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## Tumor necrosis factor alpha derived from classically activated “M1” macrophages reduces interstitial cell of Cajal numbers

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### Abstract

**Background**—Delayed gastric emptying in diabetic mice and humans is associated with changes in macrophage phenotype and loss of interstitial cells of Cajal (ICC) in the gastric muscle layers. In diabetic mice, classically activated M1 macrophages are associated with delayed gastric emptying whereas alternatively activated M2 macrophages are associated with normal gastric emptying. This study aimed to determine if secreted factors from M1 macrophages could injure mouse ICC in primary culture.

**Methods**—Cultures of gastric ICC were treated with conditioned medium (CM) from activated bone marrow-derived macrophages (BMDMs) and the effect of CM was quantified by counting ICC per high-powered field.

**Key Results**—BMDMs were activated to a M1 or M2 phenotype confirmed by qRT-PCR. CM from M1 macrophages reduced ICC numbers by 41.1%, while M2-CM had no effect as compared to unconditioned, control media. Immunoblot analysis of 40 chemokines/cytokines found 12 were significantly increased in M1-CM, including tumor necrosis factor alpha (TNF $\alpha$ ). ELISA detected  $0.697 \pm 0.03$  ng mL<sup>-1</sup> TNF $\alpha$  in M1-CM. Recombinant mouse TNF $\alpha$  reduced Kit expression and ICC numbers in a concentration-dependent manner (EC50 = 0.817 ng mL<sup>-1</sup>). Blocking M1-CM TNF $\alpha$  with a neutralizing antibody preserved ICC numbers. The caspase inhibitor Z-VAD.fmk partly preserved ICC numbers (cells/field;  $6.63 \pm 1.04$ ,  $9.82 \pm 1.80$  w/Z-VAD.fmk, n=6, P < 0.05).

**Conclusions & Inferences**—This work demonstrates that TNF $\alpha$  secreted from M1 macrophages can result in Kit loss and directly injure ICC in vitro partly through caspase-dependent apoptosis and may play an important role in ICC depletion in diabetic gastroparesis.

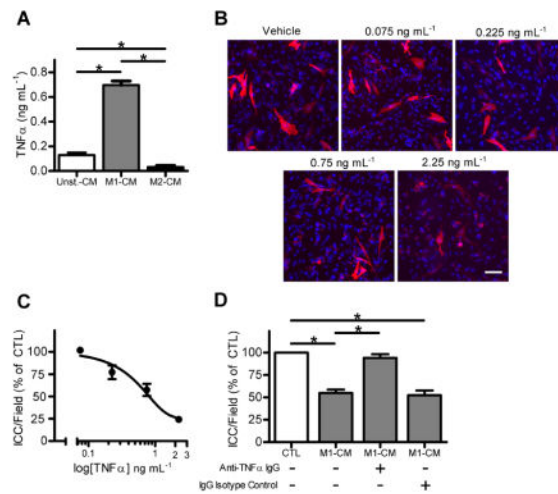
### Abbreviated abstract

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In diabetic mice, M1 macrophages are associated with delayed gastric emptying whereas M2 macrophages are associated with normal gastric emptying. TNF $\alpha$ , a factor present in M1 conditioned medium, reduced Kit-positive ICC numbers and TNF $\alpha$  neutralizing antibodies blocked the effect of M1 medium. TNF $\alpha$  derived from M1 macrophages injures ICC in vitro and TNF $\alpha$  may be important in diseases like diabetic gastroparesis.

## Keywords

diabetic gastroparesis; caspase-mediated apoptosis; cell death; cytokines; gastrointestinal motility

## Introduction

Interstitial cells of Cajal (ICC) are multi-functional cells that contribute to normal gastrointestinal function including generation of the electrical slow wave, mediation of neuromuscular signaling, setting membrane voltage gradients across muscle wall and mechanosensation.<sup>1–3</sup> Damage to ICC or a reduction in ICC has been described in many gastrointestinal motility disorders.<sup>2, 4–8</sup> Gastroparesis, defined as delayed emptying of stomach contents in the absence of a mechanical obstruction,<sup>9–12</sup> is one well-studied motility disorder where changes to ICC networks have been reported.<sup>13, 14</sup> It is a significant cause of morbidity in a subset of people with diabetes and animal models of diabetes develop delayed gastric emptying, a main feature of gastroparesis. Loss of Kit-positive ICC is associated with the development of delayed gastric emptying in both humans and diabetic mice.<sup>13, 15–17</sup>

The mechanism for damage to ICC networks in diabetic gastroparesis has not been established. However there is a close association between changes to ICC and alteration of the number and type of macrophages in the gastric muscularis propria in mice and humans with delayed gastric emptying.<sup>18, 19</sup> Recent data suggest that macrophages can directly affect the integrity of ICC networks in diseases like diabetes.<sup>20</sup> There is significant diversity in the patterns of macrophage activation<sup>21, 22</sup> but they can be broadly classified into two main groups: the pro-inflammatory, classically activated macrophages (M1) and the anti-

inflammatory, alternatively activated macrophages (M2). Activation of macrophages results in a phenotypic change affecting their physiology, expression of surface proteins, and secretome profile.<sup>23</sup> Macrophages in the gastrointestinal muscularis propria of mice have been characterized extensively.<sup>24</sup> In response to disease, injury or infection, resident macrophages in the muscularis propria are replaced by activated macrophages.

The phenotypic changes that occur to macrophages in the gastric muscularis propria when delayed gastric emptying develops in diabetic humans and mice are consistent with a role for those macrophages in directly affecting ICC networks.<sup>18, 19</sup> In diabetic mice with normal ICC networks and normal gastric emptying, mannose receptor (CD206, MRC1) positive M2 macrophages express cytoprotective factors including heme oxygenase-1 and interleukin-10 (IL-10).<sup>18</sup> Whereas, in mice with damaged ICC networks and delayed gastric emptying, increased levels of inducible nitric oxide synthase (iNOS, NOS2), a marker of M1 macrophages, are found.<sup>18</sup> The factors generated by M1 macrophages that contribute to ICC network damage and the mechanism by which ICC networks are damaged in diabetic mice with delayed gastric emptying have not been determined. It is known that CD206-negative macrophages are required for development of delayed gastric emptying in diabetic mice, as diabetic osteopetrotic (CSF1<sup>OP/OP</sup>) mice, which lack gastric macrophages due to a mutation in the macrophage colony stimulating factor (CSF1), never develop delayed gastric emptying and have normal ICC networks.<sup>17</sup> These data suggest that macrophages are needed to induce damage to ICC networks, resulting in delayed gastric emptying.

This study aimed to determine if secreted molecules from bone-marrow derived macrophages (BMDMs) activated to an M1 phenotype could injure dissociated mouse primary ICC in vitro, to identify factors responsible and determine the mechanisms for their effects.

## Materials and Methods

### Animals

Mice were maintained and the experiments were done with approval from the Institutional Animal Care and Use Committee of the Mayo Clinic. BALB/c mice (Harlan Sprague-Dawley; Indianapolis, IN, USA) were used for all experiments except cell lineage tracing. For cell lineage tracing of ICC, R26<sup>mT-mG/+</sup> (Jackson Lab, Bar Harbor, ME, stock#007576) were interbred with Kit<sup>CreERT2/+</sup> (Gift from Dr. Dieter Saur) knockin line as previously described.<sup>25</sup> All mice were killed by CO<sub>2</sub> inhalation followed by cervical dislocation.

### Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were generated as previously described.<sup>26</sup> This protocol yields macrophages that are >90% pure and have the ability to respond to exogenous stimuli. Briefly, BMDM were obtained from femurs and tibias using aseptic techniques. Marrow cores were flushed into sterile tubes using syringes fitted with 25 gauge needles and filled with Dulbecco's phosphate-buffered saline without Ca<sup>2+</sup>/Mg. Cells were washed once in media then plated and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with F12 (Invitrogen; Carlsbad, CA, USA) supplemented with 1% penicillin/

streptomycin (Life Technologies; Carlsbad, CA, USA), 10 mM L-glutamine (Invitrogen), 10% characterized fetal bovine serum (Hyclone; Logan, UT, USA), and 40 ng mL<sup>-1</sup> recombinant mouse M-CSF (ProSpec; East Brunswick, NJ, USA). Isolated BMDM were cultured for 7 days then split and plated at 0.5×10<sup>6</sup> cells per well (9.5 cm<sup>2</sup>) and exposed to either 40 ng mL<sup>-1</sup> recombinant mouse interferon- $\gamma$  (IFN- $\gamma$ ) (R&D Systems; Minneapolis, MN, USA), or to 20 ng mL<sup>-1</sup> recombinant human IL-10 (Gibco; Carlsbad, CA, USA) and 10 ng mL<sup>-1</sup> recombinant human transforming growth factor  $\beta$  (TGF $\beta$ ) (Gibco). Control cells were not treated with IFN- $\gamma$ , IL-10 or TGF $\beta$ . Macrophages were treated with exogenous stimuli for 24 hours. Macrophages were then harvested for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis to determine macrophage phenotype or used to generate macrophage conditioned medium (CM).

### Quantitative RT-PCR

Reverse transcription reactions were conducted using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). 500 ng of RNA for each sample was used. cDNA was then used for real-time PCR using the LightCycler 480 system with SYBR Green I Master Mix (Roche, Indianapolis, IN, USA). The program used for all reactions was 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. Gene expression was normalized to  $\beta$ -actin expression and assessed using the  $(-C_t)$  calculation method. Primers were designed with the Primer3 software and obtained from Integrated DNA Technologies (Coralville, IA, USA) or commercially predesigned primers were obtained. In house designed primer sequences are listed in Supplemental Table 1. IL-4 (cat# PPM03013F), IL-5 (cat# PPM03014F) and IL-10 (cat# PPM03017C) primers were obtained from Qiagen (Valencia, CA).

### Interstitial cells of Cajal Primary Culture

Primary ICC cultures enriched for gastric ICC were obtained by enzymatic dissociation of the mouse gastric body, fundus, and pylorus based on a previously described protocol for small bowel ICC dissociations.<sup>27</sup> Utilizing a collagenase-based dissociation cocktail, tissues were dissociated and then plated on a feeder layer of fibroblasts expressing the membrane bound, m248 form of Kit ligand (steel factor), which is important to maintain ICC phenotype. M199 media (Invitrogen) with 1% antibiotic/antimycotic (Gibco) medium was used for optimal ICC growth. Primary ICC were allowed to attach and recover for 24 hours before treatment. For conditioned macrophage media experiments, 50% of the culture medium was replaced with macrophage-derived conditioned medium from activated BMDM. Each experimental condition was run in duplicate. Cultures were immunolabeled for Kit, a marker of ICC, using the rat monoclonal anti-Kit antibody, ACK2 (eBioscience; San Diego, CA, USA), or a goat anti-Kit polyclonal antibody (R&D). Nuclei were counterstained with 4, 6-diamidino-2-phenylindole, dihydrochloride, DAPI (Molecular Probes; Eugene, OR, USA) as previously described.<sup>28</sup> An Olympus BX51WI fluorescence microscope with a 20X objective (NA 0.5) was used to count the number of Kit-positive cells per high-power field (0.94 mm<sup>2</sup>). At least 35 high-power fields were counted per culture as this is the optimal number to minimize field-to-field variation while not over-counting fields. TNFR1 and TNFR2 localization was analyzed by co-labeling untreated

primary ICC cultures with primary antibody for Kit and primary antibodies for either TNFR1 and TNFR2 (Supplemental Table 2).

### Conditioned Media

Macrophages activated with either IFN $\gamma$  or IL-10/TGF- $\beta$  were used to generate macrophage CM. After activation of BMDM with the exogenous stimuli for 24 hours, the stimulating factors were removed and macrophages were washed three times with 1X PBS. The medium used for primary ICC cultures (M199 with 1% antibiotic/antimycotic) was then added. Activated macrophages were allowed to secrete soluble factors into the media for 20 hours. Media were then removed, filtered with a 0.2  $\mu\text{m}$  filter, flash frozen in liquid N $_2$  and stored at  $-80^\circ\text{C}$  until use.

### Fractionation of the macrophage conditioned media

Conditioned media were fractionated by molecular weight using size separating centrifugal filters. Aliquots of macrophage-conditioned media were filtered using 3 kDa Amicon Ultra centrifugal filter units as described by the manufacturer (Millipore; Billerica, MA, USA). Following fractionation, each fraction was reconstituted to the original volume to return the factors back to the original concentration.

### Cytokine Array

For the cytokine array, the Proteome Profiler Mouse Cytokine Array Kit, Panel A from R&D Systems was used and 100  $\mu\text{g}$  of total protein was incubated with the blots according to the manufacturer's specifications. Densitometric analysis of immunoblot intensity was performed using Adobe Photoshop 6.0 software (Adobe Systems; San Jose, CA, USA).

### ELISA

Macrophage-conditioned media were tested for presence of TNF $\alpha$ . Sandwich ELISA for mouse TNF $\alpha$  was done according to the manufacturer's instructions (eBioscience). This ELISA has a sensitivity of 8  $\text{pg mL}^{-1}$  and a standard curve range of 8 – 1,000  $\text{pg mL}^{-1}$ . Each biological replicate was run in duplicate.

### Detection of Senescence by Immunohistochemistry

A rabbit polyclonal antibody targeting  $\gamma$ -H2AX was used to detect double-strand breaks in DNA to serve as a marker for cellular senescence. Cells were exposed to TNF $\alpha$  for 24-hours before being processed for immunohistochemistry and analysis of  $\gamma$ -H2AX immunoreactivity. Cells that had  $\gamma$ -H2AX-positive nuclei marking persistent DNA damage foci were only counted if the cell was also positive for Kit immunoreactivity. As noted above, 35 high-power fields were counted for 2 coverslips for each condition. A list of antibodies and optimized concentrations used are listed in Supplemental Table 2.

### Cell Lineage Tracing of ICC in Primary Culture

R26<sup>mT-mG/+</sup>;Kit<sup>CreERT2/+</sup> mice, in which CreERT<sup>2</sup> is expressed in ICC and can be activated via tamoxifen administration, were used for cell lineage tracing experiments. To activate CreERT<sup>2</sup>, primary ICC cultures were incubated with a low dose of 4-hydroxytamoxifen

(100 nM) for 24 h.<sup>29</sup> Following 4-hydroxytamoxifen treatment, cells were incubated with 2.25 ng/ml TNF $\alpha$  for 24 h followed by immunohistochemistry for Kit. Cells of ICC lineage which underwent recombination were identified by GFP positivity. Total GFP-positive cells were assessed as for each condition as previously described above.

### Confocal Microscopy

To show localization of TNFR1 and TNFR2, images of immunolabeling were collected using an Olympus FV1000 laser scanning confocal microscope. Confocal images were collected with  $\times 60$  1.2-NA water objective. All images were prepared for individual figures using Adobe Photoshop CS. No 3D reconstructions, deconvolution, surface or volume rendering, or gamma adjustments were performed for the final images.

### Reagents

Recombinant TNF $\alpha$  (R&D Systems) was used at various concentrations as indicated in the results section and figure legends. For TNF $\alpha$  neutralizing experiments, a goat IgG anti-TNF $\alpha$  neutralizing antibody (10.0  $\mu$ g/ml, R&D Systems, AF-410-NA)<sup>30</sup> was incubated with conditioned medium for 1 hour prior to treatment of cells along with control conditions using a goat IgG isotype control (10.0  $\mu$ g/ml, R&D Systems, AB-108-C). Z-VAD.fmk (Promega; Madison, WI, USA) was used at a concentration of 40  $\mu$ M.<sup>31</sup> Z-VAD.fmk was added to primary culture at the same time as TNF $\alpha$  and incubated with the ICC for a total of 24 hours. Necrostatin-1 (nec-1) in both active and inactive (Millipore) forms was used at a concentration of 30  $\mu$ M.<sup>32, 33</sup> Nec-1 was added to ICC primary cultures 1 hour before the addition of TNF $\alpha$  and incubated with ICC for a total of 24 hours.

### Statistical analysis

Data are expressed as mean with the standard error of the mean. The significance of differences between groups was examined using either Student t test or one-way ANOVA, as appropriate.  $P < 0.05$  (\*) was considered statistically significant in all analyses.

## Results

### Macrophage Phenotype

The phenotype of activated BMDMs was determined following treatment with IFN $\gamma$  or IL-10/TGF $\beta$ . Expression of iNOS and IL-6 RNAs were used as indicators of a classically-activated M1 macrophage phenotype (Fig. 1). Expression of Arg1, FIZZ, IL-4, IL-5, and IL-10 were used as indicators of an alternatively-activated M2 macrophage phenotype (Fig. 1). Data were normalized to expression of  $\beta$ -actin. Macrophages activated with IFN $\gamma$  showed significantly higher expression of iNOS and IL-6 mRNA, significantly lower expression of IL-4 and IL-5, and there was no significant difference in expression of Arg1, FIZZ and IL-10 mRNA compared to cells that were not treated (Fig. 1). In contrast, macrophages activated with IL-10/TGF $\beta$  expressed significantly less iNOS, IL-6, and IL-10 mRNA than unstimulated cells, but significantly higher expression of Arg1, FIZZ, and IL-4 mRNA (Fig. 1). There was no significant change in the expression of IL-5. We concluded that treatment of BMDMs with IFN $\gamma$  yields an activated macrophage with a phenotype similar to that of classically-activated M1 macrophages, while IL-10/TGF $\beta$  treatment

activates macrophages into a phenotype similar to that of alternatively-activated M2 macrophages. For this study, BMDM activated by IFN $\gamma$  will be referred to as M1 macrophages, BMDM activated by IL-10/TGF $\beta$  will be referred to as M2 macrophages, and BMDM that were not treated will be referred to as M(Unst.).

### **M1-conditioned media reduced ICC numbers in primary culture**

Macrophages secrete a variety of soluble chemokines and cytokines that can affect the survival and fate of adjacent cells in tissues. We tested whether soluble factors secreted by activated macrophages would affect ICC in primary culture. Gastric ICC were dissociated and enriched in our primary culture system and then incubated for 24 hours in the presence of media conditioned by incubation with activated M1 or M2 macrophages. Incubation of ICC with M1-CM caused a decrease in the number of Kit-positive ICC whereas there was no effect of M2-CM as shown in the representative images in Fig. 2A. This effect was reproducible with M1-CM causing a 41.1% decrease ( $P < 0.05$ ) in ICC numbers (Fig. 2B). In order to identify the active molecule in M1-CM that decreased Kit-positive ICC numbers, we used size-separation spin columns to fractionate the components in the media by molecular weight. M1-CM media separated into greater-than 3 kDa and less-than 3 kDa fractions had the same effects as the complete M1-CM medium on ICC numbers (Fig 2C). This indicated that more than one active molecule was present in M1-CM. To test whether there was a non-specific effect of fractionating the medium, due to an effect such as dilution of important growth factors, we tested M2-CM fractionated by the same method. The fractions of M2-CM had no effect on ICC numbers (Fig 2C). Mixing M1-CM and M2-CM and applying it to ICC cultures resulted in loss of the negative effect of M1-CM on ICC numbers (2D).

### **Identification of pro-inflammatory cytokines and chemokines in M1-conditioned media**

To identify possible bioactive components present in M1-CM, an immunoblot was done that probed for 40 different pro-inflammatory cytokines and chemokines. Representative images of the immunoblot show the presence of numerous factors present in M1-CM while there were few pro-inflammatory factors present in the M2-CM (Fig. 3A). Of the factors that were present in M1-CM, 12 were significantly increased as compared to M2-CM (Fig. 3B,  $n = 5$  independent experiments, adjusted  $P < 0.05$ ). The factors that were significantly enriched in M1-CM were known markers of a pro-inflammatory phenotype, including the interleukin IL-6, the chemokines; chemokine (C-C motif) ligand 2 (CCL2), the chemokine (C-C motif) ligand 3 (CCL3), the chemokine (C-C motif) ligand 4 (CCL4), the chemokine (C-C motif) ligand 5 (CCL5), the chemokine (C-X-C motif) ligand 1 (CXCL1), the chemokine (C-X-C motif) ligand 2 (CXCL2) and the chemokine (C-X-C motif) ligand 10 (CXCL10), as well as TNF- $\alpha$ . The anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra) was also detected as well as IFN $\gamma$  and M-CSF, which were used to activate the BMDM cells into M1 macrophages. These data demonstrate that the BMDM treated with IFN $\gamma$  were macrophages activated towards an M1 phenotype and expressed well known pro-inflammatory markers.

### Recombinant TNF $\alpha$ decreased Kit-positive ICC numbers and neutralizing TNF $\alpha$ in M1-conditioned media preserved Kit-positive ICC numbers

The presence of TNF $\alpha$  in M1-CM was of interest as this cytokine is a known mediator of cell death and injury in diseases associated with inflammation and TNF $\alpha$  had one of the highest pixel densities of the immunoblot. Therefore we determined the concentration of TNF $\alpha$  in Unst-CM, M1-CM and M2-CM by ELISA. M1-CM had contained  $0.697 \pm 0.03$  ng mL $^{-1}$  of TNF $\alpha$ , which was significantly higher than the levels of TNF $\alpha$  in the other two media (Fig 4A,  $n = 7$ ,  $P < 0.05$ ). We then tested the effect of recombinant mouse TNF $\alpha$  on primary ICC cultures. TNF $\alpha$  caused a concentration dependent decrease in Kit-positive ICC numbers at concentrations of TNF $\alpha$  from  $0.075$  ng mL $^{-1}$  to  $2.25$  ng mL $^{-1}$  (Fig. 4B). The EC $_{50}$  value for the effect of TNF $\alpha$  was  $0.817$  ng mL $^{-1}$  (Fig. 4C), which was similar to the concentration in the M1-CM. Lastly, we tested whether blocking TNF $\alpha$  signaling by incubating the M1-CM with a neutralizing antibody against TNF $\alpha$  would inhibit the effect of the medium on Kit-positive ICC numbers. The larger fraction of M1-CM ( $> 3$  kDa) was used as TNF $\alpha$  (17 kDa TNF monomer, 51 kDa trimer) would be retained within this fraction. The TNF $\alpha$  neutralizing antibody significantly inhibited the effect of M1-CM ( $> 3$  kDa) on Kit-positive ICC numbers (Fig 4D). The effect of M1-CM ( $> 3$  kDa) was not altered by incubation with IgG isotype control (Fig 4D).

### Reduced Kit-positive ICC numbers in primary culture by TNF $\alpha$ is partially mediated by caspase-dependent apoptosis

TNF $\alpha$  exerts a broad range of biological effects via two primary transmembrane receptors; TNFR1 and TNFR2. Immunohistochemistry on primary ICC cultures showed that TNFR1 colocalizes with Kit-positive ICC (Fig. 5A) while TNFR2 was not found to be present on ICC (Fig. 5A). These findings are in agreement with previous reports showing that TNFR1 is expressed on almost all cell types, while TNFR2 is primarily expressed in cells of the immune system.<sup>34–36</sup> TNF $\alpha$  signaling through TNFR1 leads to initiation of cell death pathways including apoptosis<sup>37, 38</sup> and necroptosis<sup>38, 39</sup> and TNF $\alpha$  has also been linked to the promotion of cellular senescence.<sup>40–42</sup> To test whether the effect of TNF $\alpha$  on Kit-positive ICC numbers was due to caspase-dependent apoptosis, we used the pan-caspase inhibitor Z-VAD.fmk. Incubation of ICC cultures with medium containing Z-VAD.fmk and a maximally effective concentration of TNF $\alpha$  that was 3-fold higher than the EC $_{50}$  value ( $2.25$  ng mL $^{-1}$ ), significantly but not completely reduced the effect of TNF $\alpha$  on Kit-positive ICC numbers (Fig. 5B, cells/field;  $6.63 \pm 1.04$ ,  $9.82 \pm 1.80$  w/Z-VAD.fmk,  $n=6$ ,  $P < 0.05$ ) consistent with a role for caspase-dependent apoptosis in the reduction in ICC numbers by TNF $\alpha$ . In order to test whether caspase-independent cell death contributed to the effects of TNF $\alpha$  on Kit-positive ICC numbers, we used necrostatin 1, a small molecule that allosterically blocks the kinase activity of RIP1, which is essential for caspase-independent necroptosis.<sup>32, 43, 44</sup> We found that both active necrostatin 1 and the inactive form of the compound did not alter the effect of TNF $\alpha$  on Kit-positive ICC numbers and had no effect when added alone (Fig. 5C). To examine whether TNF $\alpha$  increased cellular senescence in ICC, we used the senescence marker  $\gamma$ H2AX, a phosphorylated histone variant which is associated with double-strand breaks,<sup>45</sup> and found no difference in expression of  $\gamma$ H2AX-positive foci in Kit-positive ICC in control and TNF $\alpha$ -treated conditions (Fig. 5D, 5E). This indicates that there is no apparent role for cellular senescence in the reduction of Kit-positive



ICC numbers with TNF $\alpha$  treatment. We next quantified the total number of DAPI-positive nuclei per field. We found that there was no significant difference in total nuclei in control conditions as compared to TNF $\alpha$ -treated conditions (Fig. 5F). This indicated that the stem cell factor secreting fibroblast feeder layer and other cells surviving from the dissociated gastric muscle were not depleted by TNF $\alpha$  treatment. Lastly, we used an ICC lineage tracing model to track the fate of ICC in primary culture after treatment with TNF $\alpha$ . Immunohistochemistry for Kit showed that in the TNF $\alpha$ -treated condition there are many GFP+ cells remaining, however, they are largely Kit-negative (5G). Quantification of GFP+ cells showed no significant difference in GFP+ cells in the TNF $\alpha$ -treated condition as compared to control (5H), suggesting that the Kit-negative cells remain in the culture.

## Discussion

Our primary finding was that TNF $\alpha$  derived from classically-activated M1 macrophages reduced the number of Kit-positive ICC in vitro via caspase mediated apoptosis and loss of Kit expression, a marker of functional ICC. This suggests that in diabetic gastrointestinal tissues where activated macrophages lie in close proximity to ICC, there is a potential role for M1 macrophages to alter ICC networks and potentially other cell types and thus alter gastrointestinal contractility. Previous studies have shown that in diabetic mouse models of gastroparesis, animals that retain normal gastric emptying have normal ICC networks and CD206-positive, HO1-positive M2 macrophages in the muscularis propria, while in animals that developed delayed gastric emptying, ICC networks were damaged and the macrophages were classically activated M1 macrophages, based on being CD206-negative and HO1-negative with increased levels of iNOS mRNA in the tissue.<sup>16, 18</sup> Furthermore, we recently reported that diabetic CSF1<sup>op/op</sup> mice, which lack macrophages, never develop delayed gastric emptying and have normal ICC networks.<sup>17</sup> Based on these previous observations, we hypothesized that activated M1 macrophages secrete injurious pro-inflammatory mediators that can damage ICC. In this study, we provided evidence supporting the hypothesis that activated M1 macrophages can directly alter ICC survival and contribute to the pathophysiology of motility disorders such as diabetic gastroparesis where damage to ICC networks is observed.<sup>13, 14, 19</sup>

For this study we used M-CSF-cultured macrophages from mouse bone marrow, which remains the most commonly used in vitro system for generating macrophages. We ensured that the macrophages were activated towards a M1 and a M2 phenotype. IFN $\gamma$  treated macrophages showed high levels of iNOS and IL-6 while IL-10/TGF $\beta$  treated macrophages had high levels of Arg1, FIZZ and IL-4, confirming activation towards a M1 and M2 phenotype, respectively.<sup>46-48</sup> Macrophage polarization status is diverse and alternative methods, such as treating with IL-4 to polarize to M2 macrophages, have been previously used to obtain M2 macrophage phenotypes. We chose a method that resulted in a macrophage phenotype more closely resembling the M2C phenotype as that phenotype was associated with preservation of ICC numbers and gastric emptying in NOD diabetic mice.<sup>18</sup> This study focused on injurious factors from M1 macrophages, and did not determine which factors from M2 macrophages are protective or how M2 macrophages might protect ICC from depletion by M1 macrophages. The primary culture system used here was chosen because of the capacity to do comparatively high throughput experiments on regulators of

ICC proliferation and survival, which were validated as representative of the changes found in whole animals and in organotypic cultures.<sup>27, 49–51</sup> Therefore, we predict that these results are representative of what M1 macrophages might do in more intact systems including organotypic cultures or, more importantly, in our animal models of delayed gastric emptying in diabetic mice and that it will be important to test for these effects in the in vivo preclinical models.

As hypothesized, conditioned media from M1 macrophages decreased Kit-positive ICC numbers in vitro suggesting that a soluble bioactive factor is released into the medium by the activated macrophages. Importantly, conditioned medium from M2 macrophages had no effect on Kit-positive ICC numbers in vitro although mixing M2 media with M1 media eliminated the effect of M1 media, further supporting the hypothesis that M1 macrophages are injurious to ICC while M2 macrophages are not, but can protect against injury when present. The premise that soluble macrophage factors can alter cell survival has been investigated previously. Soluble factors in conditioned media from activated microglia, resident macrophages found in the brain, can trigger apoptosis in hippocampal neurons<sup>52</sup> and conditioned medium from M1 macrophages derived from human blood monocytes reduced cell numbers in the colon cancer cell lines HT-29 and CACO-2.<sup>53</sup>

Identification of bioactive factors present in complex media can be challenging as the secretome of macrophages is large and diverse. Meissner et al. found that activated bone marrow-derived macrophages had the capacity to release at least 775 proteins, of which 364 were proteins with annotated extracellular functions and 52 were known cytokines.<sup>54</sup> Our initial approach to identifying the active factor that reduced Kit-positive ICC numbers in our culture system was to determine the approximate molecular weight of the bioactive factor. Size separation of the M1-conditioned media showed that bioactive factors were present in both a small molecular fraction (< 3kDa) and a large molecular fraction (> 3kDa). For this study, we focused on identifying the larger molecule, which we hypothesized was a cytokine or chemokine. We have not yet identified the smaller molecule or molecules.

M1 and M2-conditioned media were studied using a protein array for expression of 40 of the most commonly investigated pro-inflammatory cytokines and chemokines. We found that 12 were increased in M1 conditioned media as compared to M2-conditioned media. The M1 conditioned media had significant amounts of the pro-inflammatory cytokines IFN $\gamma$ , IL-6, and TNF $\alpha$  as well as chemokines involved in immune cell recruitment including CCL2, CCL3, CCL4, CCL15, CXCL1, CXCL2, and CXCL10. The presence of these mediators further strengthens the conclusions that the macrophages used in this study were of a M1 phenotype. Of the 12 factors identified in M1 conditioned media, TNF $\alpha$  was of immediate interest, as it is a secreted molecule associated with a number of diseases including cancer, rheumatoid arthritis, type II diabetes, Crohn's disease, and multiple sclerosis.<sup>55</sup>

We tested TNF $\alpha$  as a potential bioactive factor by adding it directly to primary ICC cultures. We found that rmTNF $\alpha$  reduced Kit-positive ICC numbers in a dose dependent manner. The EC50 value for the effect of TNF $\alpha$  (0.817 ng mL<sup>-1</sup>) on Kit-positive ICC numbers in vitro is relatively low in comparison to concentrations of TNF $\alpha$  used in other studies on other cell types. Studies on human and rat vascular and intestinal smooth muscle found that 100 ng

mL<sup>-1</sup> TNF $\alpha$  did not reduce cell numbers but rather induced proliferation of these cell types.<sup>56-58</sup> In addition, a concentration of 40 ng mL<sup>-1</sup> of TNF $\alpha$  was needed to reduce tyrosine-hydroxylase-positive neurons by 50% in rat primary culture<sup>59</sup> and in rat enteric neuron primary cultures the number of neurons was unchanged after treatment with 50 ng mL<sup>-1</sup> TNF $\alpha$ .<sup>60</sup> The apparently higher sensitivity of ICC to TNF $\alpha$  exposure as compared to other cell types is of interest because in diabetic gastroparesis the predominant cellular defect noted among humans and animal models is damaged ICC networks more than other cell types. The relatively low concentrations of TNF $\alpha$  used in our study had profound effects on Kit-positive ICC numbers in primary culture and suggests that ICC may be especially susceptible to loss of Kit and damage by TNF $\alpha$  secreted from M1 macrophages, especially because macrophages lie in close proximity to ICC.<sup>61</sup>

Further supporting a role for TNF $\alpha$  in reducing Kit-positive ICC numbers in culture is that depleting TNF $\alpha$  in the large molecular weight fraction of conditioned media using a neutralizing antibody prevented the effect of M1-CM on Kit-positive ICC numbers. This indicates that TNF $\alpha$  was the large molecular weight factor that was necessary and sufficient for the effect of M1-CM on ICC. Given the presence of many other pro-inflammatory mediators in M1 conditioned media, we were surprised that targeted depletion of TNF $\alpha$  fully preserved Kit-positive ICC numbers. Data from neutralizing TNF $\alpha$  in M1 conditioned media, along with the effect of rmTNF $\alpha$  on ICC numbers are both strong evidence that TNF $\alpha$  can alter the survival of Kit-positive ICC and understanding the mechanism of action is important.

We found that ICC in vitro express TNFR1, but not TNFR2. Therefore we explored the possibility that the effects of TNF $\alpha$  on ICC were due to signaling through TNFR1. TNF $\alpha$  signaling via TNFR1 commonly results in cell death through caspase-mediated apoptosis or necroptosis,<sup>62, 63</sup> but other studies have suggested that TNF $\alpha$  may induce cellular senescence via the NF- $\kappa$ B pathway.<sup>40-42</sup> We looked at these different pathways by utilizing the pan-caspase inhibitor Z-VAD.fmk, the necroptosis inhibitor necrostatin-1, and a commonly used marker of cellular senescence,  $\gamma$ H2AX. We found that the reduction in Kit-positive ICC in our primary system is partly due to caspase-mediated apoptosis, as the pan-caspase inhibitor partly blocked the effect of TNF $\alpha$  on Kit-positive ICC numbers in vitro. We assume that the incomplete inhibition of the effects of the caspase inhibitor are due to our inability to either completely block caspase activation with the inhibitor, the saturating concentration of TNF $\alpha$  that was used in these experiments or the transformation of ICC into Kit-negative cells that are resistant to apoptosis. Furthermore, we found no change in Kit-positive ICC numbers after blocking necroptosis and found no change in  $\gamma$ H2AX-positive ICC after TNF $\alpha$  exposure, suggesting that necroptosis and cellular senescence play no role in the reduction of ICC in this model.

Gibbons et al. found that 1-2% of ICC are dead or dying by apoptosis in the normal human colon,<sup>64</sup> suggesting that ICC have the ability to undergo caspase-mediated apoptotic cell death and that regeneration is required for maintenance of normal ICC networks even in healthy tissue. Small perturbations to the microenvironments surrounding ICC, such as increased levels of TNF $\alpha$ , could lead to increased rates of apoptotic ICC and contribute to the development of motility disorders.

ICC lineage tracing showed that a majority of ICC exposed to TNF $\alpha$  lost Kit immunoreactivity. Kit is essential for the ICC phenotype.<sup>28, 65</sup> It's been previously shown that hypomorphic mutations in Kit protein (W/Wv mice)<sup>66</sup> or Kit ligand (Sl/Sld mice),<sup>67</sup> or block of Kit signaling with neutralizing antibodies,<sup>68</sup> result in disrupted ICC networks and defects in gastrointestinal motility. Damage to Kit-positive ICC or a reduction in Kit-positive ICC population via the chronic effects of low concentrations of macrophage-derived TNF $\alpha$  could alter motility. Others have shown effects of acute administration of TNF $\alpha$  at high concentrations on gastrointestinal smooth muscle contractility.<sup>69</sup> Our data indicate that long-term administration of low concentrations of TNF $\alpha$  would also change contractility given the levels of Kit-positive ICC loss seen in our culture system, but it will require further experimentation to confirm this.

In summary, our data demonstrate that TNF $\alpha$  derived from activated M1 macrophages reduces Kit-positive ICC in vitro and its effects are largely a result of the loss of Kit and partly mediated through caspase-dependent apoptosis. These observations indicate that activated M1 macrophages associated with diseases such as diabetic gastroparesis can secrete factors that have the ability to alter ICC network integrity and function. While we have identified TNF $\alpha$  as a bioactive factor that can alter Kit-positive ICC numbers in vitro, there remains an unidentified smaller component or components that will need to be identified. In addition, the injurious effect of the M1 conditioned media after transfer from the macrophages to the ICC cultures may be underestimated because some toxic mediators released by macrophages are labile with short half-lives (e.g. nitrogen oxide free radicals). Regardless, we show that TNF $\alpha$  injures Kit-positive ICC in vitro and warrants further investigation into its role in the pathophysiology of diseases such as diabetic gastroparesis. If in vivo experiments confirm a role for TNF $\alpha$  in the development of human diabetic gastroparesis, available therapeutics targeting this molecule may be of use.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>M2</b>	Alternatively-activated macrophage
<b>Arg1</b>	arginase-1
<b>BMDM</b>	bone marrow-derived macrophages
<b>M1</b>	classically-activated macrophage
<b>CM</b>	conditioned medium

<b>FIZZ</b>	found in inflammatory zone
<b>iNOS</b>	inducible nitric oxide synthase
<b>IFN-<math>\gamma</math></b>	interferon- $\gamma$
<b>IL-4</b>	interleukin 4
<b>IL-5</b>	interleukin 5
<b>IL-6</b>	interleukin 6
<b>IL-10</b>	interleukin 10
<b>ICC</b>	interstitial cells of Cajal
<b>CD206</b>	mannose receptor
<b>TGF<math>\beta</math></b>	transforming growth factor $\beta$
<b>TNF<math>\alpha</math></b>	tumor necrosis factor alpha

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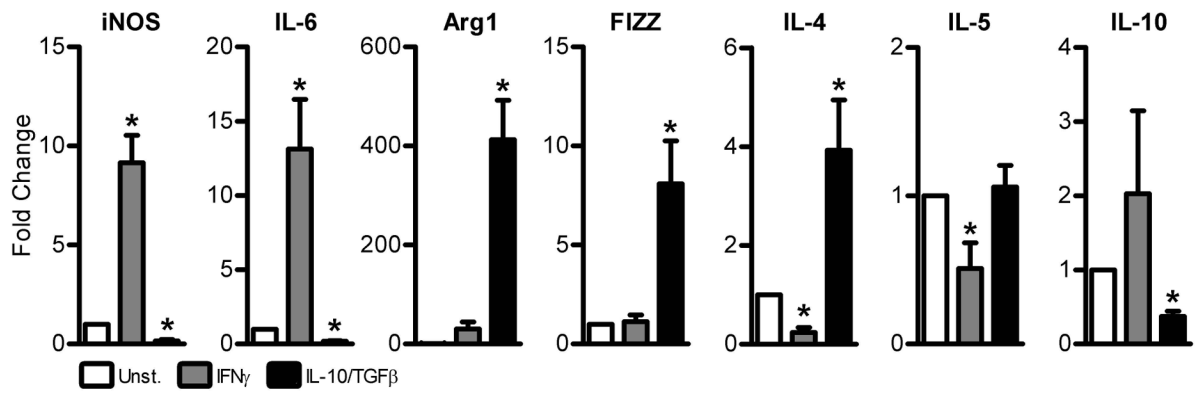
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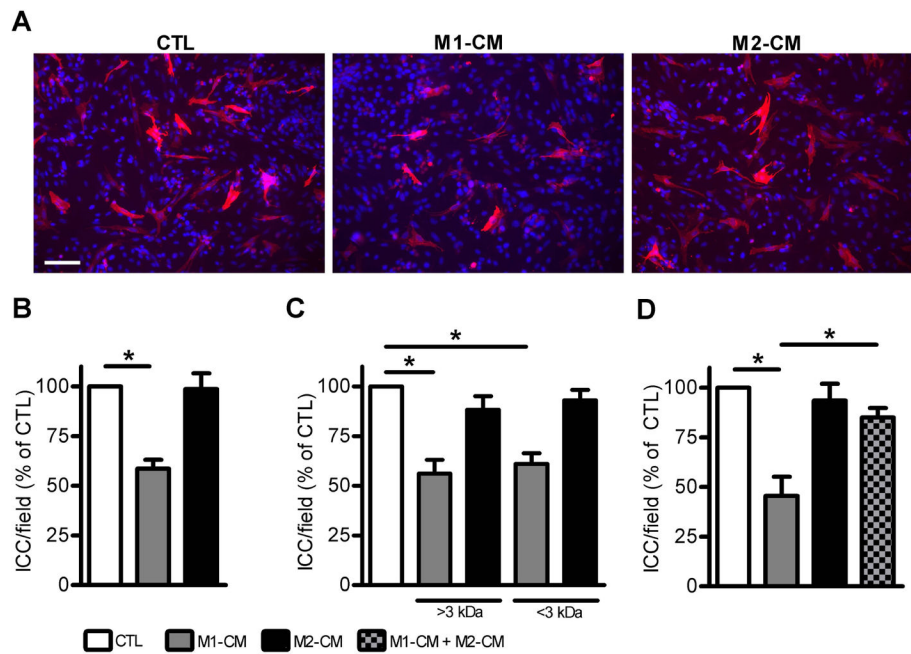
### Key Points

- In diabetic mice, M1 macrophages are associated with delayed gastric emptying whereas M2 macrophages are associated with normal gastric emptying. We determined whether secreted factors from M1 macrophages injured interstitial cells of Cajal (ICC) in vitro.
- Conditioned medium from M1 macrophages reduced ICC numbers. TNF $\alpha$ , a factor present in M1 medium, reduced Kit expression and ICC numbers and TNF $\alpha$  neutralizing antibodies blocked the effect of M1 medium.
- TNF $\alpha$  derived from M1 macrophages injures ICC in vitro and TNF $\alpha$  may be important in diseases like diabetic gastroparesis.



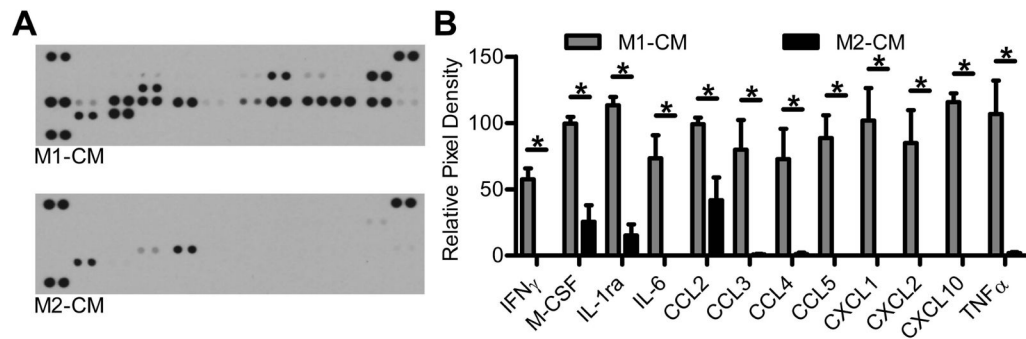
**Figure 1. Bone marrow-derived macrophages can be activated to a classically-activated M1 or alternatively-activated M2 macrophage phenotype by external stimuli**

Expression of iNOS and IL-6 RNA, markers of classically-activated M1 macrophages and Arg1, FIZZ, IL-4, IL-5, and IL-10 RNA, markers of alternatively-activated M2 macrophages in bone marrow-derived macrophages following treatment with IFN $\gamma$  or IL-10/TGF $\beta$ . Data were normalized to expression in unstimulated control macrophages (Unst). Beta-actin was used as a housekeeping control. Data are the means  $\pm$  SEM of  $n = 5$  independent experiments, \* indicates  $P < 0.05$  vs unstimulated cells, one-sample t-test.



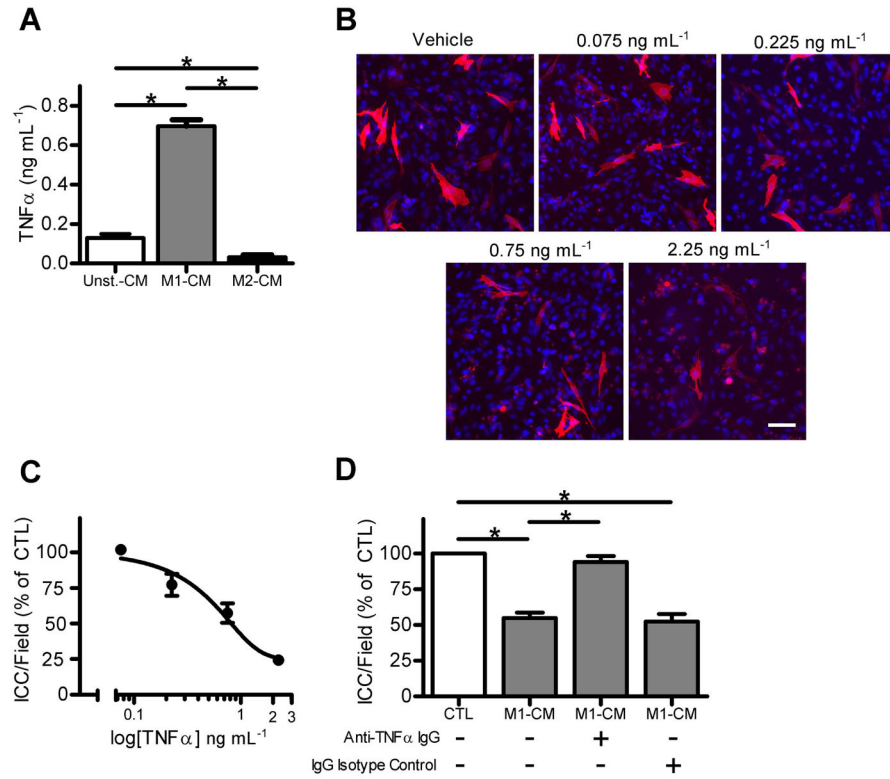
**Figure 2. M1-conditioned medium (M1-CM) has large and small molecular weight bioactive components that reduce ICC numbers in primary culture**

Effect of conditioned medium (CM) on ICC in primary culture. M1-CM but not M2-CM caused a decrease in the number of Kit-positive ICC after 24 hours of exposure to the medium. (A) Representative images showing Kit-positive ICC treated with unconditioned medium as a control (left panel), M1-CM (middle panel) and M2-CM (right panel). Note that fewer ICC are seen in the middle panel. (DAPI nuclear counterstain is shown in blue, scale bar = 50  $\mu$ m). (B) Quantification of the effects of the media on ICC numbers. Data are shown as ICC per high-powered field. M1-CM caused a significant reduction in ICC numbers (n = 7 independent experiments, \* indicates P < 0.05, 1way ANOVA with Dunnett's Post Test). (C) The activity of the M1-CM was retained after fractionating the medium into >3 kDa and <3 kDa fractions. Size separation did not alter the effect of M2-CM on ICC numbers (n = 4 independent experiments, \* indicates P < 0.05, 1way ANOVA with Dunnett's Post Test vs unconditioned medium). (D) Mixing of M1-CM and M2-CM prevented the loss of ICC numbers (n = 4 independent experiments, \* indicates P < 0.05, 1way ANOVA with Dunnett's Post Test).



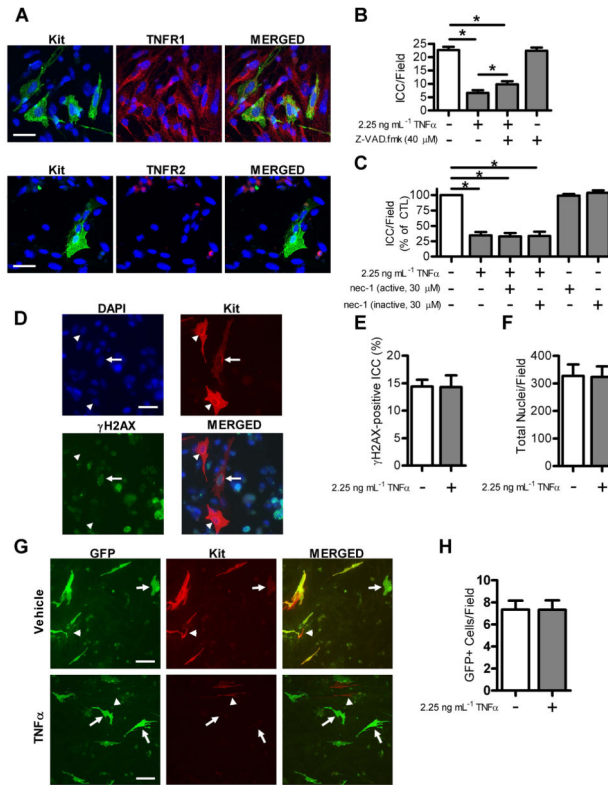
**Figure 3. M1-CM contains numerous pro-inflammatory cytokines and chemokines**

Detection of cytokine and chemokine expression in M1-CM and M2-CM by immunoblotting. (A) Representative blots showing expression of cytokines and chemokines in M1-CM (upper panel) and M2-CM (lower panel). (B) Relative expression of the identified proteins as determined by densitometry. Data are the means  $\pm$  SEM of  $n = 5$  independent experiments, \* indicates adj  $P < 0.05$ , 2way ANOVA with Bonferroni's correction for multiple comparisons.



**Figure 4. rmTNF $\alpha$  reduced Kit-positive ICC numbers in a concentration-dependent manner and neutralizing TNF $\alpha$  blocks the effect of M1-CM on Kit-positive ICC numbers**

(A) M1-CM contains significantly more TNF $\alpha$  than Unst-CM and M2-CM by ELISA. Data are means  $\pm$  SEM,  $n = 7$  independent experiments, \* indicates  $P < 0.05$ , 1way ANOVA with Dunnett's Post Test. (B) Images of Kit+ ICC after being treated for 24 hours with the indicated concentrations of rmTNF $\alpha$  (DAPI nuclear counterstain shown in blue, scale bar = 50  $\mu$ m). (C) Concentration response curve for the effect of rmTNF $\alpha$  on ICC in primary culture. Fitted line reports an EC50 value of 0.817 ng mL<sup>-1</sup> ( $n = 4$ ). (D) A TNF $\alpha$ -neutralizing antibody inhibits the effect of M1-CM (> 3 KDa fraction) on ICC in primary culture. IgG isotype control had no effect. Data are the means  $\pm$  SEM of  $n = 4$  independent experiments, \* indicates  $P < 0.05$ , ANOVA with Dunnett's Post Test.



**Figure 5. TNF $\alpha$ -mediated loss of Kit-positive ICC numbers is partially due to a caspase-dependent apoptotic mechanism**

(A) Single confocal images showing the ICC marker Kit (green, top left panel) and TNFR1 (red, top middle panel). The merged image (top right panel) indicates the presence of TNFR1 on ICC. Images showing the ICC marker Kit (green, bottom left panel) and TNFR2 (red, bottom middle panel). The merged image (bottom right panel) indicated that TNFR2 is not localized to ICC (scale bar = 30  $\mu$ m). Cells were stained with nuclear counterstain DAPI (blue). Dissociated cells from three separate primary cultures were examined for the presence of TNFR1 and TNFR2. (B) Reduced effect of TNF $\alpha$  on Kit-positive ICC numbers following incubation with the pan-caspase inhibitor Z-VAD.fmk (40  $\mu$ M) (Means  $\pm$  SEM of  $n = 6$  independent experiments, \* indicates  $P < 0.05$ , ANOVA with Dunnett's Post Test). (C) Necrostatin-1 (30  $\mu$ M), an inhibitor of caspase-independent necroptosis, did not significantly affect TNF $\alpha$ -treated Kit-positive ICC numbers (Means  $\pm$  SEM of  $n = 4$  independent experiments, ANOVA with Dunnett's Post Test). (D) Immunohistochemistry for  $\gamma$ H2AX, a marker of cellular senescence. DAPI nuclear counterstain is shown in blue (top left panel), ICC labeled for Kit are shown in red (top right panel), and  $\gamma$ H2AX is shown in green (bottom left panel). The merged image (bottom right panel) shows  $\gamma$ H2AX-positive foci in the nucleus of a Kit-positive ICC (arrow), while there are also ICC without  $\gamma$ H2AX-positive foci in the nucleus (arrowheads) (scale bar = 30  $\mu$ m). (E)  $\gamma$ H2AX-positive ICC were unaltered in TNF $\alpha$ -treated Kit-positive ICC as compared to untreated control (Means  $\pm$  SEM of  $n = 4$  independent experiments, one-sample t-test). (F) Total nuclei per high-powered field were quantified to determine if there was a loss of the feeder cell fibroblasts. Total nuclei/field were unaltered in TNF $\alpha$ -treated condition as compared to control (Means

± SEM of n = 5 independent experiments, one-sample t-test). (G) ICC lineage tracing. GFP+ cells having ICC origin (green, left panel), and immunohistochemistry for Kit (pseudo-colored red, middle panel). The merged image (right panel) shows cells that are Kit-positive and GFP-negative (arrow heads) and cells that are GFP-positive and Kit-negative (arrows) (scale bar 50 μm). (H) Total GFP-positive cells were quantified and there was no significant difference between the TNFα-treated condition compared to vehicle control condition (Means ± SEM of n = 3 independent experiments, one-sample t-test).