Evaluation of immunomodulatory properties of feline mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSC) offer a novel approach to treatment of inflammatory disorders in humans and companion animals. Cats spontaneously develop a wide variety of inflammatory disorders and may potentially benefit from MSC-based therapies. Multiple genes are involved in immunomodulation by MSC and interspecies differences between expressions of these genes exist. The goals of the study were to characterize the expression of genes known to be involved in MSC-based immunomodulation and determine the effect of MSC on proliferation of T-cells in co-culture experiments with PBMC. Unstimulated MSC expressed all immunomodulatory genes studied except for IL-10. Levels of iNOS and FASL were low or undetectable at the RNA level. INFγ stimulation resulted in significant dose dependent upregulation of IDO1, PD-L1, IL-6, COX2 and HGF. Levels of kynurenine were increased after three-day incubation with INFγ. TNFα stimulation increased expression of IL-6 at both RNA and protein level as well as upregulated COX2 gene expression and PTGES1.Stimulation with both INFγ and TNFα resulted in significant increase in PGE2 levels in cell culture medium. MSC significantly decreased proliferation of ConA stimulated PBMC in co-culture experiments at 1:5 ratio. Our results suggest that feline MSC have similar immunomodulatory gene expression and react to inflammatory cytokines in a manner similar to human MSC. Thus, MSC may play an important role in treatment of feline disease as well as serve as an important translational species to evaluate MSC-based therapies of diseases common to both humans and cats.

Introduction

Mesenchymal stem cells (MSC) have been intensively studied since their first characterization in 1970s [1]. The more recent discovery that MSC are capable of modulating a wide array of immune cells including T-cells, natural killer (NK) cells, dendritic cells, macrophages, B-cells, mast cells and neutrophils [2-9] of and induction of T-regulatory and B-regulatory cells [10,11] has led to interest in their use in patients with inflammatory disorders. MSC-based therapy has been successfully implemented in the settings of both induced inflammatory disorders in rodents,[12,13] as well as spontaneously occurring disorders [11,14,15]. Many questions still remain unanswered such as the optimal route of injection, the dose to be used and diseases that can be targeted. Use of large animal models such as cats may assist in the preclinical phase of investigations for humans. Cats spontaneously develop a variety of inflammatory disorders such as asthma [14], chronic idiopathic (interstitial) cystitis [16], chronic pancreatitis [17], chronic kidney disease [18] or inflammatory bowel disease [19], which can serve as potential translational models for cell based therapies. Many inflammatory mediators have been implicated in immunomodulatory properties of MSC. These factors include idoleamine 2,3 dioxigenase (IDO), inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), interleukin 10 (IL10), hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ), interleukin 6 (IL6), programmed death ligand 1 (PD-L1), Fas ligand (FASL), heme-oxygenase 1 (HMOX1) [3,4,8,13,15,20-23]. Interspecies differences exist in immunomodulation between human MSC and murine MSC [20,21,24]. In mice, the immunomodulation is considered to be iNOS dependent [25], while in humans it is IDO1 dependent and iNOS is expressed at very low levels [20,24]. Information on expression of immunomodulatory genes in cats is lacking. Therefore, the current study was undertaken to characterize the immunomodulatory properties of feline MSC.

Materials and Methods

Isolation of feline MSC

MSC were isolated from adipose tissue of adult cats [20.7 (±5.7) months] as previously described by our group MSC [26,27]. Tissues were collected from clinically healthy research colony based animals under approved procedures of the Institutional Animal Care and Use Committee at Michigan State University (AUF#: 09/12-171-00). In brief, adipose tissue (2-4 grams) was sterilely excised from subcutaneous tissues, minced and incubated in Collagenase I (Sigma-Aldrich, St.Louis, MO) at a concentration of 1 mg/ml in HBSS (Sigma-Aldrich, St.Louis, MO) for 2 hours at 37°C with 5% CO_2. The digested tissue was then passed through 70µm cell strainer in order to create single cell suspension and washed in sterile PBS (Sigma-Aldrich, St.Louis, MO). The cell pellet was then suspended in KNAC medium [Keratinocyte SFM medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) supplemented with 2mM of N-acetyl-L-cysteine (Sigma-Aldrich, St.Louis, MO) and 0.2 mM of L-ascorbic acid 2-phosphate (Sigma-Aldrich, St.Louis, MO)] with 5% of MSC-grade FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and plated in a T25 flask. Cells were trypsinized with 0.05% trypsin after reaching 80% confluency and subsequently passaged using KNAC medium. At each passage, half of the cells were cryopreserved utilizing in Basal Medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) supplemented with 20% of FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and 10% DMSO (Sigma-Aldrich, St.Louis, MO). Cells utilized in the experiments described in the manuscript were from passages 3-8. A total of 9 cell lines established from different cats were used interchangeably between the experiments.

Phenotypic characterization

98 All MSC cell lines were characterized for tri-lineage differentiation as well as expression
99 of CD90, CD44, MHCII and CD105 on cell surface of the cells. Trilineage differentiation
100 was accomplished through utilization of Stempro (Gibco, Life Technologies, Thermo Fisher
101 Scientific Inc, Grand Island, NY) differentiation medium specific for each lineage.
102 Experiments were carried out according to manufacturers recommendations in 24 well plates
103 (USA Scientific, Ocala, FL) for adipogenesis and osteogenesis. For chondrogenesis, instead
104 of using a plate, a 15 ml spinning tube was utilized to form micromass of 10^6 number of
105 cells/tube. Cells were incubated in differentiation media for at least two weeks before
106 staining for adipogenesis, utilizing Oil-o-red stain. Two-week incubation period was also
107 used for characterization of chondrogenesis and subsequent Alcian Blue staining. Cells in the
108 osteogenesis group were incubated for 3 weeks and stained with Alizarin red stain.

109 Expression of cell surface markers CD90, CD44, CD105, MHCII was analyzed with flow
110 cytometry using previously described [28,29] antibodies: CD90 (clone 5E10, BD Biosciences,
111 San Jose, CA), CD44 (clone IM7.8.1, Invitrogen, Life Technologies, Thermo Fisher
112 Scientific Inc, Grand Island, NY), CD105 (clone SN6, Invitrogen, Life Technologies,
113 Thermo Fisher Scientific Inc, Grand Island, NY), MHCII (clone Tu39, BD Biosciences, San
114 Jose, CA). All samples were characterized using LSRII flow cytometer (BD Biosciences, San
115 Jose, CA). Data was then analyzed using commercially available software (FlowJo, LLC,
116 Ashland, OR).

117 Peripheral blood mononuclear cells (*PBMC) isolation*

118 Peripheral blood was collected into EDTA tubes. Peripheral blood mononuclear cells
119 (PBMC) were isolated using gradient centrifugation with Histopaque-1077 (Sigma-Aldrich,
120 St.Louis, MO) in Accuspin tubes (Sigma-Aldrich, St.Louis, MO) according to manufacturer
121 recommendations. Isolation of feline PBMCs using Histopaque-1077 has been previously
122 described [30]. The cellular interface was collected, washed twice with PBS (Sigma-Aldrich,

123 St.Louis, MO) and cryopreserved in Basal Medium (Gibco, Life Technologies, Thermo
124 Fisher Scientific Inc, Grand Island, NY) supplemented with 20% of FBS (Gibco, Life
125 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and 10% DMSO (Sigma-
126 Aldrich, St.Louis, MO).

127 Interferon gamma (*INFγ) and Tumor necrosis factor alpha (TNFα) stimulation*

128 In order to evaluate the effects of inflammatory cytokines INFγ and TNFα on gene
129 expression of feline MSC, $2x10^5$ cells were plated in a 6 well plate in KNAC medium,
130 allowed to attach overnight and stimulated with increasing concentrations of feline
131 recombinant INFγ (R&D Systems, Minneapolis, MN), ranging from 0.25 to 50 ng/mL, or of
132 feline recombinant TNFα (R&D Systems, Minneapolis, MN), ranging from 0.25 to 10 ng/mL.
133 Cells were incubated for 24 hours for gene expression studies. Both cells and supernatant
134 were collected and frozen for subsequent analysis at -80° C. For protein/metabolite assays
135 cells were stimulated for both 24 and 72 hours. In each experiment MSC isolated from 5
136 different donors were utilized.

137 *Quantitative Real Time PCR*

138 For gene expression analysis, RNA was isolated using mirVana Isolation kit (Life
139 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY). RNA was quantified on
140 Qubit 2.0 Fluorometer using Qubit RNA HS assay kit (Life Technologies, Thermo Fisher
141 Scientific Inc, Grand Island, NY). Quality of RNA was evaluated using Bioanalyzer (Agilent,
142 Santa Clara, CA) or Caliper LabChip GX (PerkinElmer, Waltham, MA). Only RNA with
143 RNA integrity number higher then 8 was used for subsequent analyses.

144 Reverse transcription was accomplished using Superscript III reverse transcription kit
145 (Invitrogen, Thermo Fisher Scientific Inc, Grand Island, NY) and random primer hexamers
146 (Promega, Fitchburg, WI).

147 QPCR was performed using custom designed TaqMan primers (Supplemental Table 1;
148 Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and TaqMan Universal
149 Master Mix (Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) on ABI
150 7900 HT RT-PCR system or StepOnePlus system (Life Technologies, Thermo Fisher
151 Scientific Inc, Grand Island, NY). Each assay was run in triplicate.

152 Data analysis was performed using relative quantification method ($_{\Delta\Delta}$CT method) [31].
153 Relative quantification data was log2 transformed (Log2RQ) for normalization and displayed
154 as such in all figures [32].

155 *Protein quantification*

156 IL10 and HGF concentrations were assed using feline specific ELISA kits (R&D Systems,
157 Minneapolis, MN and MyBioSource, Inc., San Diego, CA respectively). Feline IL6 and
158 multispecies TGFβ1 were used to quantify the respective proteins using Milliplex magnetic
159 bead based assays (Millipore Billerica, MA) according to the manufacturers'
160 recommendations. ELISA plates were analyzed in triplicate using a plate reader (PerkinElmer,
161 Waltham, MA), while bead based assays were analyzed in duplicate using Luminex 100
162 machine (Luminex Corporation, Austin, TX).

163

164 *PGE2 quantification*

165 A 50-μL aliquot of each cell culture medium was transferred to a polypropylene
166 microcentrifuge tube. Then 200 μL of methanol with 0.01% BHT (Tokyo Kasei Kogyo Co.,
167 Tokyo, Japan) and PGE_2-d_9 (Cayman Chemical, Ann Arbor, MI) as the internal standard was
168 added. After vortexing for 1 min, the mixture was centrifuged at 10,000 × *g* for 10 min at 4˚C.
169 One hundred μL supernatant was transferred to an amber autosampler vial with 250-μL glass
170 insert in preparation for LC-MS/MS analysis. Liquid chromatography separations were

171 performed using an Ascentis Express C18 column (10 cm×2.1 mm; 2.7 μm particles, Sigma-
172 Aldrich, St. Louis, MO) maintained at 50 ˚C on a Waters ACQUITY UPLC system (Waters,
173 Milford, MA). The autosampler was cooled to 10 ˚C, and injection volume was set to 5 μL.
174 Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. The
175 flow rate of the LC gradient was 0.3 mL/min. The mobile phase gradient began at 1% B,
176 followed by a linear increase to 40% B at 2 min; then to 80% B at 8 min, and 99% B at 9 min,
177 at which the composition was held until 13 min; then returned to 1% B until 15 min. PGE_2
178 eluted at 4.32 min. The LC column was connected to a Waters Xevo TQ-S triple quadrupole
179 mass spectrometer. PGE_2 and PGE_2-d_9 were analyzed with electrospray ionization (ESI)
180 multiple reaction monitoring (MRM) in negative-ion mode at transitions of m/z 351.2 → 271
181 for PGE_2 and 360.2 → 280 for PGE_2-d_9. Each transition was monitored with a 0.163s dwell
182 time. The optimized cone voltages of 55 V and 21 V, and collision energy of 16V were
183 selected for both PGE_2 and PGE_2-d_9. Other MS parameters were as follows: capillary voltage,
184 2.5 kV; source temperature, 150 ˚C; desolvation temperature, 500 ˚C; desolvation gas flow
185 rate, 600 L/h; cone gas flow rate 150 L/h. Calibration solutions were prepared by serial
186 dilution of PGE_2 (Cayman Chemical, Ann Arbor, MI) stock solution by methanol to
187 concentrations of 20, 4, 0.8, 0.2, 0.05, 0.02, and 0.01 ng/mL. Internal standard PGE_2-d_9 was
188 added to each calibration solution with the same amount as added in cell culture medium. For
189 quantification, the ratio of PGE_2 peak area to its internal standard peak area was plotted
190 against the concentration, fitted by a weighted (1/x) linear regression using the TargetLynx
191 component of MassLynx 4.1 software (Waters, Milford, MA).

192 *IDO activity measurement*

193 $5x10^5$ MSC were plated in a T25 flask in KNAC medium with 5%FBS and allowed to
194 attach overnight. Cell culture medium was then changed to KNAC medium with 5% FBS
195 supplemented with 100μM Tryptophan (Sigma-Aldrich, St.Louis, MO). After a 3 day

196 incubation, the cell culture supernatant was collected and analyzed. To measure the IDO
197 mediated tryptophan catabolism, a spectrophotometric method was utilized to detect
198 kynurenine, a stable catabolite of IDO, as previously described [33] with slight modifications.
199 Cell culture medium was mixed with 30% trichloroacetic acid at a 2:1 ratio, mixed and spun
200 down at 10000 rpm. Supernatant was then mixed in equal volume with Ehrlich reagent in a
201 96 well microplate (R&D Systems, Minneapolis, MN). Optical density was then read at 492
202 nm utilizing a plate reader (PerkinElmer, Waltham, MA).

203 *Co-culture experiments*

204 MSC were plated at a density of 10^4 cells per well in KNAC medium with 5% FBS
205 and irradiated with 30Gy using X-RAD320 irradiator (Precision X-ray, North Branford, CT)
206 in order to stop proliferation. Previous reports have indicated that pre-stimulation with
207 inflammatory cytokines potentiate the immunomodulatory properties of MSC [20,25]
208 therefore, we wanted to investigate if that's the case in feline MSC. In order to assess the
209 effect of prestimulation, cells were prestimulated with the cytokines INFγ (50 ng/mL) , TNFα
210 (10 ng/mL) or both INFγ and TNFα (50 and 10 ng/mL respectively) for 2 hours prior to
211 addition of PBMCs. Isolated allogenic PBMCs were thawed, washed in PBS. Subsequently
212 cells were quantified and assessed for viability utilizing Countess cell counter (Life
213 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY). All PBMCs were
214 cryopreserved only short-term for 48-72 hours. PBMCs were then suspended in RPMI1640
215 (Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) with 10% FBS (Life
216 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and added at ratio of 1:5 of
217 MSC to PBMC. Proliferation of lymphocytes was induced by 5 ng/mL of ConA (Sigma-
218 Aldrich, St.Louis, MO). Cells were pulsed with EdU (Life Technologies, Thermo Fisher
219 Scientific Inc, Grand Island, NY) 24 hours before the end of the 3-day incubation period.
220 Fluorescence intensity was then measured using fluorescent plate reader (PerkinElmer,

221 Waltham, MA). Background fluorescence assessed from wells containing only MSC was
222 subtracted from the total fluorescent values in co-culture wells. Experiments shown represent
223 a single allogenic PBMC donor co cultured with MSC derived from four different donors.

224 *Statistical analysis*

225 Each data set was analyzed for normality using Shapiro-Wilk normality test. Gene
226 expression data was analyzed using ANOVA with post-hoc Dunnett test for normally
227 distributed data, while Friedman test with post hoc Dunn's test was used for non-parametric
228 data. Protein, metabolite and cellular response data were analyzed using t-test or non-
229 parametric Wilcoxon-signed rank test. All analyses were performed using GraphPad Prism 6
230 (GraphPad Software, Inc, La Jolla, CA)

231 **Results**

232 *Isolation and characterization of MSC*

233 All MSC cells isolated strongly expressed the cell surface markers CD90, CD105,
234 CD44 and did not express MHCII (Figure 1). They also underwent trilineage differentiation
235 into chondrocytes, adipocytes and osteocytes as previously described by our group [27]
236 (Figure 2).

237 *Quantitative real time PCR*

238 All cell lines constitutively expressed IDO1, COX2, PTGES1, PTGES2, PTGES3,
239 HGF, TGFβ1, IL6, PD-L1, and HMOX1. IL10 was undetectable at RNA level, while levels
240 of iNOS and FASL were low or undetectable. Stimulation with varying concentrations of
241 INFγ resulted in increased expression of IDO1 (8.5 to 12.3 Log2-fold increase, $p<0.001$),
242 which reached plateau at concentrations at 5 ng/ml (Figure 3A). Expression of IL-6 was

243 upregulated after exposure to INFγ as well (Figure 3A, 0.3 to 2.1 Log2-fold increase,
244 $p<0.001$), with increased expression being statistically significant at levels as low as 1 ng/ml
245 of cytokine. Concentrations of INFγ as low as 0.1 ng/ml significantly upregulated expression
246 of PD-L1 (Figure 3A, 1.5 to 3.7 Log2-fold increase, $p<0.001$), while expression of HGF rose
247 gradually with cytokine concentration increment (Figure 3A, 0.29 to 1.8 Log2-fold increase,
248 at 0.25 to 50 ng/ml of INFγ, $p<0.001$), with statistically significant result at as low 1 ng/ml of
249 INFγ. Stimulation with as low as 5 ng/mL of INFγ resulted in significant increase in COX2
250 expression compared to unstimulated cells (0.98 to 1.62 Log2-fold increase, $p<0.05$ to
251 $p<0.01$, Figure 3A). Interestingly levels of PTGES1 decreased significantly after stimulation
252 with 50 ng of INFγ (-0.45 Log2-fold difference, $p<0.05$, Figure 3A).
253 Stimulation of MSC with as low as 0.25 ng/ml of TNFα resulted in significantly
254 increased expression of IL6 (Figure 3B, 2.7 to 5.2 Log2-fold difference, $p<0.001$).
255 Expression of COX2 and PTGES1 significantly increased at doses 2.5 and 10 ng/mL of
256 TNFα (COX2: 2.08 Log2-fold increase, $p<0.01$ and 2.014 Log2-fold increase, $p<0.05$
257 respectively; PTGES1: 0.758 Log2-fold increase, $p<0.05$ and 0.8 Log2-fold increase, $p<0.01$
258 respectively; Figure 3B).
259 In order to determine if uPA levels were responsible for decrease of levels of HGF in
260 our protein assay, we investigated uPA levels after stimulation with a single dose of INFγ.
261 This stimulation resulted in significant upregulation of uPA at gene expression level (2.47
262 Log2-fold increase, $p<0.05$, Figure 3A).
263 All other genes tested remained unaltered by stimulation with inflammatory cytokines.
264 In summary, we have identified that 24-hour stimulation INFγ results in significant
265 dose dependent upregulation of IDO1, IL6, PD-L1, COX2 and uPA, while decreased
266 PTGES1. TNFα stimulation resulted in upregulation of COX2 and PTGES1.

267 *Protein and metabolite quantification*

Levels of IL6 increased after stimulation with each cytokine over 72 hours (TNFα, $p<0.01$; INFγ, $p<0.05$; both, $p<0.001$; Figure 4A). Levels of IL6 were also affected by inflammatory environment after 24-hour cytokine stimulation (Supplemental Figure 3).

After 72 hours of stimulation with TNFα amount of TGFβ1 significantly increased ($p<0.05$; Figure 4B), while stimulation with both INFγ and TNFα decreased the concentration of secreted TGFβ1 as compared to unstimulated cells ($p<0.01$; Figure 4B).

Interestingly, there were no differences in the concentration of HGF between different conditions at 72 hours, but levels of HGF decreased significantly after three days of incubation compared to day 1 (Supplemental Figure 1), and where on the lower end of detection of the assay.

Levels of PGE2 significantly increased after stimulation of cell with both INFγ and TNFα for three days ($p<0.05$, Figure 5A).

IL10 values were below the detection limit of the only commercially available assay for feline IL10.

IDO activity

INFγ stimulation significantly increased kynurenine levels in cell culture supernatants after 3 days of incubation compared to unstimulated cells ($p<0.001$, Figure 5B).

Co-culture experiments

Co-culture of irradiated MSC with ConA stimulated PBMCs at 1:5 ratio significantly blocked the proliferation of PBMCs as measured by EdU incorporation assay ($p<0.001$; Figure 6). Prestimulation of MSC with either INFγ or with both INFγ and TNFα did not change this effect.

290 # Discussion

291 The data presented in this study document that feline MSC constitutively express
292 IDO1, PTGES1, PTGES2, PTGES3, HGF, TGF-beta, IL6, PD-L1, HMOX1. Significantly,
293 stimulation with INFγ for 24hrs, resulted in up to 12- Log2-fold increase in IDO1 expression
294 in MSC, in a manner similar to those observed in human MSC upon stimulation with this
295 cytokine [34]. The upregulation was confirmed through measurement of kynurenine, an
296 IDO1-mediated tryptophan metabolite. The increase in this metabolite was clearly detectable
297 after 3-days of INFγ incubation. Interestingly, IDO1 is strongly upregulated and functional in
298 human MSC [20,34], whereas in the mouse, this gene is not responsive to INFγ [3,21,24,35].
299 Mouse MSC induce iNOS upon stimulation with INFγ, one of the key molecules involved in
300 murine immunomodulation [25,36]. Expression of iNOS by feline MSC in our experimental
301 conditions was low or undetectable, which closely parallel human MSC [24].

302 Expression of IL6 was upregulated by both INFγ and TNF α stimulation at the RNA
303 level and that was further confirmed by protein assays. Constitutive expression of IL6 is
304 characteristic for MSC [37,38].

305 FASL and IL10 transcripts were undetectable in the feline MSC and stimulation using
306 INFγ and TNFα did not change this. FASL expression has been recently put forth as being
307 important for MSC mediated T-cell apoptosis [15]. Interestingly the authors of that study did
308 not detect expression of PD-L1 in MSC. However, we have observed strong induction of PD-
309 L1 after stimulation with INFγ. Our findings are concordant with data reported from placenta
310 derived MSC [39,40], where expression of PD-L1, but not FASL, has been reported Thus,
311 MSC may use different effectors to induce apoptosis of T-cells. The differences may arise
312 from culture conditions, source of MSC (adipose, placenta, bone-marrow). In previously
313 described studies, bone marrow derived [15,40] and placenta derived cells were utilized [22].
314 Expression of IL10 by feline MSC was not detectible in our study both at the gene and

315 protein expression level. At the protein level, the signals generated were below the limit of
316 detection of the commercially available feline IL10 assay (<125 pg/ml,). Reports on the
317 production of IL10 by MSC are not concordant. In some studies with human MSC, IL10 was
318 detectable at the gene expression level [41] and at the protein level [22,35], while in others,
319 no IL10 was detected [3,42]. Both the source of MSC and culture conditions may affect the
320 production of IL10.

321 We have also identified that feline MSC upregulated COX2 in response to both INFγ
322 and TNFα stimulation, although all of the PGE2 synthases were strongly expressed in our
323 data set we identified significant increase in PTGES1 expression only after TNFα stimulation,
324 while stimulation with INFγ resulted in significant decrease in expression at the highest dose
325 given. The results on PTGES1 have to be treated with caution as the changes, although
326 statistically significant, may not be biologically effective due to low level of changes. We
327 also identified significant increase in PGE2 levels in cell culture medium after stimulation of
328 MSC with both INFγ and TNFα, while this effect was not identified after stimulation with
329 only a single cytokine. One potential hypothesis would be that increased degradation of
330 PGE2 through upregulation of enzyme responsible for PGE2 degradation HPGD
331 (Hydroxyprostaglandin Dehydrogenase 15-(NAD)). We have investigated this possibility and
332 no increase in expression of HPGD was identified after stimulation with cytokines studied
333 (data not shown). Thus we hypothesize that strong expression of PGE2 syntheses in our
334 experimental setting have precluded additional induction through stimulation. Another
335 explanation could be potential species-specific differences in prostaglandin metabolism and
336 further work would need to be done in order to answer this question.

337 Release of HGF by feline MSC into the supernatant was significantly decreased in all
338 of the conditions the amount of HGF further decreased at 72 hours. In a study with human
339 MSC, 24 hr stimulation with INFγ was reported to increase HGF release by 10 times [43],

340 however, the INF γ concentration used was four times higher (200ng/ml) as compared to our
341 study. Thus, different doses of INFγ may induce release of HGF at different time points.
342 HGF is being released as an inactive precursor, and in order for it to be fully functional, it
343 requires proteolytic cleavage through urokinase type plasminogen activator (uPA) [44]. In
344 order to investigate if this enzyme is being induced by INFγ we performed QPCR analysis of
345 uPA after cytokine stimulation. We found significant 2- Log2-fold induction of uPA after
346 stimulation of MSC with INFγ dose used in ELISA studies (50ng/mL,). One could speculate
347 that this allows for HGF to be bound by its receptor MET. Interestingly we found that after 3
348 days of incubation the levels of HGF were hardly detectable in all of the conditions measured,
349 which may suggest that HGF is maybe being cleaved by uPA in all of the conditions.
350 The levels of TGFβ1 were significantly higher after stimulation with TNFα for 3 days,
351 while lower after stimulation with both INFγ and TNFα for 3 days. The reason for this
352 disparity is unknown. In one study on human MSC the levels of TGFβ1 almost doubled after
353 stimulation with INFγ [43].In another study, stimulation of human MSC with INFγ and/or
354 TNFα resulted in decreased production of TGFβ1 [45]. Importantly we also identified that
355 TGFβ1 is constitutively expressed by feline MSC and TGFβ1was detected as early as after 24
356 hours of stimulation (Supplemental Figure 2).
357 Feline adipose derived MSC significantly blocked proliferation of ConA stimulated
358 PBMCs both in naïve state as well as post prestimulation with INFγ, TNFα or both. No
359 difference was noted between the prestimulated and naïve cells in our experiments and mixed
360 results have been previously reported in this aspect [25,43,46]. The MSC mediated blockade
361 of ConA PBMC proliferation was previously described [47] and our results are similar to
362 these previously presented.
363 Significant reduction of stimulated PBMCs seen in co-culture studies described above,
364 along with the gene expression of immunomodulatory molecules documented, provides a

strong rationale for clinical utilization of MSCs in treatment of feline inflammatory disease as well as for the utilization of cats in translational studies.

The current study documents that feline MSC share many similarities in immunomodulatory properties as human MSC and adds to the limited information available from large animal systems. Spontaneously occurring chronic inflammatory disorders in cats and dogs may serve as translational models of MSC-based therapy of human diseases [48]. Studies of MSC in mice have previously reported differences in mice as compared to humans especially with regard to expression of INOS vs IDO [21,24]. The appropriateness of mice models for human immune disorders has recently been challenged [49-51]. Although mouse models will remain important tools in the biomedical field [52], use of spontaneously occurring disorders in cats can potentially successfully fill in the gap between mouse and human in development of new treatment strategies for a variety of disorders[48,53].

The immunomodulatory properties of feline adipose derived MSC documented in this study, provide the rational for the utilization of MSC based approaches to feline diseases with inflammatory components for the treatment of both the feline diseases as well as an informative translational system, valuable for development of human therapies.

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390 # Author disclosure statement

391 No competing financial interests exist

392

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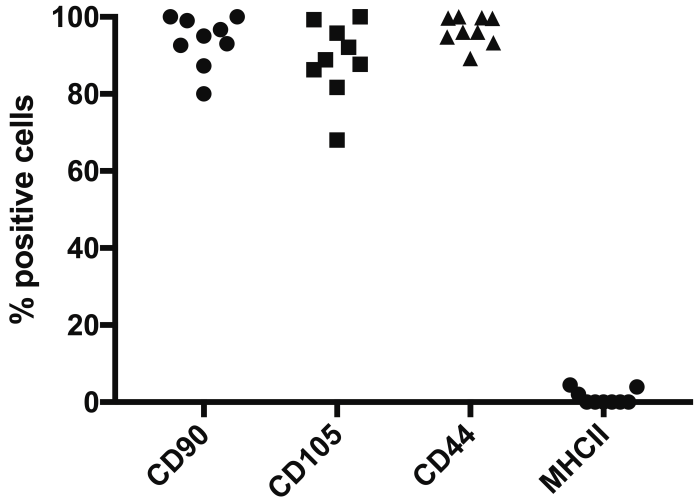
567

568 # Figure legends:

569 Figure 1. Flow cytometric characteristics of feline MSC used in this study. Note that all of
570 the cell lines are strongly expressing MSC markers CD90, CD44 and CD105, while lack
571 expression of MHCII



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573 Figure 2. Representative pictures presenting differentiation potential of isolated MSC into
574 adipocytes (A), chondrocytes (B) and osteocytes (C)

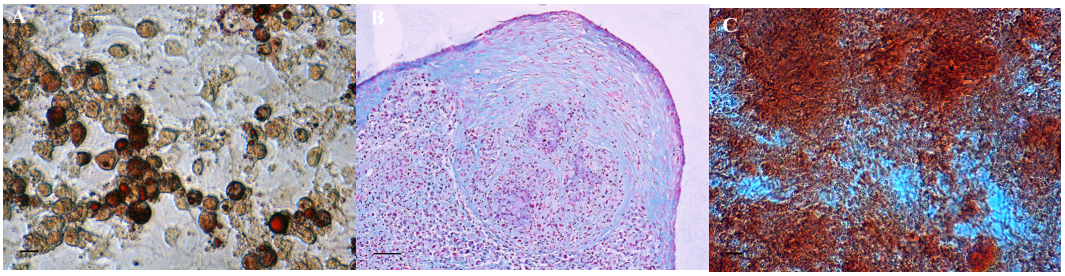
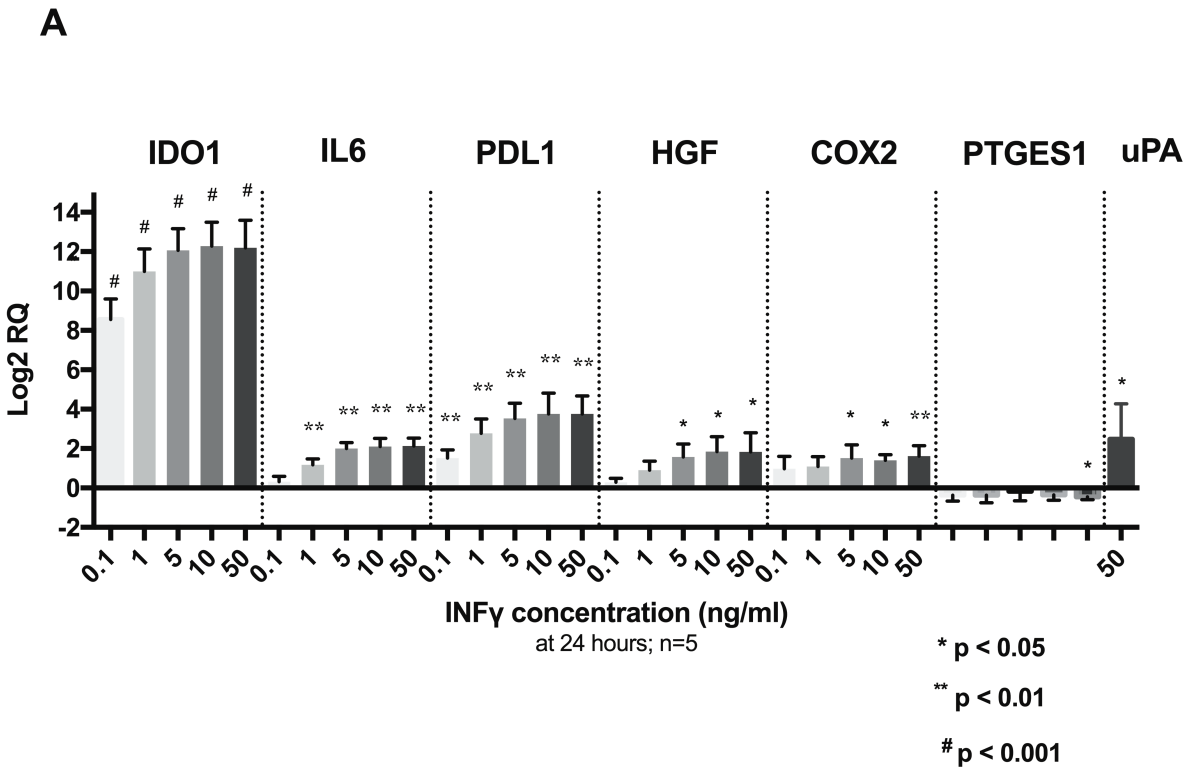


Figure 3. A) Increasing concentrations of INFγ significantly increased the expression of IDO1, IL6, PD-L1, HGF and COX2 in dose dependent fashion, while decrease in PTGES1 was identified. Stimulation of feline MSC with a single dose of 50 ng/mL of INFγ results in significant increase in uPA expression; B) Stimulation with TNFα significantly increased the expression of IL6, COX2 and PTGES1. n=5



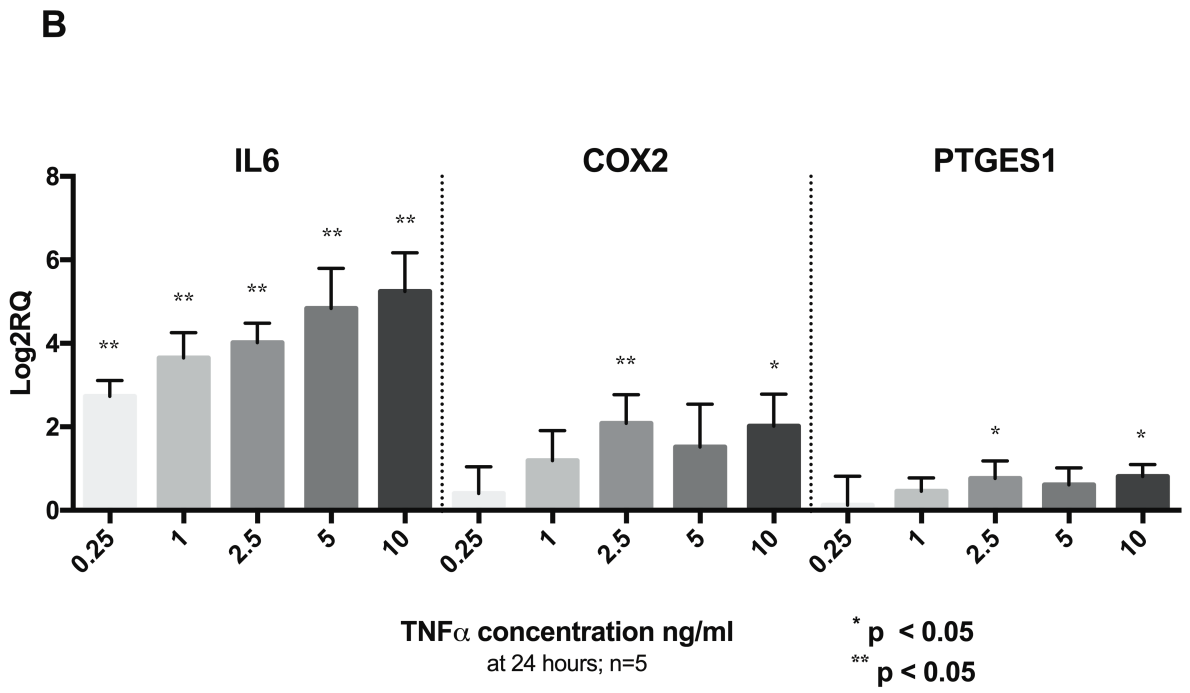
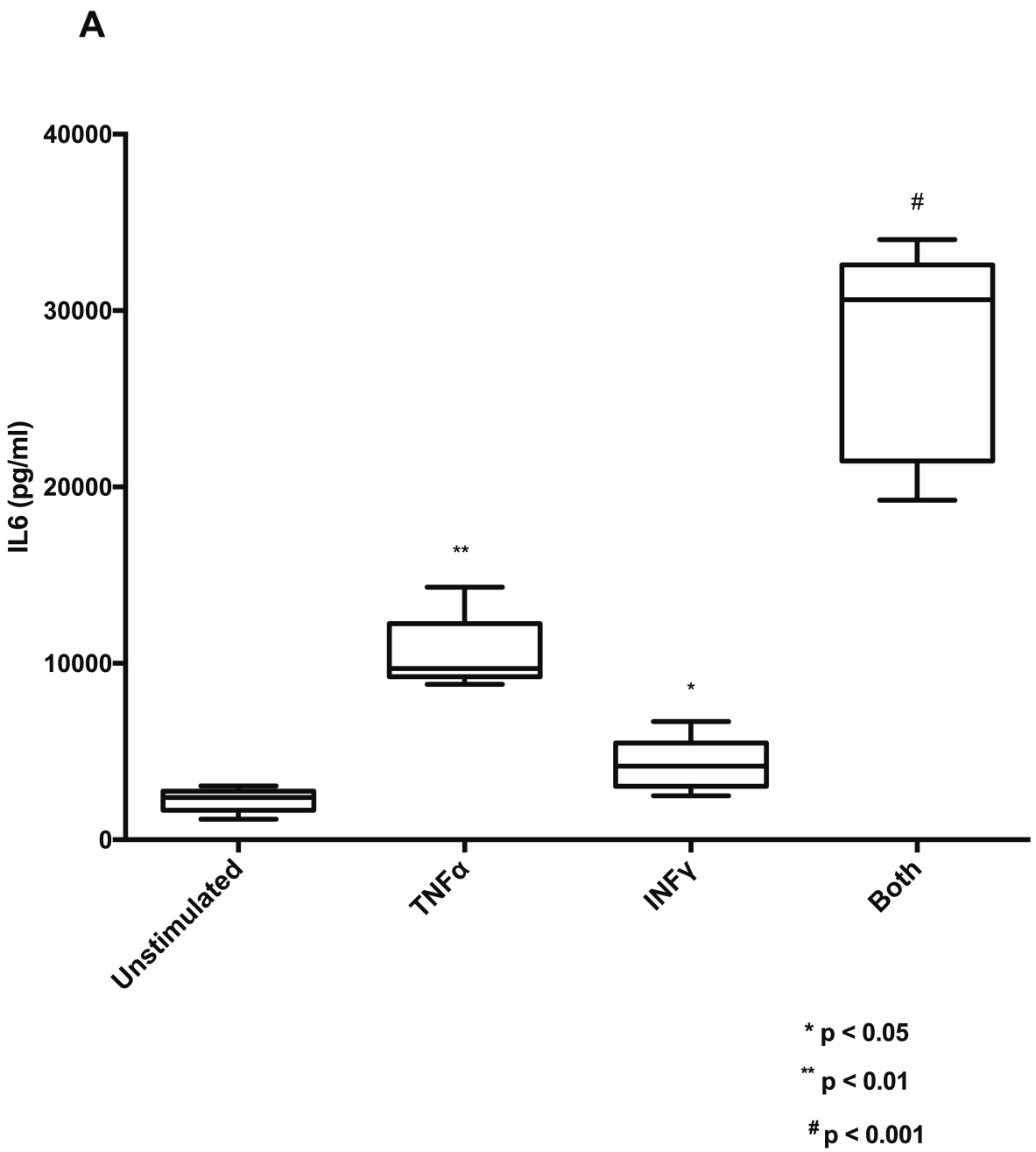
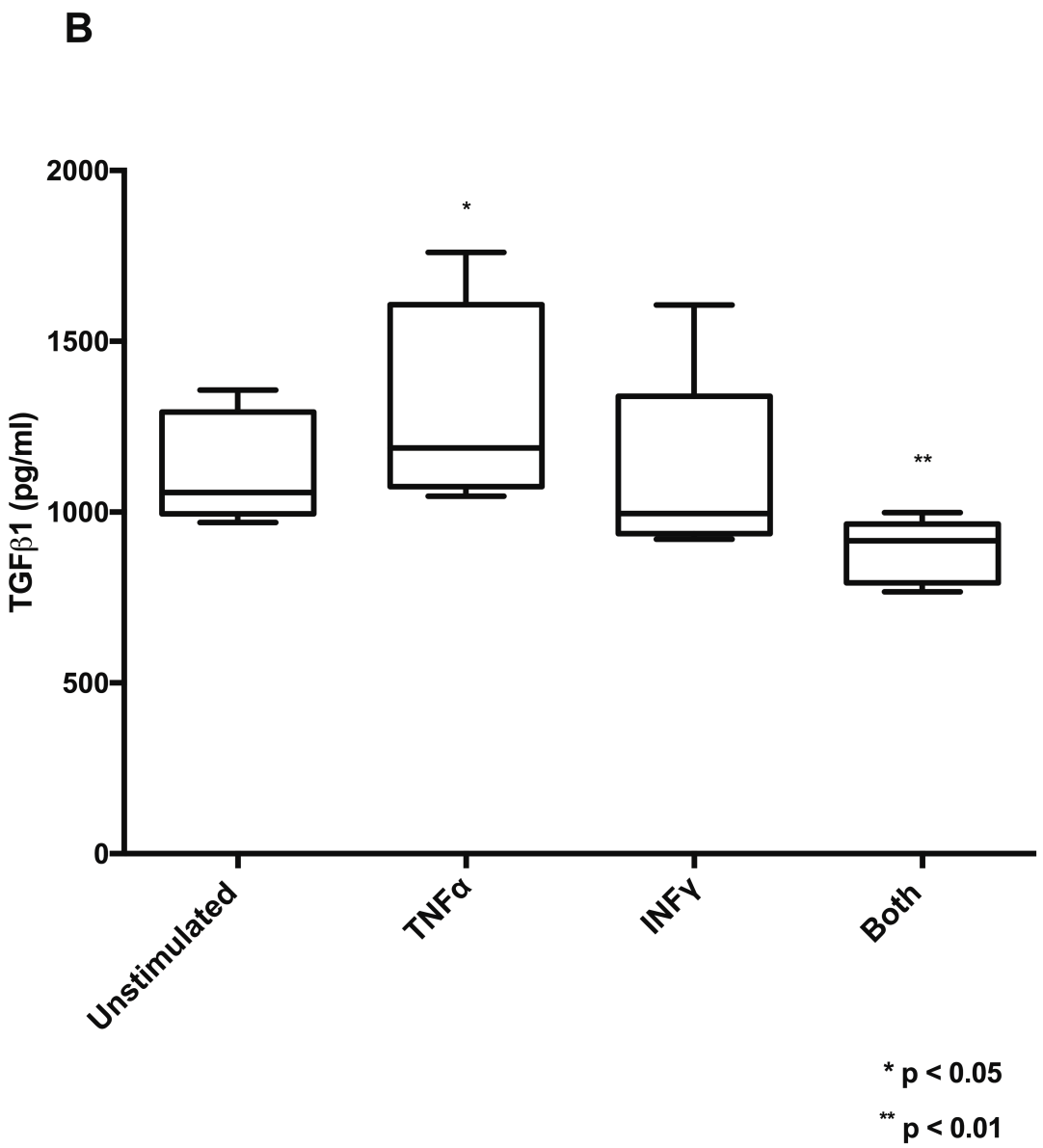


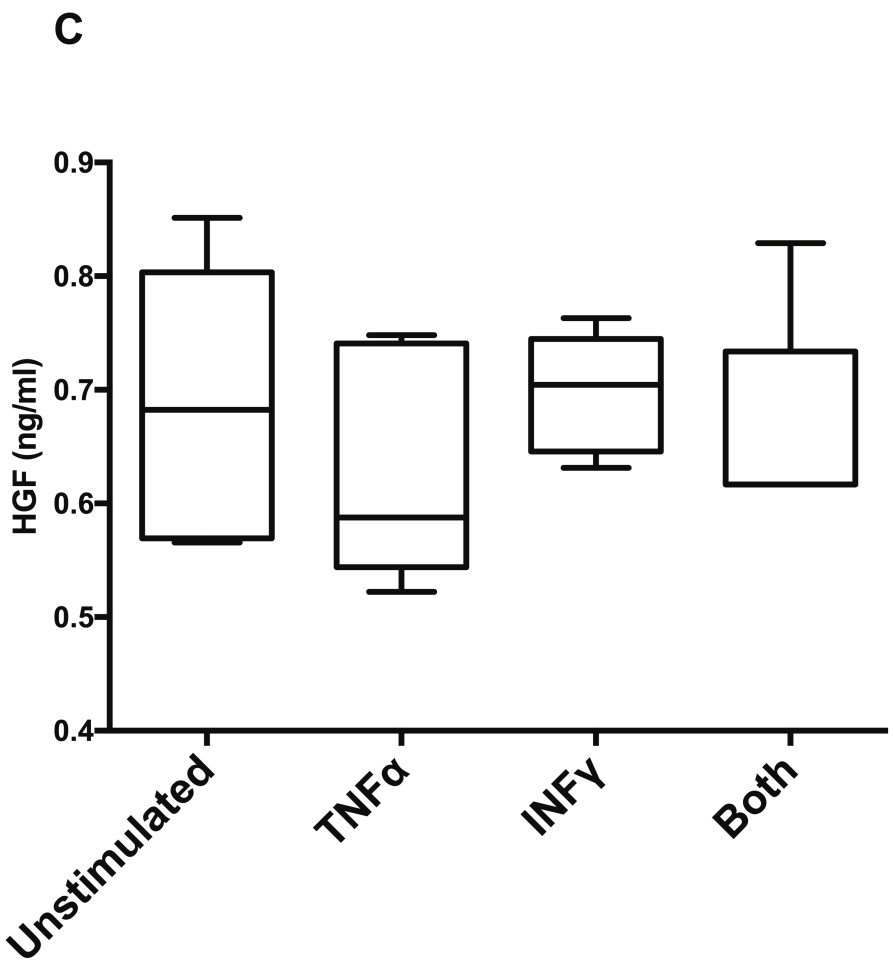
Figure 4. A) IL6 concentration in cell culture medium as measured by ELISA. Note that stimulation with a single dose of TNFα or both TNFα and INFγ resulted in significant upregulation of IL6 production; B) Levels of TGFβ1 significantly increased after 3-day incubation with TNF and significantly decreased after stimulation with both INFγ and TNFα; C) No changes in HGF expression after 3 days in culture. Interestingly the levels of HGF decreased at 3 days to levels on borderline of detection compared to levels after 1 day (Supplemental materials). n=5 per group, each assay was run in triplicate,


A
40000
30000
20000
10000
0
IL6 (pg/ml)
#
**
*
Unstimulated
TNFα
INFγ
Both
* p < 0.05
** p < 0.01
# p < 0.001

590


B
2000
1500
1000
500
0
TGFβ1 (pg/ml)
*
**
Unstimulated
TNFα
INFγ
Both
* p < 0.05
** p < 0.01

591



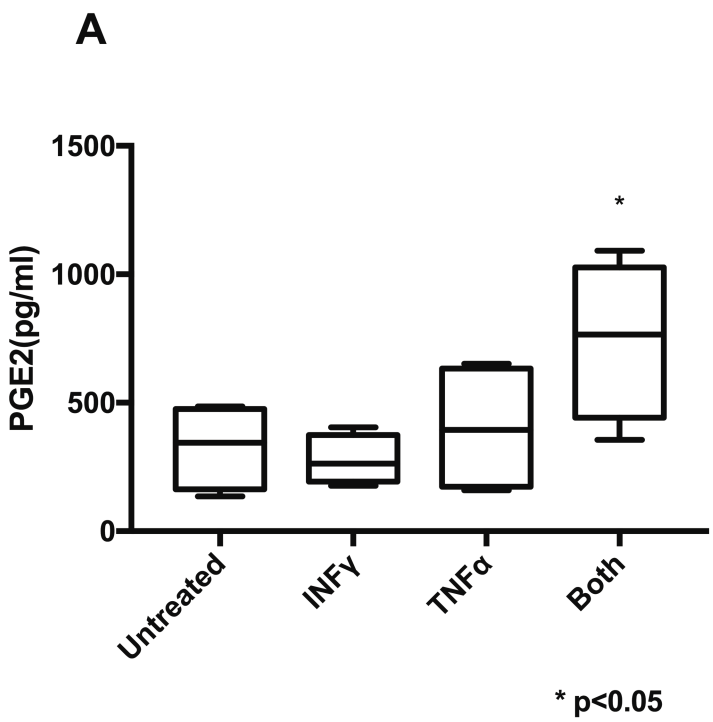
592

593 Figure 5. A) Significant increase in PGE2 levels after 3 day stimulation with both INFγ and

594 TNFα, n=5 ; B) Cells incubated for 3 days with 50 ng/mL of INFγ in medium supplemented

595 with tryptophan significantly increased the amount of kynurenine in the cell culture

596 medium.n=3



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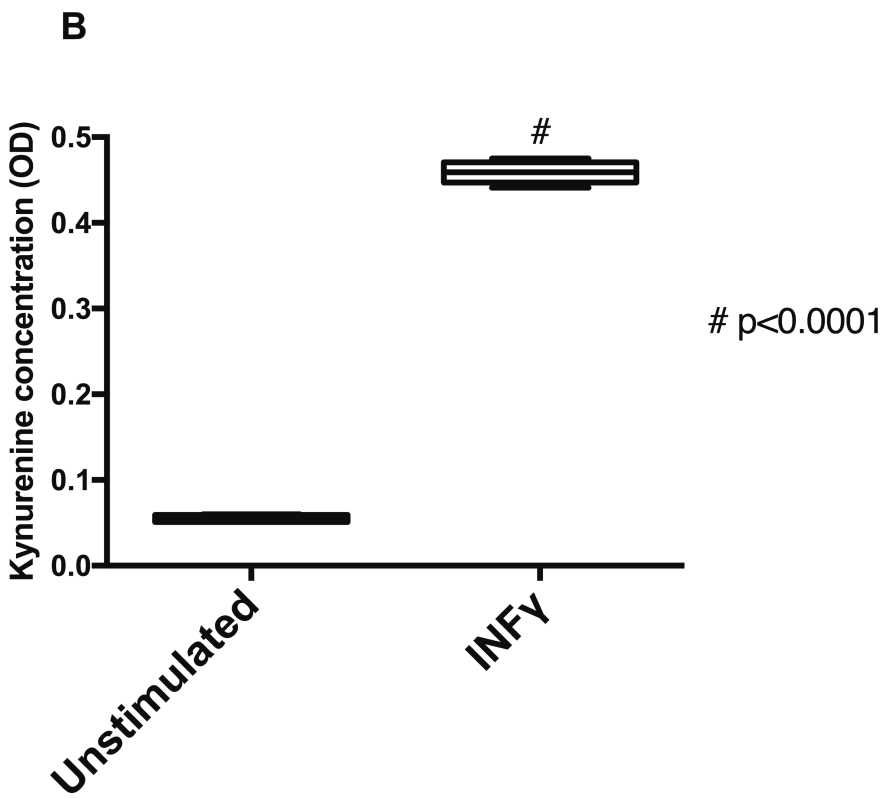

598

599 Figure 6. Co-culture experiment revealed a strong decrease in PBMC proliferation at 1 to 5

600 ratio as measured by fluorescence intensity after subtraction of background proliferation of

MSC. No effect of prestimulation with INFγ, TNFα or both INFγ and TNFα was identified n=4, each assay was run in triplicate. p<0.0001