: ACCGCAGCTAGGAATAATGGA and GCCTCAGTTC-GACATTCA
- **IL-33**: ACCCAGATGCTCAGCTGA and TCCACACATGC-ACTCCAGATGCTCAGCTGA

### Mouse:

- **HPRT-1**: GACCAGTCACAGGGGACAT and CTTGCAGAC-CTTGCACATCTT

### STATISTICAL ANALYSIS

All data are expressed as mean ± SE. A two-tailed, unpaired Student’s t-test or Mann–Whitney test was used to determine a significant difference between two groups. The Kruskal–Wallis ANOVA test with Dunn’s post-test was used for three or more groups. A p-value <0.05 was considered statistically significant.

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