

**Metabolite releasing polymers control dendritic cell function
by modulating their energy metabolism**

Journal:	<i>Journal of Materials Chemistry B</i>
Manuscript ID	TB-COM-03-2020-000790.R1
Article Type:	Communication
Date Submitted by the Author:	02-May-2020
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1 **Metabolite releasing polymers control dendritic cell function by modulating their energy**
2 **metabolism**

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24 **Keywords:** immunometabolism, immunoengineering, dendritic cell, lymphocytes, metabolites

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1 **Metabolites control immune cell functions, and delivery of these metabolites in a**
2 **sustained manner may be able to modulate function of the immune cells . In this study,**
3 **alpha-ketoglutarate (aKG) and diol based polymeric-microparticles (termed paKG**
4 **MPs) were synthesized to provide sustained release of aKG and promote an**
5 **immunosuppressive cellular phenotype. Notably, after association with dendritic cells**
6 **(DCs), paKG MPs modulated the intracellular metabolic-profile/pathways, and**
7 **decreased glycolysis and mitochondrial respiration *in vitro*. These metabolic changes**
8 **resulted in modulation of MHC-II, CD86 expression in DCs, and altered the frequency**
9 **of regulatory T cells (Tregs), and T-helper type-1/2/17 cells *in vitro*. This unique**
10 **strategy of intracellular delivery of key-metabolites in a sustained manner provide a new**
11 **direction in immunometabolism field-based immunotherapy with potential applications**
12 **in different diseases associated with immune disorders.**

13

14 **1. Introduction**

15 Immunometabolism reprogramming is an emerging and exciting new field that is involved
16 in the induction, progression, and therapy of several diseases such as cancer, infections,
17 autoimmune disorders, and Alzheimer's among others¹⁻⁵. Notably, modulation of
18 immunometabolism can be performed by delivery of cell permeable metabolites (e.g., 2-
19 hydroxyglutarate)² or enzymatic inhibitors (e.g., 2-deoxyglucose)⁶. For example, regulatory T
20 cell (Treg - immunosuppressive) and T-helper type 17 (Th17 - pro-inflammatory)
21 differentiation can be controlled by modulating the glutamate oxaloacetate transaminase 1
22 (GOT1) enzyme, which has direct implications in immunosuppressive applications. On the
23 other hand, metabolites provided via dietary interventions may improve immune cell function
24 in pro-inflammatory applications such as cancer⁷⁻⁹. Notably, increasing energy production
25 from the Krebs cycle without affecting glycolysis can reduce the function of pro-
26 inflammatory effector T-cells, without affecting the function of regulatory anti-inflammatory

1 Tregs (required for regeneration)¹⁰. These strategies can be targeted toward the adaptive
2 branch of the immune system to generate effector function. Interestingly, dendritic cells
3 (DCs) that form the bridge between innate and adaptive immune responses, are capable of
4 inducing robust adaptive and innate immune responses, which is advantageous in diseases
5 such as cancer, infections, and wound healing.

6 Interestingly, DCs play an important role in inflammatory disorders, such as wound
7 healing^{11,12}, potentially by secreting growth factors¹¹⁻¹⁴. Therefore, targeting DCs that may
8 not only modulate the innate cells (e.g., neutrophils) in inflammatory disorders, but also
9 adaptive cells (e.g. regulatory T cells). Importantly, activated immune cells (e.g.,
10 macrophages type 1, activated DCs, and Th1) have an enhanced glycolysis profile in various
11 pro-inflammatory environments, which hampers faster anti-inflammatory responses¹⁵⁻¹⁷.
12 Therefore, modulating the energy-metabolic pathways of the immune cells can be a viable
13 strategy for controlling macrophages and DC responses and affect the inflammatory
14 responses.

15 In this study, we demonstrate that delivery of aKG (a Krebs cycle metabolite, and
16 involved in immunosuppression)¹⁸ and a diol (feeds into the Krebs cycle and Fatty acid
17 oxidation involved in immunosuppression)^{1,19} after a one-time treatment lead to modulation of
18 the DC metabolism, DC function and modulation of adaptive immune responses in the form
19 of T cell function (**Figure 1**).

20

21 **2 Results**

22 **2.1 Central-carbon aKG metabolite-based microparticles (MPs) release aKG in a** 23 **sustained manner.**

24 Steady state intracellular concentrations are needed to achieve the desired immunomodulation
25 effect. Although, esterification of aKG molecules makes them cell permeable, aKG would
26 need to be given in high and frequent dosages to achieve the desired effect *in vivo*. The high

1 and frequent dosages are needed to overcome fast diffusion and elimination from the body
2 caused by the low molecular weight. Therefore, polymers of aKG were generated, which can
3 then degrade over a period of time to release aKG in a sustained manner after one-time
4 application. Specifically, aKG was reacted with 1,10-decanediol to generate a polymer with a
5 number-average molecular weight (M_n) of 15.3 – 23.9 kDa (**Figure 2a**), which was
6 determined using ^1H NMR spectroscopy and gel permeation chromatography (GPC) (**Figure**
7 **S1 and S2**).

8 Next, in order to ensure that these polymers can deliver aKG intracellularly in
9 phagocytes (e.g. DCs), these polymers were formulated into phagocytosable MPs using oil-in-
10 water emulsions (DCs can phagocytose particles $< 15 \mu\text{m}^{20}$). The formation of particles was
11 confirmed using scanning electron microscopy (SEM) and (**Figure 2b**), the average size of
12 these particles was determined to be $4 \mu\text{m}$ using dynamic light scattering, which matched
13 SEM analyses. In order to test if these particles could release aKG in a sustained manner,
14 release kinetics experiments in pH 7.4 (1X PBS, physiological pH) and in pH 5.0 (100 mM
15 acetate buffer) were performed. The amount of aKG released as a function of time was
16 determined using high-performance liquid chromatography (HPLC), which demonstrates that
17 the particles were able to release aKG in a sustained fashion for at least 30 days. Moreover,
18 the particles released aKG faster and at a higher amount at pH 5.0 as compared to pH 7.4.
19 Cumulative release of aKG from paKG MPs is shown in **Figure 2c**. Supernatant from paKG
20 particles was visualized under scanning electron microscope at increasing magnification at
21 multiple spots (representative images shown) to confirm that no particles were left in the
22 supernatant (**Figure S6**). It should be noted that the release of aKG in DCs is not required for
23 30 days, as the DCs post-phagocytosis many not live for 30 days.

24

25 **2.2 DCs are capable of associating with paKG MPs**

1 Although, DCs play an important role in modulating immune responses, the modulation in
2 DC function after exposure to TCA metabolites is not well understood. In this study, we
3 tested the ability of paKG MPs to deliver aKG, potentially a key-immunosuppressive
4 metabolite in TCA cycle, to DCs. In order for the intracellular sustained delivery of aKG to
5 occur, DCs should be able to associate the paKG MPs. To determine if DCs are capable of
6 associating with paKG MPs, bone marrow derived DCs were cultured for 60 minutes with
7 Rhodamine 6G encapsulated paKG MPs. These cells were then fixed and imaged using a
8 fluorescent microscope. It was observed that DCs were able to successfully associate with the
9 paKG MPs (**Figure 3a**). It should be noted that the particle size is much larger than the size of
10 the endosome that might contain spontaneously released dye from the particles, which
11 strongly suggests that the particles are associating or potentially internalized by DCs.

12

13 **2.3 paKG MPs modulate intracellular metabolite profile in DCs**

14 DC function can be modulated by changing the intracellular metabolite profile²¹. Since,
15 paKG MPs release aKG in a sustained manner, the ability of these particles to modulate
16 intracellular metabolite levels was measured using LC-MS²²⁻²⁸. Modulation in intracellular
17 metabolite levels in DCs was determined by culturing BMDCs with paKG MPs. The
18 intracellular metabolites were isolated, quantified using LC-MS/MS, normalized to protein
19 amount, and the levels of these metabolites were compared to no treatment control.

20 Enrichment analysis was conducted using the Kyoto Encyclopedia of Genes and
21 Genomes (KEGG) database searches and metabolite intensities for different conditions was
22 determined. The enrichment analysis of 299 reliably detected metabolites (using LC-MS)
23 showed significant ($p < 0.05$) perturbations or changes in the levels of glutamate,
24 glycine/threonine/serine, alanine/aspartate/arginine and glutathione metabolisms, among
25 others. Notably, large impact coefficient (> 0.50) was observed in glutamate pathway
26 suggesting the greatest impact of aKG delivery on the intracellular glutamate pathway

1 metabolite levels (**Figure 3b**). Specifically, L-glutamate, 4-aminobutanoate and asparagine in
2 the glutamate pathway were 3-6 fold higher in DCs treated with paKG MPs as compared to
3 the no treatment control. Moreover, in the arginine pathway, aspartate, acetyl-ornithine and
4 L-citrulline are upregulated in DCs treated with paKG MPs approximately 4-fold as compared
5 to the no treatment control (**Figure S3**). Notably, these metabolites are involved in immune
6 suppression via DCs²⁹⁻³¹.

7 Interestingly, although large impact coefficients were not observed in other metabolic
8 pathways, certain metabolites involved in DC-mediated immunosuppression such as
9 kynurenine (>6-fold increase), were significantly affected as compared to the no treatment.
10 Moreover, the intracellular levels of aKG were not significantly different when compared to
11 the no treatment control. Since glutamate and succinate were significantly upregulated, this
12 suggests quick metabolization of aKG intracellularly. Overall, these data demonstrate that the
13 paKG MPs modulate intracellular metabolism of DCs.

14 Additionally, in order to ensure that the changes in DC metabolism are not due to
15 endotoxins present in the paKG MPs, endotoxin tests were performed. Endotoxin levels were
16 found to be <0.05 endotoxin units/mL for the paKG MPs, thus suggesting that the release of
17 metabolites might be responsible for the modification in DC metabolism.

18

19 **2.4 paKG MPs reduce glycolysis and spare capacity in DCs**

20 Intracellular metabolite changes in DCs due to paKG MPs suggest that the metabolic
21 pathways in DCs might be modulated as well. Notably, upregulation of glycolysis and
22 metabolic respiration are known to play an important role in DC activation¹⁹. Therefore, in
23 order to test if the paKG MPs modulate glycolysis and metabolic respiration, Seahorse Assays
24 were performed to determine the extracellular acidification rate (ECAR) and oxygen
25 consumption rate (OCR). Lipopolysaccharide (LPS) was used as a positive control, and no

1 treatment was used as a negative control. Additionally, extracellularly added soluble aKG to
2 the cell culture was used as a control.

3 It was observed that the paKG MPs have a lower trend of OCR as compared to the
4 soluble aKG and no treatment groups. Importantly, in the presence of LPS (mimicking
5 inflammation e.g. in wound healing), paKG substantially decreased the OCR in DCs,
6 suggesting substantial decrease in the metabolic respiration (**Figure 3c, 3d**). In order to
7 further analyze this data, basal respiration without glucose, maximal respiration in the
8 presence of glucose, and spare capacity (determines the ability of the cell to respond to an
9 energetic demand) was determined. It was observed that the DCs treated with paKG MPs had
10 higher basal respiration, lower maximal respiration and lower spare capacity than the no
11 treatment control (**Figure 3e**).

12 In addition, glycolysis was significantly decreased in DCs cultured with paKG MPs as
13 compared to LPS (positive control) and no treatment (negative control). Interestingly, soluble
14 aKG decreased glycolysis in DCs compared to the no treatment control; however, soluble
15 aKG had higher glycolysis levels in DCs as compared to paKG MPs (**Figure 3f**). Taken
16 together, these data demonstrate that the paKG MPs reduce the metabolic activity of DCs
17 even in the presence of LPS. Thus, these data suggest that paKG MPs might be beneficial in
18 preventing uncontrolled inflammation due to reduced DC metabolic activity.

19

20 **2.5 paKG MPs do not activate DCs *in vitro***

21 The metabolism of DCs can play an important role in its function including changes in MHC-
22 II, CD86 expression and adaptive immune responses^{1,19,21}. In order to test if paKG MPs
23 modulated DC metabolism, can modify DC function, MHC-II, and CD86 expression was
24 determined using flow cytometry (representative flow plots - **Figure S4**).

25 It was observed that the paKG MPs do not activate DCs as compared to untreated
26 immature DCs, since frequency of MHC-II⁺CD86⁺ was not significantly different from each

1 other. On the other hand, LPS (positive control), significantly upregulated the frequency of
2 MHC-II⁺CD86⁺ in DCs (**Figure 3g**). Additionally, the controls of 1,10-decanediol, and
3 soluble aKG, added at the equivalent amount as the paKG MPs did not significantly change
4 the MHC-II⁺CD86⁺ expression in CD11c⁺ DCs as compared to the no treatment control and
5 the paKG MP condition (**Figure 3g**). Notably, in the presence of LPS, paKG MPs upregulate
6 activation of DCs, thus suggesting that the inflammatory response to DCs is not modified by
7 particles. Interestingly, addition of soluble aKG or diol (same quantity as the final paKG
8 MPs) prevented activation of DCs in the presence of LPS, which suggests that individual
9 components in large quantities added as bolus modulate the activation state in the presence
10 of inflammation. Overall, these data suggest that DCs may have an immunosuppressive
11 phenotype in the presence of paKG MPs, and therefore may modulate adaptive immune
12 responses.

13

14 **2.6 paKG MPs decreases pro-inflammatory T cell responses in allogeneic mixed** 15 **lymphocyte reaction**

16 DCs are effective modulators of T cell responses, which are essential for generating adaptive
17 immune responses. paKG MPs modulate DC phenotype, which might also modulate T cell
18 responses. Therefore, a mixed lymphocyte reaction (MLR) with BALB/c mice-derived CD3⁺
19 T-cells and C57BL/6j mice-derived DCs were cultured with the paKG MPs. Co-culture of
20 untreated DCs and T cells was used as control. These cells were cultured for 48-72 hours and
21 the frequency of T helper type 1 (Th1 - CD4⁺Tbet⁺), Th2 (CD4⁺GATA3⁺), Tc1 (CD8⁺Tbet⁺)
22 and Treg (CD4⁺CD25⁺Foxp3⁺), along with proliferation (Ki67⁺) and activation (CD25⁺) in
23 these cells (**Figure 4a**) was determined. The DCs that were treated with paKG MPs down-
24 regulated Th1, Th2, Th17 and Treg population proliferation in the MLR (**Figure 4b-e**).
25 Moreover, the DCs that were treated with soluble aKG also down-regulated Th1, Th2, Th17
26 and Treg population proliferation in the MLR. Notably, the percentage decrease in the

1 frequency of proliferating Th1 (1.7-fold decrease) and Th17 (2 fold decrease) population was
2 substantially higher as compared to the frequency of proliferating Treg population decrease
3 (1.2 – fold decrease), suggesting preferential decrease in pro-inflammatory cell type.
4 Moreover, the decrease in Treg proliferation in soluble aKG group was 8-fold lower than the
5 paKG MP group, thus suggesting the difference in exposing aKG intracellularly versus
6 extracellularly. Additionally, it was observed that the paKG MPs did not modulate the
7 frequency of CD4⁺ T cell percentage in the MLR (**Figure S5**) as compared to the no treatment
8 control or soluble aKG control. These data strongly suggest that paKG MPs by themselves
9 may prevent CD4⁺ pro-inflammatory immune activation.

10

11 **3 Discussion**

12 To the best of our knowledge, this is the first study which demonstrates that one-time
13 application of aKG metabolite-based polymeric microparticles can lead to modulation of the
14 immune system. Energy metabolic reprogramming can orchestrate immune cell polarization
15 and contribute to functional plasticity^{1,32,33}. Notably, it has been demonstrated that immune
16 cells can generate an anti-inflammatory response when cell permeable aKG is delivered to
17 macrophages¹⁸. However, delivery of these molecules *in vivo* can be challenging due to the
18 quick diffusion (within seconds) of small molecules *in vivo*³⁴. Therefore, in this work, a
19 polymer made of aKG metabolite was generated that could release aKG in a sustained
20 manner. This is the first evidence of Krebs cycle metabolite-based polymers that can be used
21 to deliver metabolites in a sustained fashion *in vitro*, and lead to modulation of the
22 metabolism of immune cells.

23 Metabolism modulating enzyme inhibitor drugs can also be used to alter the function
24 of macrophages and DCs, and thus modulate disease outcomes^{1,21,32}. For example, global
25 immune suppression can be achieved using glycolytic inhibitors 2-deoxyglucose⁶. However,
26 these inhibitors can also lead to prevention of proliferation and migration of endothelial cells

1 and hence may not be suitable for anti-inflammatory applications.³⁵ Therefore, development
2 of a strategy that does not modulate the glycolytic enzymes but still leads to local suppression
3 of immune cells can be beneficial for anti-inflammatory applications.

4 Targeting phagocytic cells (e.g. macrophages, DCs) in a tissue, where there is a
5 diverse type of cell population is challenging. Therefore, microparticles made of the aKG
6 polymer that can be picked by phagocytic cells, were utilized in this study for intracellular
7 delivery of aKG. Interestingly, the transporter of aKG, GPR99 expression on DCs is not well
8 known. Furthermore, recently it was demonstrated that dimethyl alpha-ketoglutarate when
9 added to antigen presenting cells were able to modulate these cells at the epigenetic scale and
10 also effect their metabolism¹⁸. Therefore, we expect that addition of soluble aKG should
11 modulate the metabolite levels of the DCs as well. These studies support our results that
12 intracellular delivery of aKG indeed modulate the metabolic profile of cells. Interestingly,
13 these paKG MPs also provide an added advantage of encapsulating and delivering drugs
14 intracellularly in phagocytic cells, which was demonstrated by delivering rhodamine as a
15 representative fluorescent drug molecule dye. This strategy can further be utilized to deliver
16 proteins, peptides or other drugs that can then modulate the function of these phagocytic cells.

17 In a pro-inflammatory milieu, a series of cellular and humoral immune responses are
18 involved in the recruitment of immune cells. Specifically, innate immune cells such as DCs,
19 which are professional APCs are known to infiltrate the injection sites¹². Importantly, DCs
20 form a bridge between the innate and adaptive immune system, and modulate the function of
21 lymphocytes such as T cells^{36,37}. T cells play a crucial role in defending against in both the
22 inflammatory and anti-inflammatory phases of immune responses^{13,38}. The data presented
23 herein demonstrate that paKG MPs might assist in anti-inflammatory responses by releasing
24 aKG intracellularly or extracellularly in a sustained manner.

25

26 **4 Conclusion**

1 In summary, metabolite-based polymers provide a new technique to deliver
2 metabolites intracellularly and locally to modulate the metabolism of immune cells. In this
3 study, paKG MPs were generated that could not only modulate DC function but also modulate
4 T cell functions *in vitro*.

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8

1 **5 Experimental Section**

2 ***5.1 Polymer synthesis and characterization***

3 Ketoglutaric acid and 1,10-decanediol were mixed at equimolar ratio in a round-bottom flask.
4 This mixture was stirred at 130°C for 48 hrs under nitrogen. The polymer thus generated was
5 precipitated in methanol solution, and the unreacted monomers were removed by multiple
6 (3X) precipitation steps. Residual methanol was then evaporated off using a rotary
7 evaporator, and the polymers were then dried under vacuum at room temperature for 48 hrs.

8 ¹H-NMR spectroscopy was performed on a Varian 500 MHz spectrometer using
9 deuterated chloroform (CDCl₃ - VWR, Radnor, PA), with a concentration of 5mg/ml. All ¹H
10 NMR experiments are reported in δ or parts per million (ppm) unit, and were measured
11 relative to the chloroform H-signal (7.26 ppm) in CDCl₃, unless stated differently. The
12 following abbreviations were used to indicate multiplicities: s (singlet), d (doublet), t (triplet),
13 q (quartet), m (multiplet), br (broad), and tt (triplet of triplets). Coupling constants are
14 expressed in hertz (Hz). ¹H NMR (500 MHz, Chloroform-d) δ 4.21 (t, 82H), 4.03 (t, 85H),
15 3.11 (t, 88H), 2.94 (t, 1H), 2.62 (t, 88H), 1.68 (tt, 91H), 1.57 (tt, 91H), 1.25 (br, 571H).

16 The molecular weight of the polymer was determined using GPC and ¹H NMR
17 spectroscopy. GPC was performed using a Waters Alliance e2695 HPLC system interfaced to
18 a light scattering detector (miniDAWN TREOS) and an Optilab T-rEX differential refractive
19 index (dRI) detector controlled using Astra v6.1 software. The mobile phase was
20 tetrahydrofuran (THF) Optima (inhibitor-free) at a flow rate of 1.0 mL/min and molecular
21 weights were determined either via a calibration curve prepared using Agilent low dispersity
22 polystyrene standards of 500, 200, 100, 30, 10, and 5 kDa or by determining the refractive
23 index increment using the RI detector and using the light scattering detector response to
24 determine an absolute weight-average molecular weight (M_w). The paKG samples were
25 dissolved in THF at ~1.0 mg/mL and passed through 0.22 μm filters before injection to the
26 GPC system. For molecular weight determination by ¹H NMR spectroscopy, the integration

1 of the peaks attributed to the end group protons were compared to the integrations of the
2 peaks from the main-chain backbone protons to determine the number-average molecular
3 weight (M_n).

4

5 ***5.2 Microparticle synthesis and characterization***

6 paKG polymers were utilized to generate microparticles using a water-oil-water emulsion
7 method. In order to generate microparticles, first, 50 mg of the polymers were dissolved in 1
8 mL of dichloromethane (DCM). This solution was then added to 10 mL of 2% polyvinyl
9 alcohol (PVA) solution in deionized water (DIH_2O) and homogenized at 30,000 rpm using a
10 handheld homogenizer for 2 min. This emulsion was then added to 50 mL of 1 % (vol/vol)
11 PVA solution and stirred at 400 rpm for 3 hrs to remove DCM. The particles thus formed
12 were then washed 3 times by centrifuging at 2000 X g for 5 min (Eppendorf, Hauppauge,
13 NY), removing the supernatant and resuspending in DIH_2O . These particles were then freeze
14 dried and used for next experiments.

15 The particles were imaged using a scanning electron microscope (SEM) XL30
16 Environmental FEG - FEI at Erying Materials Center at Arizona State University. Moreover,
17 the size of the particles was quantified using dynamic light scattering (Zetasizer Nano,
18 Cambridge, UK).

19 Release kinetics of the metabolites were determined by incubating 5 mg of the
20 microparticles in 1 mL of phosphate buffered saline (PBS) and placed on a rotator at 37 °C.
21 Next, triplicates of each release sample were centrifuged at 2000 X g for 5 min. After
22 centrifugation, 800 μL of the supernatant was removed and placed into a separate 1.5 mL tube
23 (Eppendorf, Hauppauge, NY) and then replaced by 800 μL of new buffer. Collection days
24 were initially everyday for the first 7 days. This then transitioned to 3-5 days a week until 30
25 days was reached.

1 The amount of metabolite released was then determined by developing a new method
2 in a high-performance liquid chromatography (HPLC, Agilent Technologies, Santa Clara,
3 CA). Specifically, the mobile phase of 0.02 M H₂SO₄ in water was used. A 50 µL of
4 injection volume was utilized in a Hi-Plex H, 7.7 × 300 mm, 8 µm column (Agilent
5 Technologies, Santa Clara, CA). A flow rate of 1.2 mL/min was utilized and the absorbance
6 was determined using a UV detector at 210 nm. The area under the curve was determined in
7 order to determine the concentration using the ChemStation analysis software as per
8 manufacturer's directions.

9

10 ***5.3 Dendritic cell isolation and culture***

11 Immature bone marrow-derived DCs were generated from 6-8 week-old female C57BL/6j
12 mice in accordance with protocol approved by the Arizona State University (protocol number
13 19-1688R) using a modified 10-day protocol^{36,39,40}. Briefly, femur and tibia from mice were
14 isolated and kept in wash media composed of DMEM/F-12 (1:1) with L-glutamine (VWR,
15 Radnor, PA), 10% fetal bovine serum (Atlanta Biologics, Flowery Branch, GA) and 1%
16 penicillin-streptomycin (VWR, Radnor, PA). The ends of the bones were cut and bone
17 marrow was flushed out with 10 ml wash media and mixed to make a homogeneous
18 suspension. Red blood cells were lysed by incubating and resuspending the pellet in 3mL 1X
19 red blood cell (RBC) lysis buffer for 3 min at 4 °C. The cell suspension was then washed
20 twice with wash media and re-suspended in DMEM/F-12 with L-glutamine (VWR, Radnor,
21 PA), 10% fetal bovine serum, 1% sodium pyruvate (VWR, Radnor, PA), 1% non-essential
22 amino acids (VWR, Radnor, PA), 1% penicillin–streptomycin (VWR, Radnor, PA) and 20
23 ng/ml GM-CSF (VWR, Radnor, PA) (DC media). All % values shown here are vol/vol. This
24 cell suspension was then seeded in a tissue culture-treated T-75 flask (day 0). After 48 hrs
25 (day 2), floating cells were collected, centrifuged, re-suspended in fresh media and seeded on
26 low attachment plates (VWR, Radnor, PA) for 6 additional days. Half of the media was

1 changed every alternate day. At the end of 6 days (day 8), cells were lifted from the low
2 attachment wells by gentle pipetting, re-suspended and seeded on tissue culture-treated
3 polystyrene plates for 2 more days before treating them. On day 10, cells were treated with
4 either 0.1 $\mu\text{g}/\text{ml}$ of paKG MPs or 0.1 $\mu\text{g}/\text{mL}$ of soluble aKG and LPS conditions were
5 administered 1 $\mu\text{g}/\text{mL}$ of LPS. Purity, yield and immaturity of DCs (CD11c, MHC-II and
6 CD86) were verified via immunofluorescence staining and flow cytometry. Dendritic cells
7 were isolated from at least 3 separate mice for each experiment.

8

9 *5.4 Mixed lymphocyte reactions*

10 Spleens were isolated from 6–8-week-old BALB/c mice. Single cell suspensions were
11 prepared by mincing the spleen through a 70 μm pore sized cell strainer. The effluent was
12 centrifuged for 5 min at 300 X g. The cells were then resuspended in 3 mL 1X RBC lysis
13 buffer for 3 min at 4 °C. Next the cells were spun down at 300Xg for 5 min and the pellet was
14 re-suspended in 4 μL of buffer (0.5% BSA and 2 mM EDTA in PBS) per million cells.
15 Negative selection of CD3⁺ T-cells was performed according to manufacturer's
16 recommendation (Miltenyi Biosciences, San Diego, CA). A biotin-labeled antibody cocktail
17 (CD8a (Ly-2) (rat IgG2a), CD11b (Mac-1) (rat IgG2b), CD45R (B220) (rat IgG2a), DX5 (rat
18 IgM) and Ter-119 (rat IgG2b); Miltenyi) was added (10 μL per 10 million cells) and
19 incubated for 15 min at 4 °C. Buffer (30 μL) and anti-biotin microbeads (10 μL) were added
20 to the mixture per 10 million cells. After 15 min incubation at 4°C, cells were centrifuged at
21 300Xg for 5 min and re-suspended in 500 μL of buffer per 100 million cells. A magnetic
22 column was then utilized to collect CD3⁺ T-cells. The CD3⁺ T-cells were centrifuged at
23 300Xg for 5 min and used in mixed lymphocyte reaction. Bone marrow derived DCs were
24 isolated from C57BL/6j mice and were treated prior to the addition of T cells. Mixed
25 lymphocyte reaction was performed at DC:T cell ratio of 1:5 for 48-72 hours.

26

1 **5.5 LC-MS metabolomics studies**

2 Bone marrow derived DCs from C57BL/6j were cultured in 6 well plates at 1 million cells per
3 well. paKG MPs were added at 50 $\mu\text{g}/\text{well}$ and no treatment was utilized as a control. After
4 24 hrs of culture, the supernatant was removed, and the cells were gently rinsed with 2 mL of
5 37 $^{\circ}\text{C}$ PBS. Next, immediately, 1 mL of 80:20 methanol:H₂O (-80 $^{\circ}\text{C}$) into the plates, and the
6 plates were then placed on dry ice to quench metabolism and perform extraction. After 30 min
7 of incubation on dry ice the cells were scraped using a cell scraper (VWR, Radnor, PA), and
8 transferred into centrifuge tubes. The tubes were then spun at 16,000 rpm for 5 min at 4 $^{\circ}\text{C}$
9 (Eppendorf, Hauppauge, NY, USA). The soluble extract was removed into a vial and
10 completely dried. The pellets were utilized to measure the total protein using Nanodrop 2000
11 (ThermoFisher Scientific, Waltham, MA, USA).

12 The LC-MS/MS method was performed according to previous protocols^{22–28}. Briefly,
13 LC-MS/MS were performed using Agilent 1290 UPLC-6490 QQQ-MS (Santa Clara, CA)
14 system. A total of 10 μL of the processed samples were injected twice, for analysis using
15 negative ionization mode and a total of 4 μL of the processes sample for analysis using
16 positive ionization mode. Both chromatographic separations were performed in hydrophilic
17 interaction chromatography (HILIC) mode on a Waters XBridge BEH Amide column (150 x
18 2.1 mm, 2.5 μm particle size, Waters Corporation, Milford, MA). The flow rate utilized in
19 these studies was 0.3 mL/min, auto-sampler temperature was kept at 4 $^{\circ}\text{C}$, and the column
20 compartment was set at 40 $^{\circ}\text{C}$. The mobile phase was composed of Solvents A - 10 mM
21 ammonium acetate, 10 mM ammonium hydroxide in 95% H₂O/5% ACN and Solvent B - 10
22 mM ammonium acetate, 10 mM ammonium hydroxide in 95% acetonitrile (ACN)/5% H₂O.
23 After the initial 1 min isocratic elution of 90% B, the percentage of Solvent B decreased to
24 40% at t=11 min. The composition of Solvent B maintained at 40% for 4 min (t=15 min), and
25 then the percentage of B gradually went back to 90% to prepare for the next injection.

1 The mass spectrometer is equipped with an electrospray ionization (ESI) source. Targeted
2 data acquisition was performed in multiple-reaction-monitoring (MRM) mode. A total of
3 ~320 MRM transitions in negative and positive modes were observed. The whole LC-MS
4 system was controlled by Agilent Masshunter Workstation software (Santa Clara, CA). The
5 extracted MRM peaks were integrated using Agilent MassHunter Quantitative Data Analysis
6 (Santa Clara, CA).

7

8 **5.6 Seahorse assay**

9 Glycolysis and oxidative phosphorylation were measured with the Seahorse Extracellular
10 Flux XF-96) analyzer (Seahorse Bioscience, North Billerica, MA) as previously described⁴¹.
11 Briefly, cells were seeded in Seahorse XF-96 plates at a density of 50,000 cells per well and
12 cultured for 24 hrs in the presence of 10 µg/well of paKG MPs or equivalent amount of
13 soluble aKG, in the presence of absence of 1 µg/mL of LPS. After 24 hrs, cells were changed
14 to unbuffered DMEM in the absence of glucose. Sequential injections were performed with
15 D-glucose (10 mM), oligomycin (1 mM), and 2-deoxyglucose (100 mmol/L). The
16 extracellular acidification rates (ECAR) after the injection of D-glucose was a measure of
17 glycolysis, and the ECAR after the injection of oligomycin represented maximal glycolytic
18 capacity. Non-glycolytic activity was quantified by the measure of ECAR after the injection
19 of 2-deoxyglucose. Samples were analyzed with 10 technical replicates.

20 For oxidative phosphorylation, 24 hrs after cell seeding, media was changed to
21 unbuffered DMEM containing 2mM glutamine, 1mM pyruvate, and 10 mM glucose.
22 Sequential injections were performed with oligomycin (2 mM), 7 Carbonyl cyanide-4
23 (trifluoromethoxy) phenylhydrazone (FCCP) (1 mM), and antimycin/rotenone (1 mM) to
24 modify mitochondrial membrane potential. The oxygen consumption rate (OCR) after the
25 injection of oligomycin was a measure of ATP-linked respiration and the OCR after the
26 injection of FCCP represented maximal respiratory capacity. Basal respiration was quantified

1 by the measure of OCR prior to the injection of oligomycin. Samples were analyzed with 10
2 technical replicates.

3

4 **5.7 Flow cytometry**

5 All the antibodies were purchased and used as is (BD biosciences, Tonbo Biosciences,
6 BioLegend, Thermo Scientific, Invitrogen). Flow staining buffers were prepared by
7 generating 0.1% bovine serum albumin (VWR, Radnor, PA), 2mM Na₂EDTA (VWR,
8 Radnor, PA) and 0.01% NaN₃ (VWR, Radnor, PA). Live/dead staining was performed using
9 fixable dye eF780 (ThermoFisher Scientific, Waltham, MA, USA). Flow cytometry was
10 performed by following manufacturer's recommendations using Attune NXT Flow cytometer
11 (ThermoFisher Scientific, Waltham, MA, USA). The antibodies used in these studies are
12 shown in the table below.

	Target	Fluorophore	Company	Catalog #	Clone
1	CD4	BB700	BD Biosciences	566407	RM4-5
2	CD8	APC-R700	BD Biosciences	564983	53-6.7
3	CD25	PECy7	BD Biosciences	552880	PC61
4	CD11c	PE	BioLegend	117308	N418
5	CD86	SB600	ThermoFisher Scientific	63-0862-82	GL1
6	MHC	APC	BioLegend	107614	M5/114.15.2
7	Tbet	BV785	BioLegend	644835	4B10
8	FoxP3	eF450	Invitrogen	48-5773-82	FJK-16s
9	ROR γ T	BV650	BD Biosciences	564722	Q31-378
10	Ki67	FITC	Invitrogen	11-5698-82	SolA15
11	GATA3	BV711	BD Biosciences	565449	L50-823

1

2 ***5.11 Statistics***

3 Data are expressed as mean \pm standard error. Comparison between two groups was
4 performed using Student's t-test (Microsoft, Excel). Comparisons between multiple treatment
5 groups were performed using one-way ANOVA, followed by Bonferroni multiple
6 comparisons, and p-values ≤ 0.05 was considered statistically significant. Statistical tests
7 were performed using GraphPad Prism Software 6.0 (San Diego, CA).

8

1

2 **Acknowledgements** – We would like to thank Dr. Seo, School of Molecular Sciences, ASU
3 for providing access to DLS. The authors would also like to acknowledge the start up funds
4 provided by ASU for completion of this study.

5

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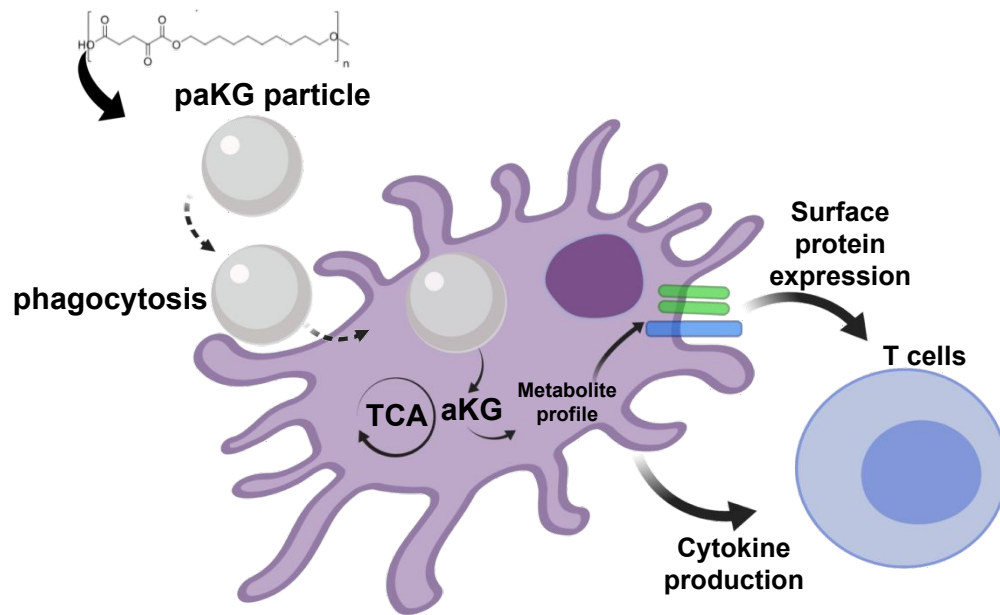
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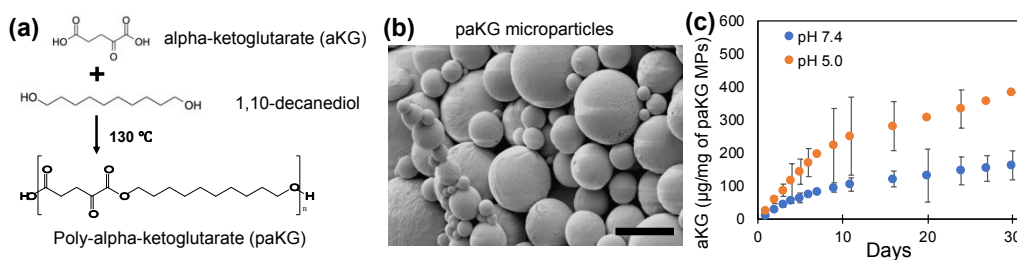
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5 **Figure 1:** Central-carbon metabolite-based polymers modulate adaptive immune responses

6 by changing the intracellular metabolites of the dendritic cells.

7

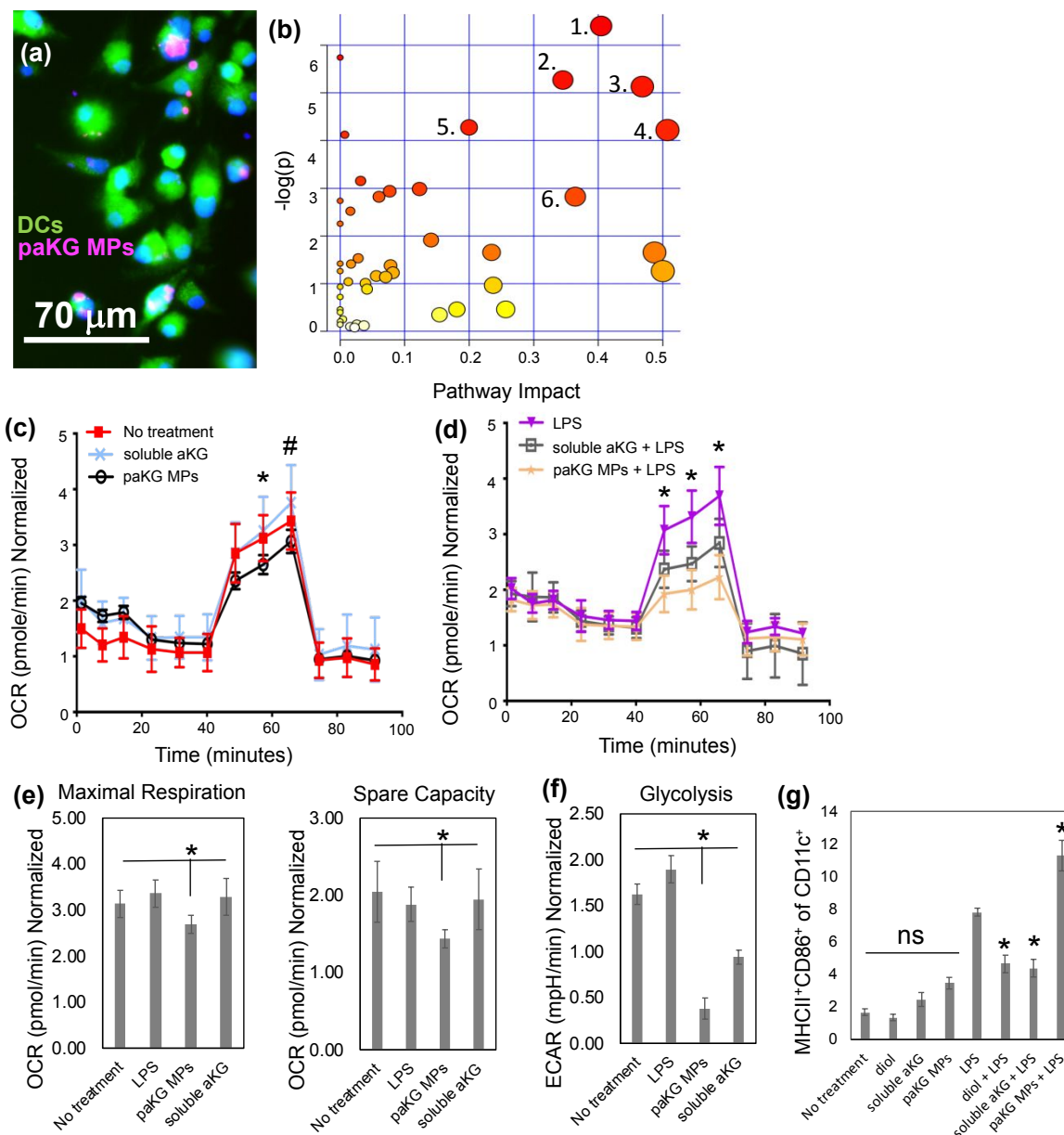
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2 **Figure 2: Central-carbon metabolite-based microparticles release metabolites in a**
3 **sustained manner. (a)** Schema of paKG synthesis. **(b)** Scanning electron microscope
4 micrograph of paKG microparticles (MPs) (scale bar = 5 μm). **(c)** Cumulative release kinetics
5 of alpha-ketoglutarate (aKG) from paKG MPs determined via high performance liquid
6 chromatography (n = 3, avg ± SD).

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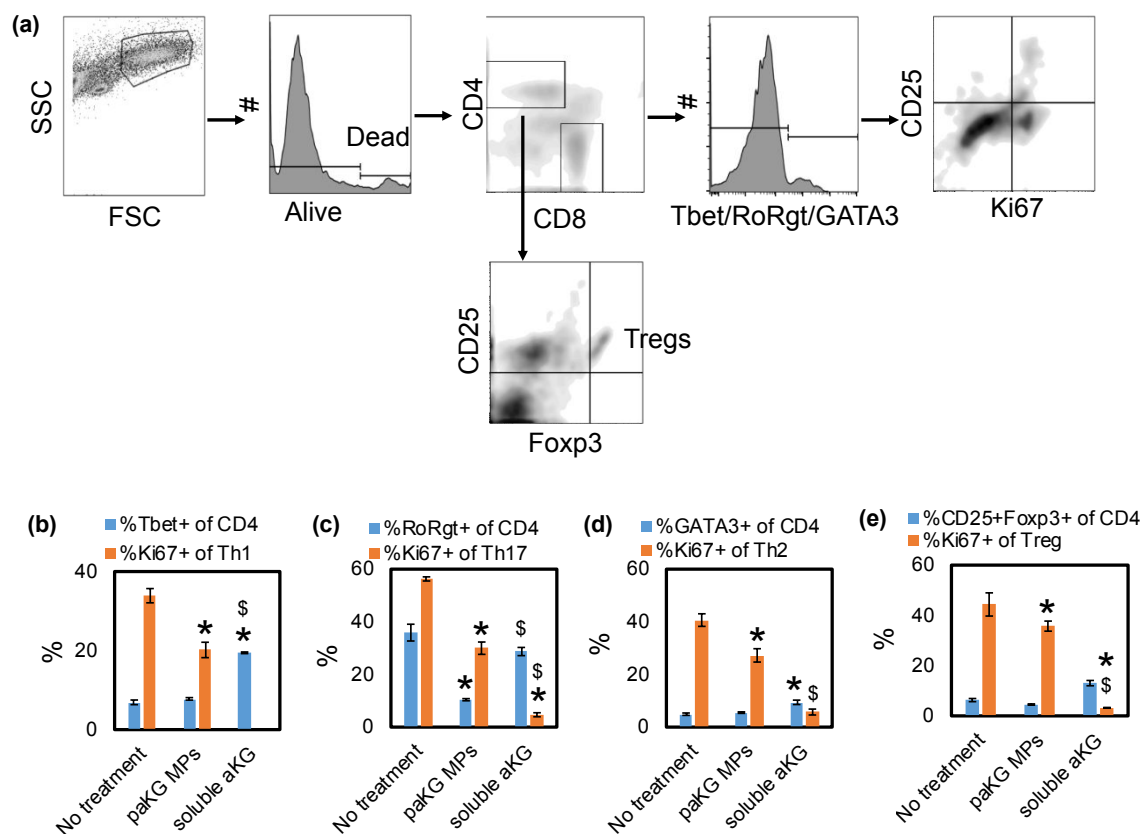
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 2 **Figure 3: paKG microparticles affect function of dendritic cells by modulating their**
 3 **metabolism. (a)** Bone marrow-derived dendritic cells (BMDCs) are able to associate with
 4 paKG microparticles, as observed by fluorescent micrograph (paKG MP – magenta, cytosol –
 5 green, nucleus – blue; scale bar = 70 μm). **(b)** paKG MPs modulate intracellular metabolite
 6 levels as observed by significant ($p < 0.05$) changes in 113 metabolites (out of 299 analyzed
 7 using LC-MS/MS), and their respective signaling pathways. - 1 – Glycine/Serine/Threonine 2
 8 – Arginine biosynthesis, 3 – Glyoxylate metabolism, 4 – Glutamate metabolism, 5 – Arginine

1 metabolism, 6 – Glutathione metabolism. Pathway impact – number of metabolites modified
2 significantly in a pathway; log(p) – level of modulation (n=3). **(c,d)** Oxygen consumption
3 rate (OCR) of DCs is reduced when cultured with paKG MPs in the presence of LPS **(d)** (* -
4 p<0.05; all groups significantly different than each other) or **(c)** absence of LPS (*, # -
5 p<0.05, * - paKG MPs significantly different than soluble aKG and no treatment; # - paKG
6 MPs significantly different than soluble aKG). **(e)** Maximal respiration and spare capacity of
7 DCs when cultured with paKG MPs, and **(f)** glycolysis of DCs in the presence of paKG MPs
8 (n = 10, avg ± SEM, * - p<0.05). **(g)** Activation of DCs in the presence of paKG MPs as
9 indicated by frequency of MHCII⁺CD86⁺ in CD11c⁺ cells. (n = 5, avg ± SEM, * - p<0.05 –
10 significantly different than LPS; ns = not significant).

11



1
2 **Figure 4: paKG microparticles modulate allogeneic adaptive immune responses *in vitro***
3 **(a)** Schematic of flow plot analysis. **(b)** T helper type 1 cell frequency (Th1). **(c)** T helper type
4 17 cell frequency (Th17). **(d)** T helper type 2 cell frequency (Th2). **(e)** Regulatory T cell
5 frequency (Treg). (n = 3-6, avg ± SEM, * - p<0.05 - significantly different than no treatment
6 control; \$ - significantly different than paKG MPs).

7

8

9