Oxidized-ATP Attenuates Kidney Allograft Rejection By Inhibiting T-Cell, B-Cell, and Macrophage Activity

Xiang Ding,1 Nancy A. Wilson,2,3 Robert R. Redfield III,4,5 Sarah E. Panzer,2,3 Bret Verhoven,4,5 Shannon R. Reese,2,3 Weixiong Zhong,6 Lei Shi,7 William J. Burlingham,4,5 Loren C. Denlinger,7 and Arjang Djamali6,2,3

Abstract
Background Extracellular ATP binds to purinergic receptors and promotes inflammatory responses. We tested whether oxidized ATP (oATP), P2X7 receptor antagonist can attenuate acute kidney allograft rejection.

Methods Brown Norway kidney allografts were transplanted into Lewis recipients. Three groups were defined: oATP (n=8), cyclosporine A (n=6), and no treatment (n=8). On day 7, we assessed kidney allograft survival, function, and rejection characteristics. We further determined T-cell, B-cell, and macrophage response to oATP in vivo and in vitro and examined intragraft inflammatory gene transcripts.

Results Kaplan–Meier survival analyses demonstrated significantly better graft survival rates in oATP and CsA groups compared with no treatment (P<0.05). Similarly, serum creatinine (Scr) and BUN levels were significantly lower in oATP and CsA groups (P<0.05). oATP reduced both T cell-mediated rejection and antibody-mediated rejection, inhibited B-cell and T-cell activation, and downregulated intragraft IL-6 mRNA levels (P<0.0001). In vitro, oATP prevented proliferation in mixed lymphocyte reaction assays, and inhibited macrophage P2X7R activity in a dose-dependent manner.

Conclusions Our findings suggest that oATP mitigates kidney allograft rejection by inhibiting T-cell, B-cell, and macrophage activity and indicate a potential role for the purinergic system and oATP in solid organ transplantation.

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Introduction
Extracellular ATP may serve as a mediator of cell-to-cell communication by interacting with specific cell-surface molecules known as P2 purinergic receptors; it can also be a mediator of cytokotic cell-dependent lysis (1). Activation of ATP receptor P2X7 (P2X7R) leads to ion-channel activity, which can depolarize the cell and activate several signaling cascades including protein kinase C and mitogen-activated protein kinase pathways (2,3). Oxidized ATP (oATP) acts as an antagonist of P2X7R (3,4). oATP has demonstrated anti-inflammatory effects in experimental models of asthma, neurodegenerative disease, infections, and autoimmunity (3,5–9).

Recent evidence supports a protective role for oATP in renal ischemia-reperfusion injury (10) and islet allograft rejection (11). Intraperitoneal (i.p.) delivery of oATP before ischemia-reperfusion injury in mice decreased serum creatinine (Scr), BUN, tubular injury scores, and apoptosis of tubular epithelial cells after injury (10). The infiltration of dendritic cells, neutrophils, macrophages, and CD69+CD4+ and CD44+CD4+ T cells was attenuated; but renal forkhead box P3 (FoxP3)+CD4+ regulatory T cell infiltration was increased by oATP. It was concluded that oATP attenuated acute renal damage and facilitated renal recovery in ischemia-reperfusion injury by expansion of regulatory T cells (10). Similarly, in vivo short-term P2X7R targeting with oATP delayed islet allograft rejection in mice, reduced the ratio of T helper 1 (Th1)/Th17 cells, and induced hyporesponsiveness toward donor antigens (11). The combination of oATP and rapamycin synergized in inducing long-term islet function in 80% of transplanted mice and resulted in reshaping of the recipient immune system (11).

These observations led us to believe that oATP may be used in solid organ transplantation to attenuate the alloimmune response. We tested this hypothesis in a robust model of acute kidney allograft rejection using Brown Norway (BN) donor kidneys and Lewis recipients. We observed that oATP therapy improved graft survival and function, reduced both T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR), inhibited B-cell and T-cell activation, and downregulated intragraft IL-6 mRNA levels. In vitro, oATP prevented macrophage and splenocyte proliferation in mixed lymphocyte reaction (MLR) assays, and inhibited macrophage P2X7R activity in a dose-

1Department of Organ Transplantation, Xiangya Hospital, Central South University, Changsha, China; and 2Divisions of Nephrology, 3Transplantation, and 4Pulmonary and Critical Care, 5Departments of Medicine, 6Surgery, and 7Pathology and Laboratory Medicine, University of Wisconsin, Madison, Wisconsin

Correspondence: Dr. Arjang Djamali, Division of Nephrology, Department of Medicine, University of Wisconsin, 5142 MFCB, 1685 Highland Avenue, Madison, WI 53705. Email: axd@medicine.wisc.edu
dependent manner. Altogether, our findings suggest that oATP mitigates kidney allograft rejection by inhibiting T-cell, B-cell, and macrophage activity; the anti-inflammatory effects of oATP may be in part mediated via P2X7R.

Materials and Methods

Animal Model

Adult (175–200 g) male Lewis (RT1+) and BN (RT1n) rats were purchased from Envigo Laboratories (Madison, WI) and were housed in the animal care facility at the William S. Middleton Veterans Affairs Hospital (Madison, WI). All procedures were performed in accordance with the Animal Care and Use Policies at the Veterans Affairs Hospital and University of Wisconsin. Guidelines from The Guide for the Care and Use of Laboratory Animals: Eighth Edition (12) were followed. This model of acute rejection including allogeneic kidney transplantation with simultaneous bilateral native nephrectomy has been previously reported by our laboratory (13,14). We defined three groups: oATP 3 mg/kg per day i.p. 7 days (A6779; Sigma Aldrich; diluted with normal saline and filter sterilized), cyclosporine A (CsA) 10 mg/kg per day i.p. 7 days, and no treatment (Figure 1A). The dose of oATP was selected based on previous studies in mice where the dose ranged from 150 μg/day to 5 mg/kg per day (3,8,10). Blood and tissue samples were collected and analyzed on day 7 or earlier if animals died sooner from rejection.

Kidney Function

BUN and Scr were measured in serum samples using VetTest Analyzer technology (IDEXX Laboratories, Westbrook, ME) as previously described (13).

Histology

Formalin-fixed, paraffin-embedded kidneys were cut into 5-μm sections. Slides were deparaffinized, rehydrated from xylene through a graded ethanol series to double-distilled water, and subsequently treated as described below. Slides were viewed on an Olympus BX52 microscope equipped with an Olympus DP70 camera and software. All hematoxylin-eosin and C4d (split product of complement C4 protein) slides were reviewed by Dr. Weixiong Zhong (MD, PhD, transplant pathologist) and scored according to Banff 2015 (15).

Immunostaining

Immunoperoxidase studies were done as previously described (16–18). The following antibodies were used for immunohistochemical studies: rabbit anti-C4D (used at a 1:200 dilution, catalog number 12-5000; American Research Products) and anti-rat P2X7R (catalog number APR-004; Alamone).

MLR

BN and Lewis splenocytes were purified using standard protocols. Target BN cells were irradiated with 2000 rad to prevent proliferation. A total of 1×10^6 Lewis cells and irradiated BN cells were mixed in a six-well plate and incubated for 3 days with or without 50 μM oATP. At the end of the assay, cells were tested for proliferation using a cell-counting assay (code CK04; Dojindo Molecular Technologies). Cell death was assessed using a Cytotoxicity LDH assay (code CK12; Dojindo Molecular Technologies).

Figure 1. | Oxidized ATP was associated with improved graft survival and function. (A) We compared animal survival and serum creatinine (Scr)/BUN levels on day 7 between three groups: oxidized ATP (oATP; n=8), cyclosporine A (CsA; n=6), and no treatment (n=6). Kaplan–Meier survival analyses demonstrated significantly better graft survival rates in oATP and CsA groups compared with no treatment. Allo + oATP; allogenic transplant with oATP; Allo+CsA, allogenic transplant +CsA; Allogeneic, Brown Norway donor kidney into fully mismatched MHC Lewis recipient. (C) Similarly, Scr and BUN levels were significantly lower in oATP and CsA groups. *P<0.05.
Flow Cytometry

Splenocytes, bone marrow (BM) cells, lymph node (LN) cells, and peripheral blood mononuclear cells (PBMCs) were isolated and 500,000 cells were aliquoted per tube. Cells from the MLR were washed off the plate, washed, and 500,000 cells were aliquoted per tube. All assays included a viability dye (GhostRed 780; Tonbo), although cells were typically >95% viable. The B-cell stain antibodies included IgG (MCA190B-biotinylated; BioRad) with phycoerythrin (PE)-CF594 streptavidin (562284; BD Biosciences), FITC IgM (clone G53-238; BD Biosciences), PE CD138 (clone B-A38; Abcam), PE CD38 (clone 14.27; BioLegend), PECy7 CD45R (B220) (clone HIS24; eBioscience), BV605 or BV510 CD27 (clone LG.3A10; BD Biosciences), and APC CD4 (W3/25; BioLegend). The T follicular helper cell stain antibodies included: AlexaFluor 488 rCCR4 (FAB1567G; R&D Systems), BV421 polyclonal goat anti-rabbit IgG (catalog number 565017; BD Biosciences), and AlexaFluor647 CD3 (clone 1F4; BioLegend). All flow cytometry was gated first through the live gate (GhostRed 780 negative), then gated for singlets before gating for lymphocytes or plasma cells. Flow data were analyzed in FlowJo version 10.2. All flow cytometry was performed at the University of Wisconsin Carbone Cancer Center Flow Cytometry Laboratory, either on the LSR II or LSR Fortessa.

Donor-specific antibody (DSA) analysis was performed essentially as previously described (13,14). Brieﬂy, donor (RT1n) splenocytes were freshly isolated from spleen, macerated through a 40-µm sieve, and washed. After red blood cell lysis, cells were resuspended and 500,000 cells were aliquoted into cluster tubes. Splenocytes were incubated for 30 minutes in a 37°C carbon dioxide incubator with 50 µl of a 1:4 dilution of plasma, washed, and stained for rat antibody isotypes. Antibodies used for the rat DSA included: AlexaFluor647 CD3 (clone 1F4), PECy7 CD45R (B220) (clone HIS24), FITC IgG1 (clone RG7/1.30; BD Biosciences), PE IgG2a (clone RG7/1.30; BD Biosciences), FITC IgG2b (clone RG7/11.1; BD Biosciences), biotinylated IgG2c (clone A92-1; BD Biosciences), Streptavidin Pacific Blue (S11222; Life Technologies, Grand Island, NY), and PE IgM (clone G53-238). Cells were put through a singlet gate, gated through either CD3+ cells (for MHC class I and MHC class II alloantibodies) or CD45R+ cells (for MHC class I and MHC class II alloantibodies), and then mean ﬂuorescence intensity was determined for each isotype.

Real-Time PCR

Real-time PCR analyses were completed as described previously (13,16). Brieﬂy, total RNA was extracted from snap-frozen kidney tissue using Trizol (Life Technologies). RNA was further puriﬁed using the RNAeasy Kit (Qiagen, Venlo, the Netherlands). cDNA was created using the SuperScript IV First Strand Synthesis system (18091050; Invitrogen, Carlsbad, CA). Taqman gene expression assays were ordered from ThermoFisher. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize gene expression.
YOPRO Pore Assay

The YOPRO dye uptake assay was described previously (6,19). Briefly, 1×10⁶ purified Lewis splenic macrophages per well were stimulated for 30 minutes with increasing concentrations of oATP or A740003 in the presence of 250 μM 20-30-O-(4-benzoylbenzoyl) adenosine 5’ triphosphate (BzATP; Sigma Chemical, St. Louis, MO) with 10 mM YO-PRO-1 (Invitrogen) and then read on a plate reader at OD<sub>excitation</sub>=491 nm and OD<sub>dmission</sub>=509 nm.

Statistical Analyses

The t test and Mann–Whitney rank sum test were used to compare parametric and nonparametric continuous data, as appropriate. One-way ANOVA was used when comparing between multiple groups simultaneously. Chi-squared or Fisher exact tests were used to compare categoric data between groups. A Kaplan–Meier analysis was used for the survival data. P values of ≤0.05 were considered significant.

Results

oATP Was Associated with Improved Graft Survival and Function

To determine the effect of oATP on kidney transplant survival and function, we compared animal survival and Scr and BUN levels on day 7 between three groups: oATP (n=8), CsA (n=6), and no treatment (n=6) (Figure 1). Kaplan–Meier survival analyses demonstrated significantly better graft survival rates in oATP and CsA groups compared with no treatment (P<0.05; Figure 1B). Similarly, Scr and BUN levels were significantly lower in oATP and CsA groups (P<0.05; Figure 1C).

Table 1. oATP attenuated T cell-mediated rejection and antibody-mediated rejection

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oATP, oxidized ATP; TCMR, T cell–mediated rejection; ABMR, antibody-mediated rejection; NA, not assessed due to diffuse kidney necrosis; CsA, cyclosporine A. Ia, IIa, IIb, III are grades of rejection, with Ia being the least amount of rejection and III being the most in this table. Ia<IIa<IIb<III.

oATP Attenuated TCMR and ABMR

To define the specific effect of oATP on kidney allograft rejection, we compared the histopathologic findings between the three groups according to Banff (15). All six surviving animals from the untreated group demonstrated severe mixed rejection (TCMR and ABMR) with large areas of interstitial hemorrhage and cortical necrosis on day 7 (Figure 2, A and B, Table 1). No recipient in the oATP or CsA group had evidence of cortical necrosis/interstitial hemorrhage (P<0.001). Neither oATP nor CsA were completely effective in preventing rejection; however, oATP reduced the severity of tubulitis (t score, P<0.001) and endarteritis.
were isolated from BN and Lewis spleens. Lewis splenocytes were incubated with irradiated Lewis* cells (negative control, Syn) or irradiated BN* cells (positive control, Allo). In a third group, Lewis splenocytes were coincubated with irradiated BN* splenocytes and 50 µM oATP (Allo+oATP). As noted in Figure 4A, Lewis splenocytes proliferated significantly in the presence of irradiated BN* splenocytes. However, splenocyte proliferation was significantly inhibited by oATP (P<0.05). A similar result was obtained looking only at macrophages (Figure 4B).

**oATP Therapy Was Associated with a Decline in B Cells and T-Cell Activation after Renal Transplantation**

We next examined the effect of oATP on B-cell and T-cell phenotypes in *vivo* after kidney transplantation. We directly tested the status of the cell populations in the treated versus nontreated allograft recipients on day 7 post-transplant for activation status; the cell populations were isolated from the recipient spleen, BM, LNs, and PBMCs and tested for activation markers. The gating strategies used are demonstrated in Supplemental Figure 1. These studies showed that oATP treatment was associated with a significant decline in CD45R+B cells in the spleen and BM (P<0.05; Figure 5A). Furthermore, transitional B cells (CD45R+CD24+CD38+) were reduced in the spleen, LNs, and PBMCs (P<0.05; Figure 5B), and activated B cells (CD45R+CD27+) were reduced in the spleen (P<0.05; Figure 5C). The total number of T cells did not change significantly (Figure 5D); however, both CD3+CD4+ICOS+ T cells (Figure 5E) and CD3+CD4+CCR6+ T cells (Figure 5F) declined significantly in the LNs, PMBCs, and spleen (P<0.05).

**oATP Treatment Was Associated with a Decline in DSAs Post-transplant**

To determine the effect of oATP treatment on DSAs, we examined IgM, IgG2b, and IgG2c alloantibodies on day 7.
Normal Lewis plasma alone served as a negative control for the assay. These studies demonstrated that DSAs were significantly reduced in animals treated with αATP. This observation was particularly evident for IgM DSAs in the absence of previous exposure and memory response.

oATP Significantly Reduced IL-6 mRNA in the Kidney Allograft

To determine the effect of oATP on intrarenal proinflammatory gene expression, we compared IL-1β, IL-2, IL-6, IL-10, IL-17, and IFN-γ mRNA levels using real-time PCR analyses, normalizing gene expression in oATP-treated kidneys to controls (Figure 7). These studies showed that oATP therapy was associated with a drastic downregulation of IL-6 mRNA (approximately 80-fold, \( P < 0.0001 \)) in kidney allografts.

Figure 5. | oATP therapy was associated with a decline in B cells and T-cell activation in vivo. (A) Seven days post-transplant, oATP treatment was associated with a significant decline in CD45R+ B cells in the spleen and bone marrow (BM; \( P < 0.05 \)). (B) Transitional B cells (CD45R+CD24+CD38+) were reduced in the spleen, lymph node (LN), and peripheral blood monocytes (PBMCs; \( P < 0.05 \)). (C) Activated B cells (CD45R+CD27+) were reduced in the spleen (\( P < 0.05 \)). (D) The total number of T cells did not change significantly; however, both (E) CD3+CD4+iCOS+ T cells and (F) CD3+CD4+CCR6+ T cells declined significantly in the LN, PBMCs, and spleen (\( P < 0.05 \)). *\( P < 0.05 \).

oATP Inhibited Macrophage P2X7R in Lewis Rats

Because macrophages are an important source of IL-6, we sought to examine whether oATP inhibited Lewis (recipient) macrophages through their P2X7R. Activation of the P2X7R leads to the formation of a membrane pore that allows molecules up to approximately 900 Da to permeate upon ATP binding (20,21). We first used increasing
and lymphocyte proliferation while inhibiting macrophage activity. The inhibition of oATP may attenuate kidney allograft rejection by inhibiting the receptor, suggesting that some anti-inflammatory effects of oATP may not be due to blockade of the P2X7R. Consistent with this observation, oATP can reduce nuclear factor κB activation and IL-8 release in cells lacking P2X7R (22). Similarly, other investigators have reported inhibitory effects of oATP on proinflammatory responses in three human cell types lacking the expression of P2X7R: umbilical vein endothelial cells (HUVEC), HEK293 cells, and 1321N1 astrocytes (23). In these studies, oATP decreased by 40%–70% the secretion of IL-8 stimulated by TNF-α in all three cell types, by IL-1β in HUVEC and 1321N1 cells, and by endotoxin in HUVEC (23). Attenuation of TNF-α–stimulated IL-8 secretion by oATP was similar in wild-type HEK cells or HEK cells stably expressing recombinant P2X7R (23).

We further demonstrated a significant inhibition of T-cell, B-cell, and macrophage activation in vitro and in vivo. The total number of T cells did not change significantly; however, both CD3+CD4+CD25+ T cells and CD3+CD4+CCR6+ T cells declined significantly in secondary lymphoid organs. These in vivo phenotypes define memory and effector T cells with high inflammatory potential (24,25). Our findings are consistent with previous studies demonstrating the inhibition of T-cell activation and the Th1/Th17 phenotype by oATP in autoimmune disease, islet transplantation, and infection (3,9,11,25). In a mouse model of islet transplantation, P2X1R and P2X7R were induced in islet allograft–infiltrating cells, but only P2X7R was increasingly expressed during alloimmune response (11). In vivo, short-term P2X7R targeting with oATP delayed islet allograft rejection, reduced the frequency of Th1/Th17 cells, and induced hyporesponsiveness toward donor antigens (11). oATP-treated mice displayed preserved islet grafts with reduced Th1 transcripts and in vitro P2X7R targeting using oATP reduced T-cell activation and diminished Th1/Th17 cytokine production. The investigators concluded that beneficial effects of oATP treatment revealed a role for the purinergic system in islet allograft rejection, and the targeting of P2X7R could be a novel strategy to induce long-term islet allograft function (11). We add new data to these observations by demonstrating a downregulation of transitional B cells (CD45R+B220+CD24+CD38+), activated B cells (CD45R+CD27+), and DSAs in a solid organ transplant model.

Our studies did not examine the potential effects of oATP on nonimmune cells, because P2X7R expression can be upregulated in glomerular and endothelial cells during acute injury (26). However, taken together, our findings suggest that oATP can attenuate proinflammatory signaling by mechanisms that are partially mediated via P2 receptor subtypes (1,4,22,23). This observation was associated with improvement in short-term kidney allograft survival and function; attenuation of acute TCMR and ABMR; inhibition of B-cell, T-cell, and macrophage activation and proliferation; and downregulation of intragraft IL-6 mRNA levels. Further studies are needed to determine the role of the purinergic system and oATP in kidney transplantation.

[Figure 7. oATP significantly reduced IL-6 mRNA in the kidney allograft. To determine the effect of oATP on intrarenal proinflammatory gene expression, we compared IL-1β, IL-2, IL-6, IL-10, IL-17, and IFN-γ mRNA levels using real-time PCR analyses, normalizing gene expression in oATP-treated kidneys to controls. These studies showed that oATP therapy was associated with a drastic downregulation of IL-6 mRNA (approximately 80-fold, \*P<0.0001) in kidney allografts.]

Concentrations of agonistic BzATP to open P2X7R pores on macrophages isolated from the spleen (Figure 8A). We observed that BzATP opened P2X7R pores in a dose-dependent manner until a concentration of approximately 500 μM; thereafter, there was a decline in BzATP activity. Next, we compared the effects of oATP and A740003 (selective inhibitor of P2X7R) on closing the P2XR pores in the presence of a constant concentration of BzATP (250 μM; Figure 8, B and C). These studies determined that, although all concentrations of A740003 were effective in inhibiting BzATP, only higher concentrations of oATP (500 μM) inhibited P2X7R (Figure 8, B and C).

Discussion

Our studies determined that oATP improves graft survival and function, attenuates TCMR and ABMR in a robust model of acute rejection, inhibits B-cell and T-cell activation in vivo and in vitro, and downregulates intragraft IL-6 mRNA levels. In vitro, oATP prevents splenic macrophage and lymphocyte proliferation while inhibiting macrophage P2X7R activity. In aggregate, these findings suggest that oATP may attenuate kidney allograft rejection by inhibiting T-cell, B-cell, and macrophage activation. The inhibition of macrophage P2X7R indicates a potential molecular pathway regulating the anti-inflammatory properties of oATP in kidney transplantation by interfering with downstream signaling.

The anti-inflammatory effects of oATP, including the downregulation of IL-6 in an experimental model of renal injury, are well established (10). oATP is a Schiff base-forming reagent that has been used for some years as an antagonist at the P2X7R (2,22). In a mouse macrophage-like cell line, low oATP concentrations (100 μM) completely blocked early permeabilization of plasma membrane, cell swelling, vacuolization, and lysis mediated by extracellular ATP (4). Antagonism developed slowly because an incubation at 37°C for at least 2 hours in the presence of oATP was needed and was irreversible, thus suggesting that the inhibitory action was due to covalent modification of the P2X7R receptor (4). In our study, although oATP inhibited macrophage P2X7R in vitro, we did not delineate whether IL-6 inhibition was mediated through P2X7R antagonism. Furthermore, although all concentrations of A740003 (the selective inhibitor of P2X7R) were effective in inhibiting BzATP, only higher concentrations of oATP (500 μM) inhibited the receptor, suggesting that some anti-inflammatory effects of oATP may not be due to blockade of the P2X7R. Consistent with this observation, oATP can reduce nuclear factor κB activation and IL-8 release in cells lacking P2X7R (22).
Author Contributions

X. Ding and N. Wilson were responsible for formal analysis; X. Ding, N. Wilson, and A. Djamali conceptualized the study, were responsible for data curation, and wrote the original draft of the manuscript; X Ding, N. Wilson, B. Verhoven, S. Reese, W. Zhong, L. Shi, W. Burlingham, L. Denlinger, and A. Djamali were responsible for methodology; R. Redfield, S. Panzer, W. Burlingham, L. Denlinger, and A. Djamali were responsible for resources; R. Redfield, S. Panzer, S. Reese, A. Djamali, W. Burlingham, and L. Denlinger provided supervision; R. Redfield, S. Panzer, S. Reese, B. Verhoven, W. Zhong, L. Shi, W. Burlingham, L. Denlinger, and A. Djamali reviewed and edited the manuscript; and A. Djamali was responsible for funding acquisition.

Disclosures

L. Denlinger reports grants and personal fees from AstraZeneca; grants from the NIH, National Heart, Lung, and Blood Institute (NHLBI) during the conduct of the study; and personal fees from Sanofi-Regeneron outside the submitted work. In addition, L. Denlinger has been issued the following patents: United States Patent number 7,560,243 B2 and United States Patent number 7,960,131 B2. All remaining authors have nothing to disclose.

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Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0000692019/-/DCSupplemental.

Supplemental Figure 1. Gating strategies for flow cytometry.

References


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X.D. and N.A.W. contributed equally to this work.