High-affinity uptake of kynurenine and nitric oxide-mediated inhibition of indoleamine 2,3-dioxygenase in bone marrow-derived myeloid dendritic cells

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Abstract

Indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan metabolism along the kynurenine (Kyn) pathway in some dendritic cells (DC) such as plasmacytoid DC regulates T-cell responses. It is unclear whether bone marrow-derived myeloid DC (BMDC) express functional IDO. The IDO expression was examined in $CD11c^{+}CD11b^{+}$ BMDC differentiated from mouse bone marrow cells using GM-CSF. CpG oligodeoxynucleotides (CpG) induced the expression of IDO protein with the production of nitric oxide (NO) in BMDC in cultures for 24 hr. In the enzyme assay using cellular extracts of BMDC, the IDO activity of BMDC stimulated with CpG was enhanced by the addition of a NO synthase (NOS) inhibitor, suggesting that IDO activity was suppressed by NO production. On the other hand, the concentration of Kyn in the culture supernatant of BMDC was not increased by stimulation with CpG. Exogenously added Kyn was taken up by BMDC independently of CpG stimulation and NO production, and the uptake of Kyn was inhibited by a transport system L-specific inhibitor or high concentrations of tryptophan. The uptake of tryptophan by BMDC was markedly lower than that of Kyn. In conclusion, IDO activity in BMDC is down-regulated by NO production, whereas BMDC strongly take up exogenous Kyn.

Key words: Bone marrow-derived myeloid dendritic cells; Indoleamine 2,3-dioxygenase; Tryptophan; Kynurenine; Nitric oxide; Transport system L

1. Introduction

Indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan (Trp) metabolism along the kynurenine (Kyn) pathway regulates T-cell responses in some dendritic cells (DC) such as plasmacytoid DC or $CD8^+$ DC in mouse spleen cells [1,2]. Two mechanisms of IDO to inhibit T-cell responses are proposed; the local depletion of Trp required for cell proliferation and the induction of apoptosis or growth arrest by Trp metabolites [1]. Three functionally distinct subsets of DC are defined in mouse spleen cells and include the plasmacytoid DC (pDC), $CD8^+$ and $CD8^-$ conventional DC (cDC) [3]. DC generated in culture from mouse bone marrow precursors with GM-CSF [4] or GM-CSF and IL-4 are mainly $CD11c^+CD11b^+$ myeloid DC, whereas cell culture from mouse bone marrow cells with Fms-like tyrosine kinase 3 ligand (Flt3-L) allows the generation of both cDC and pDC [5-7]. Human myeloid DC differentiated from blood monocytes with GM-CSF and IL-4 or macrophages differentiated with M-CSF express functionally active IDO [8,9]. It has recently been shown that thymosin α1 activates IDO in GM-CSF/IL-4- or Flt3-L-developed DC from mouse bone marrow cells [10].

IDO is induced by inflammation or immune responses such as infectious or tumor immunity. IDO expression is induced in DC by various stimuli such as IFN-γ, toll-like receptor (TLR)-ligation by LPS or CpG oligodeoxynucleotides (CpG) or CD80/CD86-ligation by CTLA-4 expressed on the regulatory T cells [1]. CpG, which is a strong immune stimulator, also possesses immune suppressive activity through the induction of IDO [11-14]. TLR-9 signaling from CpG activates NF-κB and p38 through MyD88 and IRF-8 in DC [15]. CpG is the TLR ligand to induce IDO mRNA in bone marrow-derived myeloid DC (BMDC) [12].

IDO protein can be expressed without functional enzymatic activity. Isolated mouse splenic $CD8^+$ DC were found to catabolize Trp when exposed to IFN-γ, whereas other $CD8^-$ DC did not, even though both subsets expressed comparable amounts of

IDO protein as analyzed by Western blot [16]. It is not clear yet why IDO protein expressed in $CD8^-$ DC is not functional.

IDO activity of IFN-γ-activated murine peritoneal macrophages was induced by inhibition of nitric oxide synthase (NOS) [17]. Incorporation of the heme prosthetic group into active site is required for IDO activity, and inhibition of IDO activity by NO generators was abrogated by co-addition of oxyhemoglobin, an antagonist of NO function [17]. Both the blocking of a heme site to O_2 binding and conformational changes induced by breaking the Fe-N bond have been proposed as important mechanisms by which NO inhibits IDO [18]. NO led to an accelerated degradation of IDO protein in the proteasome [19]. In addition, a peroxynitrite generator also inhibited IDO activity through the nitration of the selective tyrosines of IDO [20]. NO production was induced in BMDC by stimulation with IFN-γ and LPS [21].

It has recently been published that BMDC expressing IDO upon IFN-γ stimulation suppress OVA-specific $CD8^+$ T cell proliferation [22,23]. However, these results are not consistent with the published report that IFN- γ enhances antigen-presenting activity in BMDC [24]. In the present study, we examined the functional expression of IDO in BMDC stimulated with CpG. BMDC expressed IDO protein upon CpG stimulation but its activity was inhibited by NO production. BMDC did not secrete Kyn upon CpG stimulation but took up exogenous Kyn.

2. Materials and methods

2.1. Reagents

The Phosphorothioate CpG1826 (5-TCC ATG ACG TTC CTG ACG TT-3), N^G-monomethyl-L-arginine acetate salt (NMA), L-Kyn, L-Trp, 2-amino-2-norbornanecarboxylic acid (BCH) and 1-methyl-DL-tryptophan (1-MT)

were purchased from Sigma-Aldrich (St. Louis, MO). PE-conjugated anti-mouse CD11c and FITC-conjugated anti-mouse CD11b antibodies were purchased from eBioscience (San Diego, CA). Anti-mouse IDO polyclonal antibody was prepared as described previously [25]. Anti-inducible NOS (iNOS) polyclonal antibody was purchased from BD Bioscience (San Diego, CA).

2.2. Preparation of BMDC

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). BMDC were generated as described previously [4]. Briefly, bone marrow cells were cultured in RPMI1640 medium (10% fetal calf serum, 300 μg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol) containing 0.3% GM-CSF supernatant (from murine GM-CSF producing Chinese hamster ovary cells, a gift from T. Sudo, Toray Silicon, Tokyo, Japan). The DC culture medium was changed every 2 days to remove nonadherent cells. Loosely adherent clustering cells were collected on day 6 and used as immature DC. BMDC were activated by stimulation with CpG (5 μg/ml) alone or together with NMA (100 μM) for 24 hr.

2.3. Flow cytometry

For detection of cell surface markers, cells were incubated with PE-conjugated anti-CD11c and FITC-conjugated anti-CD11b antibodies at 4℃ for 30 min. These cells were analyzed by an EPICS XL flow cytometer (Beckman Coulter).

2.4. Western blot

Western blot was carried out as described previously [26]. BMDC were

stimulated with CpG (5 μg/ml) with or without NMA (100 μM) for 24 hr. Then, cells were washed and lysed with 1×sample buffer and boiled for 3 min. The cell lysates were passed though a syringe with a 26G needle before being applied on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After electrophoresis, protein was transferred to a nitrocellulose membrane and the membrane was blocked with PBS plus 0.05% Tween 20 (PBST) containing 0.3% skimmed milk for 1 hr at room temperature. Then the membrane was incubated with anti-IDO, anti-iNOS or anti-actin antibody at 4℃ overnight. The membrane was then washed with PBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hr at room temperature. Finally, the membrane was washed with PBST and developed with a Western lightning chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA).

2.5. Assay of NO production

The amount of NO production in the medium was estimated by the assay of nitrite using Griess reagent [27]. Fifty microliter of each supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water). The absorbance of the mixture at 590 nm was determined by a plate reader, and the nitrite concentration was determined using standard solutions of sodium nitrite.

2.6. Enzyme assay of IDO activity

IDO activity was determined by the assay previously described [28]. After cells were sonicated, the homogenate was centrifuged 10,000 rpm for 10 min. The supernatant (100 μl) was mixed with an equal volume of 2×reaction buffer (100 mM potassium phosphate buffer pH 6.5, 40 mM sodium ascorbate, 20 μM methylene blue,

200 μg/ml catalase, 800 μM Trp). The mixtures were incubated at 37℃ for 60 min to permit IDO to convert Trp to *N*-formylkynurenine, and then 40 μl of 30% (wt/vol) TCA was added to stop the reaction. After heating at 50℃ for 30 min, the reaction mixtures were centrifuged and Kyn concentration in the supernatant was measured by high-pressure liquid chromatography (HPLC).

2.7. Assay of Kyn and Trp

Concentrations of Kyn and Trp were determined by HPLC as previously described [28], with minor modification. Before HPLC assay, culture medium was deproteinized by treatment with 86% methanol (1:6, vol/vol). Twenty-five microliter of sample was injected into a 5 μm endcapped Purospher RP-18 column (Merck, Darmstadt, Germany) and analyses were carried out at a flow rate 1.0 ml/min. The mobile phase was 10 mM acetic ammonium (pH 6.5) and 10% methanol. Kyn was detected by a UV-detector at a wavelength of 360 nm and Trp by a fluorescence detector at an excitation wavelength of 285 nm and an emission wavelength of 365 nm.

2.8. Assay of Kyn uptake

For the assay of Kyn uptake for a short time, BMDC were washed with Tris-choline buffer (150 mM choline chloride, 10 mM Tris, pH 7.4) and suspended at 3 $\times 10^5$ cells/0.2 ml/well in Tris-choline buffer or Tris-Na buffer (150 mM sodium chloride, 10 mM Tris, pH 7.4). Kyn was added into cell suspensions with or without BCH, Trp, or 1-MT in water bath at 37℃. Kyn uptake was stopped by cooling cell suspension on ice. Kyn concentrations of the culture supernatant were assayed by HPLC, and the decrease of Kyn content in the supernatant was estimated as Kyn uptake by BMDC.

3. Results

3.1. Expression of IDO protein and NO production in $CD11c^+CD11b^+$ BMDC by stimulation with CpG

BMDC were differentiated from bone marrow cells with GM-CSF for 6 days. Most (73.2%) of the BMDC were $CD11c^+CD11b^+$ myeloid DC (Fig. 1A). The expression of IDO and iNOS proteins was induced in BMDC but not original bone marrow cells stimulated with CpG for 24 hr (Fig. 1B). CpG-mediated NO production was also increased with the BMDC development (Fig. 1C). These results indicate that the ability of BMDC to express IDO and iNOS proteins upon CpG stimulation is induced in the fully developed BMDC.

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3.2. Inhibition of IDO activity by NO production in BMDC

Effects of NO production on IDO activity in BMDC were tested. IDO protein was expressed in BMDC by stimulation with CpG in the presence or absence of NMA, a NOS inhibitor (Fig. 2A). CpG-mediated NO production was inhibited by NMA (Fig. 2B). In the enzyme assay using cellular extracts of BMDC, IDO activity of BMDC stimulated with CpG was weak but enhanced by the addition of NMA (Fig. 2C). These results show that expression of IDO protein is induced in BMDC stimulated with CpG, although its activity is inhibited by NO production.

3.3. Non-secretion of Kyn by BMDC stimulated with CpG

We examined whether BMDC secreted Kyn upon stimulation with CpG. The Kyn level in the culture supernatant of BMDC was not increased significantly by stimulation with CpG even in the presence of exogenously added Trp (Fig. 3A). Correspondingly,

the concentration of Trp in the culture supernatant of BMDC stimulated with CpG was hardly decreased unless NMA was added (Fig. 3B). These results indicate that BMDC do not secrete Kyn upon CpG stimulation.

3.4. Uptake of exogenously added Kyn by BMDC without CpG stimulation

It has been recently shown that $CD8^-$ DC take up exogenously added Kyn upon IFN-γ stimulation [29]. Therefore, we examined whether exogenously added Kyn was taken up by BMDC by CpG stimulation for 24 hr. The Kyn concentration in the culture supernatant of BMDC was decreased by around 20% independently of CpG stimulation and NMA when 50 μM Kyn was exogenously added (Fig. 4A). The Kyn concentration in the culture supernatant of BMDC was decreased dependently on the concentration of exogenously added Kyn (10-100 μM) (Fig. 4B). Concentrations of Kyn in the culture supernatants of BMDC were decreased almost linearly with time for 24 hr (Fig. 4C). The ability of BMDC to take up Kyn was induced with the development from bone marrow cells (Fig. 4D). These results suggest that BMDC take up Kyn independently of IDO expression and NO production.

3.5. Inhibition of Kyn uptake by a transport system L-specific inhibitor and Trp

It has been shown that astrocytes take up Kyn through a Na^+-independent transport system L [30]. Na^+-dependency of Kyn uptake by BMDC was tested in cultures of BMDC by using Na^+ and Na^+-free buffers for a short time. Kyn uptake by BMDC in Na^+-free buffer for 15 min was higher than that in Na^+ buffer (Fig. 5A), indicating that Kyn uptake by BMDC is mainly Na^+-independent. Therefore, Kyn uptake by BMDC was tested in Na^+-free buffer. The uptake of exogenously added Kyn (10 μM) by BMDC (3×10^5 cells) increased rapidly to around 100 pmole (5% of Kyn exogenously

added) within 2 min and gradually thereafter (Fig. 5B). Effects of the addition of Trp, BCH, a specific inhibitor of the transport system L, or 1-MT, an inhibitor of IDO and the transport system L [31-33], on Kyn uptake by BMDC were tested (Fig. 5C). All the BCH, Trp and 1-MT prevented the uptake of exogenously added Kyn by BMDC. These results show that Kyn is taken up by BMDC mainly through the transport system L.

4. Discussion

The present study shows that the expression of IDO activity in BMDC is regulated at the post-transcriptional level by NO production. IDO activity of BMDC was detected only by the enzyme assay using the cellular extracts of BMDC stimulated with CpG in the presence of a NOS inhibitor. IFN-γ-activated mouse peritoneal macrophages secrete Kyn in the presence of a NOS inhibitor [17]. However, CpG-activated BMDC did not secrete Kyn even in the presence of a NOS inhibitor. This may be caused by the weak activity of IDO in BMDC. Human myeloid DC differentiated from blood monocytes with GM-CSF and IL-4 as well as macrophages differentiated with M-CSF express IDO activity [8,9]. These differences are caused by a clear species specificity regarding the induction of IDO versus iNOS in cultured cells [34]. In human monocytes/macrophages, IFN-γ or IFN-γ/LPS strongly induces IDO, but not iNOS activity, while in mouse macrophages these stimuli strongly induce iNOS, but not IDO activity. Thus, the inhibition of iNOS expression is at least required for the induction of IDO activity.

We showed the expression of IDO protein, non-secretion of Kyn and NO production in BMDC stimulated with CpG. IFN-γ-activated $CD8^-$ DC in mouse spleen express IDO protein but not IDO activity in contrast to $CD8^+$ DC [16]. $CD8^-$ DC treated with IFN-γ produce significantly higher levels of NO than the $CD8^+$ DC

counterpart [16]. Thus, activated myeloid dendritic cells such as BMDC and $CD8^-$ DC express IDO protein and produce NO but do not secrete Kyn. Unexpectedly, Park and coworkers have recently published that IDO-expressing BMDC upon IFN-γ stimulation suppress OVA-specific $CD8^+$ T-cell proliferation [22,23]. However, IFN-γ induces high levels of NO production [21], and enhances antigen-presenting activity in BMDC [24]. At present, it is difficult to reconcile these observations [22,23] with our finding that the activity of IDO is suppressed by NO production in BMDC stimulated with CpG and also with the enhancement of antigen presenting activity of BMDC stimulated with IFN-γ [24].

Bone marrow cells neither expressed IDO and iNOS proteins nor produced NO upon CpG stimulation without differentiation to BMDC with GM-CSF (Fig. 1B, C). GM-CSF induces NO production in a skin dendritic cell line and enhances IDO expression in eosinophils stimulated with IFN-γ [35, 36]. Thus, GM-CSF seems to be an important factor for the induction of IDO and iNOS. However, GM-CSF completely inhibits Flt3-L-induced pDC development from bone marrow cells [5, 6]. As far as we know, there is no report showing that high levels of NO production are induced in pDC. Therefore, GM-CSF seems to be a much more critical factor in vitro for the induction of iNOS than that of IDO with BMDC differentiation.

The development of $CD8^-$ DC from bone marrow cells in the presence of GM-CSF depends on IRF-4, whereas the development of $CD8^+$ DC and pDC in the presence of Flt3-L mainly depends on IRF-8 [37,38]. Correspondingly, the negative regulation of gene expression of IRF-8 inhibits the induction of IDO activity in $CD8^+$ DC stimulated with IFN-γ or human DC stimulated with LPS [39]. Induction of IDO by LPS but not IFN-γ in human monocytic THP-1 cells involves p38 and NF-κB pathways [40]. TLR-9 signaling from CpG activates p38 and NF-κB through MyD88 and IRF-8 in DC [15]. BMDC developed from bone marrow cells with GM-CSF express significantly IRF-8 [37,38]. Therefore, it may be possible that BMDC express IDO protein through

MyD88 and IRF-8 in response to CpG. However, it has recently been shown that LPS but not IFN-γ induces the IDO expression in BMDC through the activation of PI3 kinase and JNK [41]. Therefore, it is interesting to clarify which signal pathways are required for the induction of IDO in BMDC by stimulation with CpG.

We showed that BMDC took up exogenous Kyn independently of CpG stimulation and NO production. Na^+-independent uptake of Kyn by BMDC was bloked by BCH, a transport system L-specific inhibiter, Trp or 1-MT blocked. The transport of Kyn in BMDC is similar to that in astrocytes, which is inhibited by BCH and Trp in Na^+-free solution [30]. BCH, Trp, 1-MT and various other amino acids also inhibit Trp uptake through the transport system L [31-33]. A BCH-sensitive and Na^+-independent transport is consistent with system L, a neutral amino acid transport mechanism, being the major conduit of Trp [31-33]. Therefore, we conclude that BMDC take up Kyn mainly through the transport system L.

We showed that BMDC took up Kyn much more preferentially than Trp, indicating a higher affinity of Kyn than Trp to the transporter. A low affinity of Trp to the transporter corresponds to the expression of a low IDO activity in BMDC. In fact, the enzyme assay of IDO activity using the cellular extract of BMDC, which does not require membrane transport of Trp, demonstrated IDO activity. A high-affinity, Trp-selective amino acid transport system has been recently shown in human macrophages, and speculated that this unique transport system allow macrophages to take up Trp efficiently under low substrate concentration, such as may occur during interaction between T cells and IDO-expressing antigen presenting cells [42]. Taken together with our findings, a low affinity of Trp to the transporter in BMDC causes the expression of a low IDO activity, in addition to the suppression by NO production.

It has recently been shown that $CD8^-$ DC as well as $CD8^+$ DC take up exogenously added Kyn and secrete quinolinic acid upon IFN-γ stimulation [29]. Therefore, DC such as BMDC, $CD8^-$ DC and $CD8^+$ DC take up exogenous Kyn. However, the uptake

of Kyn by BMDC is independent of CpG stimulation. Therefore, it is suggested that CpG does not activate down stream enzymes of IDO along the Kyn pathway. On the other hand, immunogenic $CD8^-$ DC became immunosuppressive DC through the generation of Kyn metabolites such as quinolinic acid upon IFN-γ stimulation in the presence of exogenous Kyn [29]. However, we did not observe the immunosuppressive activity of BMDC stimulated with IFN-γ in the presence of exogenously added Kyn (unpublished data). Our results suggest a new possibility that BMDC counteract Kyn-mediated induction of regulatory DC or T cells by scavenging Kyn.

The utilization of Kyn by BMDC in the resting state might be physiologically important for cell survival because IDO is not constitutively activated. Moffett et al. have shown that intraperitoneal injections of Kyn did not result in any significant increase in hepatocyte immunoreactivity with quinolinate-specific antibody, but rather led to dramatic increase in immunoreactivity in tissue macrophages, splenic white pulp, and thymic medulla [43,44]. Quinolinic acid formation was also induced most strongly in spleen by systemic immune stimulation with pokeweed mitogen [45]. Therefore, it is suggested that extrahepatic Kyn is preferentially metabolized in immune cells involving BMDC. It may be possible that Kyn is utilized for NAD synthesis for the survival of BMDC as shown in RAW264.7 macrophages [46]. Trp metabolism along the Kyn pathway is also required for DC activation [47]. Further study is required in order to clarify the fate of Kyn taken up by BMDC.

GM-CSF induces in vivo as well as in vitro the development of myeloid DC, whereas Flt3-L induces the development of both myeloid DC and pDC [5-7,48,49]. Thus, the activity of GM-CSF to induce in vitro the development of immunogenic myeloid DC from bone marrow cells correlates with the physiological activity of GM-CSF in vivo. In interaction between DC subsets, otherwise immunogenic $CD8^-$ DC become tolerogenic in co-culture with $CD8^+$ DC upon IFN-γ stimulation [29]. The

present study suggests alternative possibility that myeloid DC differentiated with GM-CSF up-regulates immune responses by counteracting tolerogenic activity of IDO-expressing DC through the two independent mechanisms; the inhibition of IDO activity by NO production and scavenging Kyn secreted from tolerogenic DC.

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Legends

Fig. 1. The induction of IDO expression and NO production in BMDC stimulated with CpG. (A) Surface expression of CD11c and CD11b on bone marrow cells or BMDC was analyzed by flow cytometry. The percentage of cells in each quadrant is presented. (B, C) Cells (10^6 cells/ml) collected after cultures of bone marrow cells for the indicated days were stimulated with CpG for 24 hr. (B) IDO and iNOS expressions were assessed by Western blot. (C) Nitrite accumulation in the culture supernatant was measured using Griess reagent. Means ± SD of triplicate cultures are presented.

Fig. 2. The inhibition of IDO activity by NO production in BMDC stimulated with CpG. BMDC (10^6 cells/ml) were stimulated with CpG alone or together with NMA for 24 hr. (A) The expression of IDO and iNOS proteins was assessed by Western blot. (B) Nitrite accumulation in the culture supernatant was measured using Griess reagent. (C) IDO activity was determined via Kyn formation using cellular extract as described under Materials and methods. (B, C) Means ± SD of triplicate cultures are presented.

Fig. 3. The non-secretion of Kyn by BMDC stimulated with CpG. BMDC (3×10^5 cells/0.2 ml) were stimulated with CpG alone or together with NMA in the presence or absence of exogenously added Trp (100 μM) for 24 hr. Concentrations of (A) Kyn or (B) Trp in the culture supernatant of BMDC were measured by HPLC. Means ± SD of triplicate cultures are presented.

Fig. 4. Kyn uptake by BMDC independent of stimulation with CpG. (A, B, C) BMDC (3×10^5 cells/0.2 ml) or (D) cells collected after cultures of bone marrow cells for the indicated days were stimulated with CpG alone or together with NMA in the

presence of exogenously added (A, C, D) 50 μM or (B) the indicated concentration of Kyn for (A, B, D) 24 hr or (C) the indicated time (2-24 hr). Concentrations of Kyn in the culture supernatant were measured by HPLC. Means ± SD of triplicate cultures are presented.

Fig. 5. Kyn uptake by BMDC through transport system L. (A) BMDC (3×10^5 cells/0.2 ml) were incubated with Kyn (10 or 50 μM) in Tris-choline buffer or Tris-Na buffer for 15 min. (B, C) BMDC (3×10^5 cells/0.2 ml) were incubated with 10 μM Kyn in (B) Tris-choline buffer for 0-15 min or (C) together with 2 mM BCH, Trp or 400 μM 1-MT for 15 min. Concentrations of Kyn in the culture supernatant were measured by HPLC. Means ± SD of triplicate cultures are presented.

Cover letter

I hereby send a revised manuscript of the article entitled “High-affinity uptake of kynurenine and nitric oxide-mediated inhibition of indoleamine 2,3-dioxygenase in bone marrow-derived myeloid dendritic cells" (Ms. Ref. No.: IMLET-D-07-00091) (Toshiaki Hara, Nanako Ogasawara, Hidetoshi Akimoto, Osamu Takikawa, Rie Hiramatsu, Tsutomu Kawabe, Ken-ichi Isobe, Fumihiko Nagase) for the publication in Immunology Letters.

This article is not currently under consideration in another journal and all authors agree with the concepts of the manuscript.

Desired section of publication is Reseach Articles.

Corresponding author: Fumihiko Nagase, Department of Medical Technology, Nagoya University School of Health Sciences, 1-20 Daikominami-1-chome, Higashi-ku, Nagoya, Aichi, 461-8673, Japan. Telephone and fax numbers: +81-52-719-1189. E-mail address: nagase@met.nagoya-u.ac.jp

We answered to the comments of reviewers as follows:

I would greatly appreciate it if our article could again be considered for publication in Immunology Letters.

Answers to reviewers' comments:

Reviewer I:

This study focuses on expression of the regulatory enzyme indoleamine 2,3 dioxygenase (IDO) in murine bone marrow derived dendritic cells (BMDCs). The authors used a well-established method to culture myeloid BMDCs (GM-CSF) and showed that TLR9 ligands (CpGs) induced BMDC to express both IDO and iNOS at the protein level. In subsequent experiments, (1) iNOS inhibitors were shown to enhance IDO activity (consistent with previous reports that nitric oxide, a product of iNOS activity, blocks IDO enzyme functions) and (2) BMDCs were found to consume exogenous kynurenine (Kyn) produced by other IDO+ cells, suggesting that IDO activity in BMDCs does not lead to Kyn production. Though data largely supports these conclusions the following key points must be addressed to increase the scope of this report and strengthen the conclusions to meet minimum standards for publication.

Point 1. (general points) Analyses of the T cell stimulatory properties of BMDCs has the potential to significantly increase enthusiasm for this study since the main reason that

IDO expression in DCs is of biological significance is due to IDO-mediated suppression of T cell responses to antigens presented by DCs. In addition, the authors should address better how their findings with cultured BMDCs relate to physiologic (tissue) DCs that have been shown to express IDO by other groups. Several reports now document that functionally relevant IDO expression (that causes T cells suppression) is restricted to plasmacytoid DCs (pDCs), not myeloid DCs (mDCs) that are related to the BMDCs that emerge from GM-CSF bone marrow cultures. As stated in the introduction Flt3L induces pDCs to differentiate from bone marrow, and it is unclear why the authors did not compare parallel cultures of mDCs and pDCs to assess their abilities to express function al IDO.

Answer: Flt3L is most suitable for the comparative study of IDO-expressing myeloid DC and plasmacytoid DC derived from bone marrow cells, because these studies have not been published yet. However, it is very difficult to obtain Flt3L in our laboratory, because Flt3L is very expensive. Besides, comparative study of mDC and pDC is required repetition of all the experiment that we have done using GM-CSF. We will investigate this issue as an independent study and will report the results elsewhere. We limited our purpose to the study of IDO in BMDC in this article.

Point 2. (Figs 1-3) Though of interest in the context of BMDCs, biochemical data presented in Figures 1-3 is largely repetitive of previous work showing that IDO activity is inhibited in the presence of NO produced by iNOS activity. Hence, these data are not particularly novel. Also, it is unclear why levels of tryptophan in medium did not decrease more significantly than shown in Fig 3B when BMDCs were treated with CpGs in the presence of NMA (as would be expected if IDO is active). Can this experiment be performed using media with lower starting concentrations of tryptophan to address this question more effectively?

Answer: As pointed out by the reviewer, the inhibition of IDO activity by NO is reported in several monocyte/macrophage systems. However, before starting this study, there were no reports about the regulation of IDO in mouse BMDC. It was also unknown whether CpG induces IDO in BMDC. We therefore believe that our finding is useful for understanding of physiological function of BMDC.
The decreases of tryptophan concentration in cultures of control, CpG and CpG/NMA groups were 0.6 μM, 0.5 μM and 2.3 μM in the absence of additional tryptophan, and 14.2 μM, 15.4 μM and 21.1 μM in the presence of 100 μM tryptophan exogenously added, respectively (Fig. 3B). Tryptophan uptake by BMDC was increased by stimulation with CpG/NMA in the presence of 100 μM tryptophan exogenously added.

Thus, culture medium with high concentrations (20 plus 50-100 μM) of tryptophan is better for the assay of IDO activity than RPIM culture medium with low concentration (around 20 μM) of tryptophan.

Point 3. (Fig 4). Though apparently significant, the reduction in Kyn levels is not large, which may be due to the fact that a large excess of Kyn was added initially. Does the level of exogenously added Kyn decrease more substantially when lower amounts of Kyn are added to cultures? (note that the maximum Kyn concentrations in Figs 4A and 5 differ by an order of magnitude). In addition, it is unclear what was done to generate data shown in Fig 4C - what does 'RPMI and 'control' mean?

Answer: Kynurenine concentrations of control, CpG and CpG+NMA groups in Fig. 4A were decreased by 11.2 μM, 9.9 μM and 12.3 μM, respectively. Kynurenine concentrations of control group on day 0, 2, 4 and 6 in Fig. 4C were decreased by 0 μM, 16.3 μM, 23.4 μM and 18.0 mM, respectively. Thus, large amounts of kynurenine (20 – 46% of exogenous kynurenine (50 μM)) were taken up by BMDC for 24 hr. The extent of uptake depends on the concentration of exogenous kynurenine as shown in Fig. 5A. In the second revise of our manuscript, we added a new result confirming the relationship between exogenous concentration and uptake of kynurenine in Fig. 4B. The Kyn concentration in the culture supernatant of BMDC was much more markedly decreased dependently on the concentration of exogenously added Kyn (10-100 μM). The study in Fig. 4D was done to know how the ability to take up kynurenine is related to the differentiation of bone marrow cells to BMDC. 'RPMI's in Fig. 4A,B,D mean cell-free cultures as a control.

Point 4. (Fig 6). Data shown is impressive and convincing but additional analyses is needed to resolve key issues. In mixed cell cultures, do BMDCs express IDO (and iNOS), and what is the effect of adding LPS and IFN-g to BMDCs? These key issues need to be addressed to fully understand what is happening in mixed cell cultures to produce the outcomes shown in Fig. 5

Answer: We used LPS/IFN-γ instead of CpG in the mixed cultures of BMDC and THP-1 cells, because the former but not the latter stimulus induced IDO expression in THP-1 cells. LPS/IFN- γ induced the expression of IDO protein and NO production in BMDC. We tested effects of NO production using NMA on kynurenine uptake. Kynurenine was secreted by THP-1 but not BMDC, whereas NO was produced by BMDC but not THP-1 cells by stimulation with LPS/IFN-γ. Kynurenin was not accumulated in the culture supernatant of the mixed culture of THP-1 cells and BMDC

in the presence or absence of NMA that completely inhibited NO production by BMDC. Therefore, we concluded in the first revise of our manuscript that BMDC took up kynurenine secreted from THP-1 cells independently of the inhibition of IDO in THP-1 cells by NO production.

We further tried to confirm that THP-1 cells secreted kynurenine in the mixed culture of THP-1 and BMDC as well as the single culture of THP-1 cells by the assay of tryptophan. Tryptophan uptake by THP-1 cells was significantly decreased in the mixed culture with BMDC. These results suggest that BMDC not only take up kynurenine but also nonspecifically inhibit tryptophan metabolism in xenogenic THP-1 cells. Therefore, we decided to delete the result in Fig. 6 in the second revise of our manuscript. The conclusion of our article is not influenced by this change.

Figure 1

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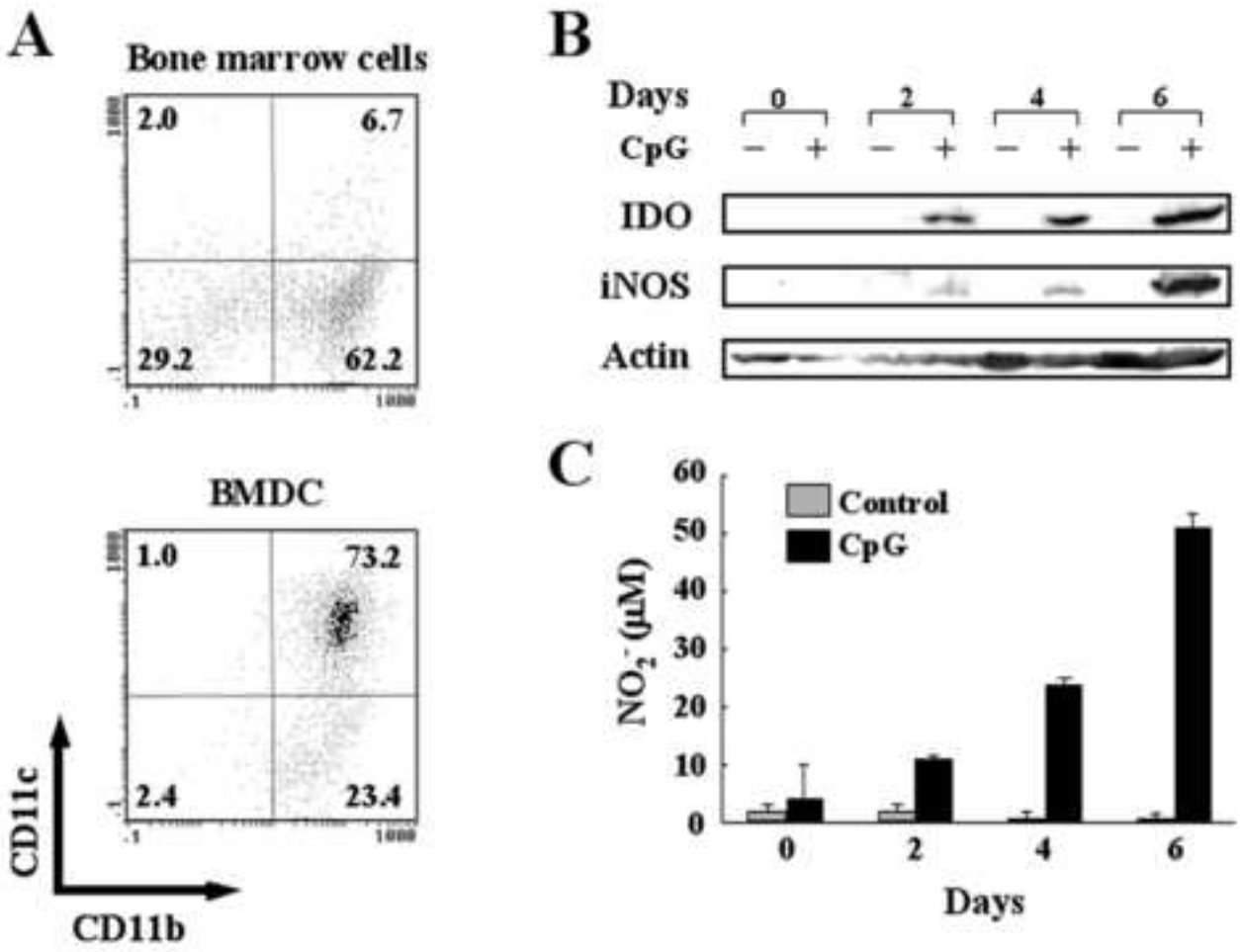

Figure 2

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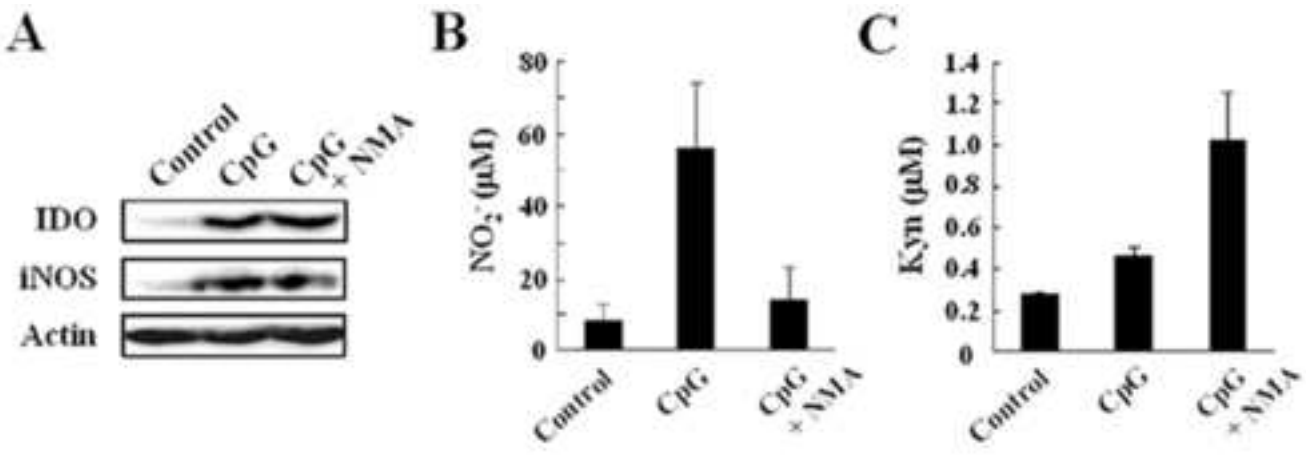

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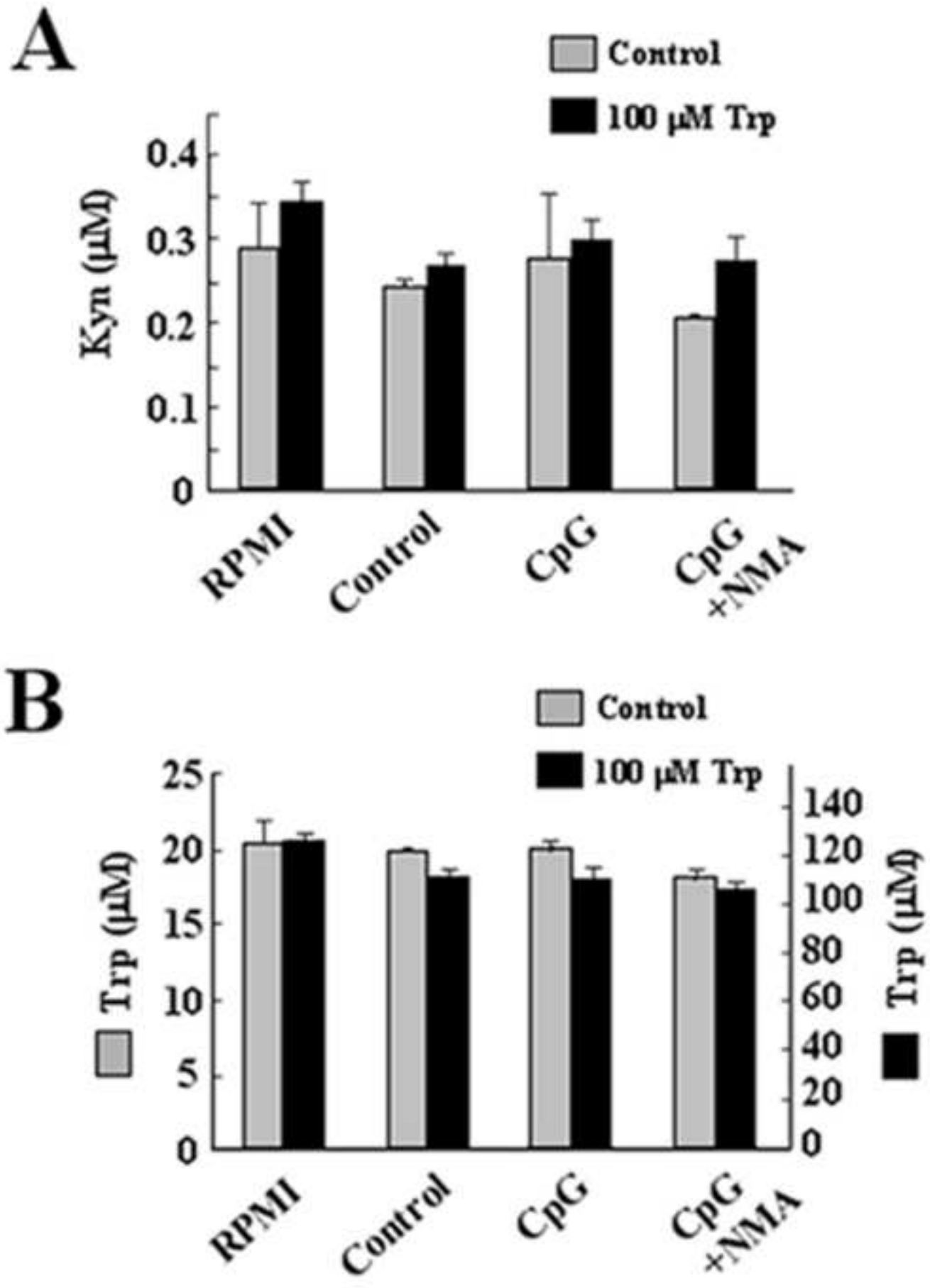

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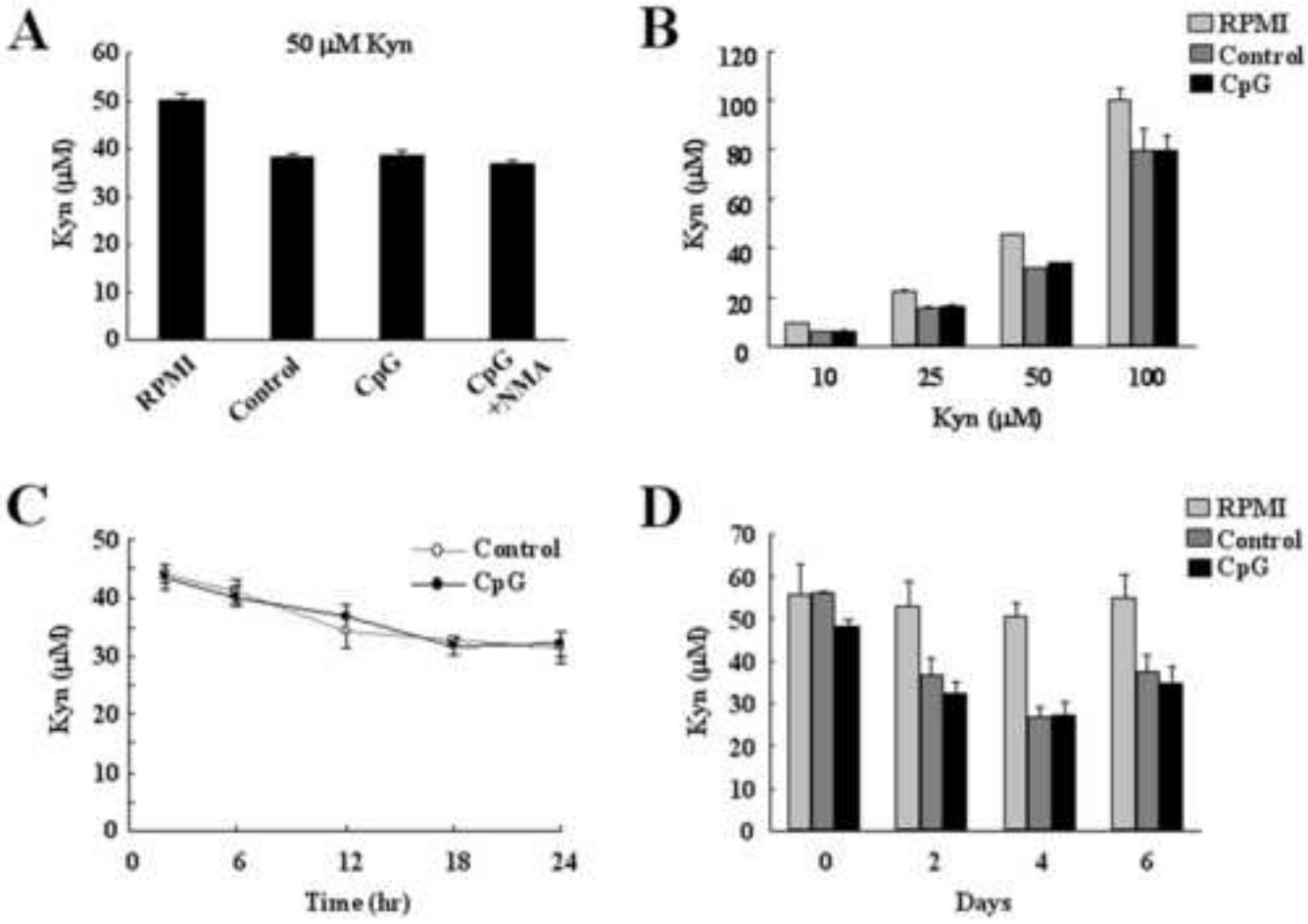

Figure 5

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