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Purinergic signaling during macrophage differentiation results in M2 alternative activated macrophages

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ABSTRACT

Macrophages represent a highly heterogenic cell population of the innate immune system, with important roles in the initiation and resolution of the inflammatory response. Purinergic signaling regulates both M1 and M2 macrophage function at different levels by controlling the secretion of cytokines, phagocytosis, and the production of reactive oxygen species. We found that extracellular nucleotides arrest macrophage differentiation from bone marrow precursors via adenosine and P2 receptors. This results in a mature macrophage with increased expression of M2, but not M1, genes. Similar to adenosine and ATP, macrophage growth arrested with LPS treatment resulted in an increase of the M2-related marker Ym1. Recombinant Ym1 was able to affect macrophage proliferation and could, potentially, be involved in the arrest of macrophage growth during hematopoiesis.
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Introduction

Macrophages are key cells of the innate immune system that initiate inflammation via the production of cytokines. Proinflammatory macrophages are referred to as M1, or classically activated macrophages, and result from activation with the Th1 cytokine IFN-γ and/or PAMPs such as LPS [1, 2]. In contrast, macrophages exposed to Th2 cytokines, such as IL-4 or IL-13, derive into the alternative activated phenotype and are termed M2. M2 macrophages are involved in both immunity against parasites and the resolution of inflammation [1, 3]. M2 macrophages differentiated in vitro resemble the phenotype of in vivo-resolving macrophages and are considered anti-inflammatory, because the NF-κB and inflammasome pathways are inhibited [4, 5]. Specific gene expression patterns have been characterized to distinguish between M1 and M2 macrophages [6, 7]. Although M1 markers are well-characterized proinflammatory genes (including *TNF-α, IL-1, IL-6, COX2*, and *iNOS*), some M2 markers constitute a group of genes with poorly studied function [6–8]. The prototypic M2 marker is the mannose receptor C1 (encoded in mice by *Mrc1*), which was used to describe for the first time the alternative activation of macrophages [9]. Several M2 markers are exclusive to mouse macrophages, because no human homologs of these particular genes exist. Examples include *Chi3l3* (which encodes for the lectin chitinase-like 3 or Ym1) and *Retnla* (which encodes for the resistin-like molecule-α or FIZZ1). Other mouse M2 markers are not upregulated by IL-4 in humans, for example *Arg1* (which encodes for arginase 1) [10].

M1 and M2 macrophages are considered extreme activation states of a wide range of different intermediate polarization subsets. Some studies have shown that, based on the expression of different M1 and M2 markers, intermediate macrophages result in complex mixed polarization subsets [11–13]. According to the results from different stimulations, macrophages could express both M1 and M2 markers [2]. For example, M2b macrophages result from stimulation of Fcγ receptors by immunocomplexes, together with Toll receptor stimulus, resulting in low IL-12 and high IL-10 production [2, 14, 15]. The different polarization subsets reflect the ability of macrophages to adapt to different environments, conferring to these cells an extremely exquisite plasticity that could drive a proinflammatory M1 macrophage to turn into a M2 proresolving macrophage after an appropriate change in the extracellular signals [11].

Although the pathways that push macrophages toward the M1 phenotype are well established and mainly involve activation of NF-κB, the activation and regulatory pathways leading to alternative macrophages are less well studied [16]. IL-4 receptor engagement leads to the recruitment and dimerization of

Abbreviations: ADA = adenosine deaminase, Ado = adenosine, AMP-PCP = β,γ-methyladenosine 5′-triphosphate, Bay-60-6583 = 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamid, BMDM = bone marrow-derived macrophage, cAMP = cyclic AMP, Chi3l3 = chitinase 3-like 3, COX = cyclooxygenase, Gr1 = PE-conjugated anti-mouse Ly6G, NECA = 5′-*N*-ethylcarboxamidoadenosine, P2X7R = P2X7 ligand-gated

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The online version of this paper, found at www.jleukbio.org, *includes supplemental information.*

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STAT6, a crucial nuclear factor that upregulates M2-related genes [17]. Also, IL-4 receptor activation leads to insulin receptor substrate phosphorylation and subsequent activation of PI3K, which is required, for example, to enhance *Arg1* expression in mouse macrophages [18]. This process could be further regulated by the degradation of the PI3K inhibitor SHIP [19].

Extracellular nucleotides and nucleosides that act on purinergic cell surface receptors in macrophages create an exquisite regulatory signaling network in diverse health and disease scenarios [20, 21]. A high concentration of extracellular nucleotides, such as ATP, functions as a danger signal that activates the P2X7R and leads to the activation of the NRLP3 proinflammatory inflammasome pathway in M1 macrophages [22–24]. A low concentration of extracellular ATP and of other nucleotides such as ADP or UTP activates the G-protein-coupled P2Y family of receptors to mediate chemotaxis of myeloid cells to areas of tissue damage [25]. Alternatively, the degradation of extracellular ATP into pyrophosphates and Ado constitutes a potent anti-inflammatory signal [4, 11, 26, 27]. The activation of Ado receptors leads to the production of cAMP and the activation of CCAAT-enhancer-binding protein-β, thereby increasing M2 macrophage polarization and dampening inflammation [28, 29].

The complex study of macrophage polarization and purinergic signaling has been poorly addressed in the context of macrophage differentiation from bone marrow precursors. However, the differentiation of macrophages in the bone marrow is a critical process during inflammation, because the number of immune cells needs to be increased at sites of infection or injury [1]. In the present study, we addressed the effects of purinergic signaling on macrophage differentiation from bone marrow precursors and studied how it alters the expression of different M1 and M2 marker genes. We found that extracellular ATP was quickly degraded by bone marrow precursors and that both ATP and Ado were potent blockers of macrophage growth, resulting in mature macrophages with upregulation of the M2 marker Ym1 and a predisposition toward M2 polarization.

MATERIAL AND METHODS

Animals

C57 BL/6 (wild-type) mice were purchased from Harlan Laboratories (Indianapolis, IN, USA). P2X7R-deficient mice in C57 BL/6 background (*P2rx7*$^{-/-}$) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) [30]. For all experiments, mice aged 8–10 wk and bred under specific pathogen-free conditions were used, in accordance with the University Hospital Virgen Arrixaca animal experimentation guidelines, and the Spanish national (RD 1201/2005 and Law 32/2007) and European Union (86/609/EEC and 2010/63/EU) legislation. According to the cited legislation, local ethics committee review or approval is not needed, because the mice were killed by CO_2 inhalation and used to obtain bone marrow. No procedure was undertaken that compromised animal welfare.

Reagents

Mouse seroblock-FcR antibody was purchased from AbD Serotec (Kidlington, UK), Alexa Fluor 488 conjugated anti-mouse F4/80 from Caltag Laboratories (Burlingame, CA, USA), APC-conjugated anti-mouse CD11b from BD Pharmingen (San Diego, CA, USA), and Gr1 from eBiosciences Inc. (San Diego, CA, USA). *Escherichia coli* LPS serotype 055:B5, ATP, ADP, UTP, UDP, Ado, Ado deaminase, theophylline, and recombinant mouse IFN-γ were purchased from Sigma-Aldrich (St. Louis, MO, USA). P2X7R antagonist A438079, NECA, PSB077, Bay-60-6583, and KT5720 were from Tocris (Bioscience, Bristol, U.K.). Recombinant mouse IL-4 was from BD Biosciences (San Diego, CA, USA). Recombinant mouse Chi3l3/Ym1 was from R&D Systems (Minneapolis, MN, USA).

Differentiation of macrophages from mouse bone marrow precursors

Bone marrow was obtained from the leg bones of mice killed by CO_2 inhalation. The femurs and tibia were removed, and the bone marrow was flushed out and resuspended in DMEM (Lonza, Walkersville, MD, USA) supplemented with M-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ, USA), 15% FCS (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin/streptomycin (Lonza), and 1% L-glutamine (Lonza). The bone marrow was then plated onto 150-mm dishes and cultured at 37°C in the presence of 5% CO_2. Purinergic agonists and antagonists or LPS were added on the first day of differentiation (d 0) and supplemented on d 2. In other experiments, after 7 d of macrophage differentiation, the resulting BMDMs were detached with cold PBS, seeded into 6-well plates at a confluence of 0.42×10^6 cells/cm^2, and used the following day (d 8 of differentiation) to polarize macrophages to M1 (by 4 h of treatment with LPS 100 ng/ml and IFN-γ 20 ng/ml) or to M2 (by 4 h of treatment with IL-4 20 ng/ml).

Mature macrophage quantification

Macrophages were washed and detached using cold PBS supplemented with 2 mM EDTA. The cells were first incubated with mouse seroblock-FcR and then stained with anti-F4/80 Alexa Fluor 488-conjugated or a mix of anti-CD11b-APC and anti-Gr1-PE for 30 min at 4°C. Finally, the cells were washed, fixed with PFA (1%) in PBS, and subjected to flow cytometry analysis using a FACSCanto flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences) by gating for BMDM cells based on FSC versus SSC parameters. F4/80$^+$ or CD11b$^+$Gr1$^{low/-}$ cells were quantified as mature macrophages.

MTT assay

Macrophage growth during in vitro differentiation with M-CSF was assessed using the MTT assay at different days as indicated in the figure legends. MTT (1 mg/ml) was incubated with the cells for 4 h at 37°C. The medium was then removed, and MTT crystals were solubilized with DMSO by agitation for 30 min at room temperature. The optical density was measured at 540 nm using a Synergy Mx plate reader (BioTek Instruments, Winooski, VT, USA). The results are expressed as the normalized percentage of cells with respect to the control untreated macrophages.

Extracellular ATP degradation

Culture media from mature BMDM (d 8) or bone marrow precursors (d 0) plated in a 24-well plate was replaced with basal salt solution containing 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Na-HEPES (adjusted to pH 7.5 at room temperature), 5 mM glucose, and 0.1% BSA. Next, 3 mM ATP was added to the wells, and the degradation of the ATP was measured at 0, 5, 15, 30, 60, and 120 min using the ATP Bioluminescent Somatic Cell Assay kit (Sigma-Aldrich) in accordance with the manufacturer's instructions.

Quantitative reverse transcriptase-PCR analysis

The detailed methods used for quantitative reverse transcriptase-PCR have been described previously [11]. Specific primers were purchased from Qiagen (QuantiTech Primer Assays; Hilden, Germany). For each primer set, the efficiency was >95%, and a single product was obtained on melt curve

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ion channel receptor, PAMP = pathogen associated molecular pattern, PFA = paraformaldehyde, PKA = protein kinase A, PSB077 = 4-[2-[(6-Amino-9-b-D-ribofuranosyl-9*H*-purin-2-yl)thio]ethyl]benzenesulfonicacid

analysis. The presented relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalizing to *Gapdh* expression levels for each treatment, and the fold increase in expression was relative to the smallest expression level or to the control basal levels [31].

Cell death measurements

Cell death was determined at 2 h, 5 h, and 8 d after treatment with Ado (0.1 mM) using the Live/Dead Fixable Dead Cell Staining Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, BMDMs were incubated with near-infrared fluorescent reactive dye for 30 min. Then, the cells were washed twice, fixed in PFA (1%), and subjected to flow cytometry using a FACSCanto flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences) by gating for BMDM cells according to FSC versus SSC parameters.

Cell cycle determination

Cell cycle was determined at 2 h and 8 d after treatment with Ado (0.1 mM) or ATP (1 mM). The cells were washed and fixed in ice-cold 70% ethanol for 1 h at 4°C. Next, 10^6 cells were centrifuged at 400*g* for 5 min and washed twice. The cells were treated for 30 min at 37°C with ribonuclease A (10 mg/ml; Roche, Indianapolis, IN, USA) and then stained for 30 min with propridium iodide (5 μg/ml, Sigma-Aldrich) before flow cytometry, as stated.

Statistical analysis

All data are shown as mean values, and error bars represent the SEM from the number of independent assays (indicated in the figure legends). For statistical comparisons, the data were analyzed using an unpaired 2-tailed Student's *t* test to determine the difference between groups using Prism software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Extracellular ATP impairs growth but not the differentiation of bone marrow precursors into macrophages

In this work, we explored the role of purinergic signaling during the differentiation of bone marrow precursors into macrophages by supplementing M-CSF media with different concentrations of ATP. After plating bone marrow precursors (d 0), we monitored cell growth at different days and found that macrophages were starting growth after d 2 of differentiation (**Fig. 1A**). Repeated ATP application at d 0 and d 2 resulted in a statistically significant decrease in macrophage growth when either 0.1 or 1 mM of ATP was used (Fig. 1A). We then analyzed whether bone marrow precursors that were differentiated for 8 d in the presence of 1 mM ATP became mature macrophages. We found a similar percentage of mature macrophages in the bone marrow cells differentiated during 8 d with M-CSF in the absence or presence of ATP (Fig. 1B). The result was the same whether detected by the expression of the surface antigen F4/80 or by the determination of $CD11b^+Gr1^{low/-}$ cells by flow cytometry. We found that approximately 80–90% of the cells at d 8 of differentiation with M-CSF were mature macrophages, irrespective of ATP treatment (Fig. 1B). This suggests that extracellular ATP does not affect macrophage differentiation but does impair macrophage growth.

High extracellular ATP concentrations activate P2X7R, and, in myeloid cells, prolonged activation of this receptor leads to cell death [32, 33]. We then tested the hypothesis that the inhibition of cell growth induced by extracellular ATP during macrophage differentiation could be due to P2X7R activation and cell death. We analyzed *P2rx7* mRNA expression using quantitative PCR through the differentiation of bone marrow precursors into mature macrophages and found that *P2rx7* expression significantly increased from d 6 of differentiation (**Fig. 2A**). Treatment with the specific P2X7R antagonist A438079 before ATP application did not recover macrophage growth (Fig. 2B). ATP treatment of bone marrow cultures inhibited the growth of both wild-type and P2X7R-deficient macrophages (Fig. 2B). We next assessed cell death and found that ATP stimulation was not inducing death in cells during macrophage differentiation (Fig. 2C). Furthermore, ATP was not arresting the macrophage cell cycle (Supplemental Fig. 1A). We therefore ruled out the involvement of P2X7R and cell death in the inhibition of the growth of macrophages with ATP treatment.

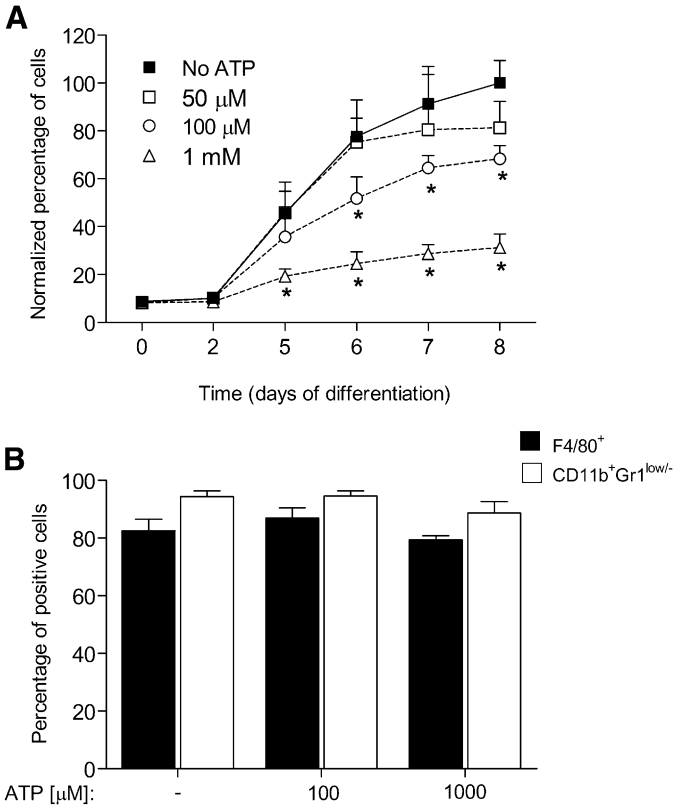

Figure 1. Effect of extracellular ATP during macrophage growth and differentiation. (A) Growth of bone marrow precursors measured by MTT assay during 8 d of differentiation in the presence of M-CSF and different concentrations of ATP, as indicated. D 0 corresponds to the day the bone marrow was isolated from the mice and plated. Data presented as mean ± SEM of 8 independent experiments. (B) Percentage of $F4/80^+$ or $CD11b^+Gr1^{low/-}$ macrophages detected by flow cytometry after 8 d of differentiation in the presence of M-CSF and different concentrations of ATP, as indicated. Data presented as mean ± SEM of 3 independent experiments. $^*P > 0.01$ but < 0.05.

Ado nucleoside reduces macrophage growth during differentiation

We next explored the action of different nucleotides on macrophage growth and found that ADP and Ado were also able

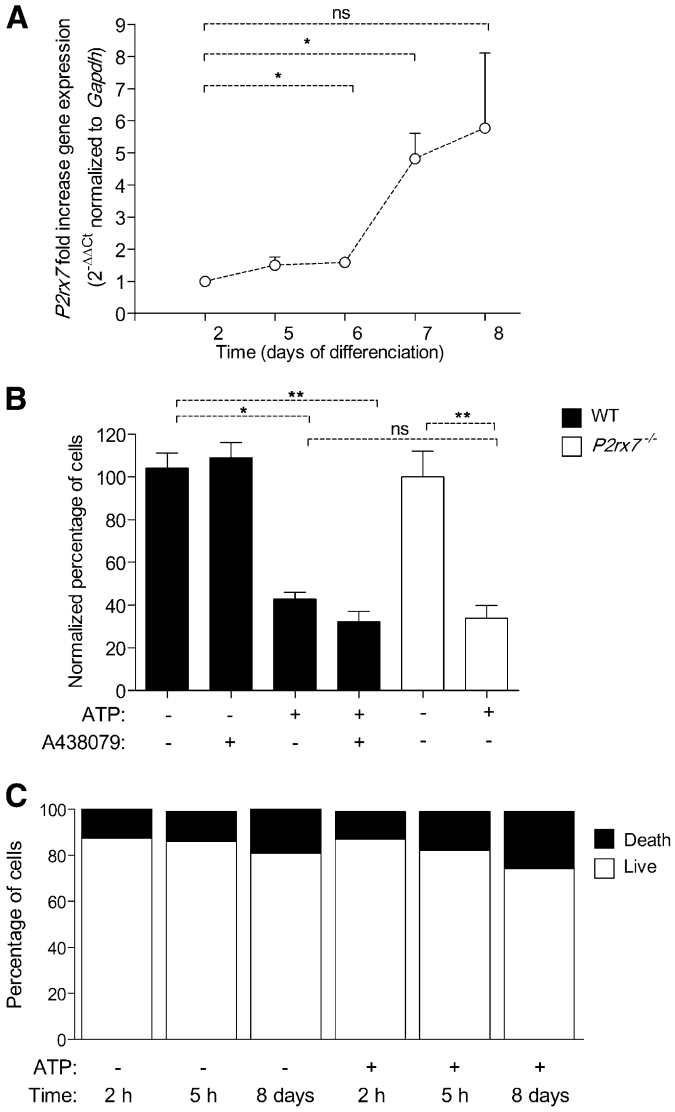

Figure 2. Macrophage growth inhibition by extracellular ATP is not dependent on P2X7R activation. (A) Expression of *P2rx7* gene analyzed by quantitative PCR during macrophage differentiation with M-CSF. Data presented as mean ± SEM of 3 independent experiments. (B) Growth of wild-type or *P2rx7*$^{-/-}$ bone marrow precursors measured by MTT assay after 8 d of differentiation with M-CSF and ATP (1 mM) in the presence or absence of A438079 (10 μM). Data presented as mean ± SEM of 4 independent experiments. (C) Percentage of viable and dead cells measured by Live/Dead Fixable Dead Cell Staining kit at different times of differentiation with M-CSF and ATP (1 mM), as indicated. Data presented as average of 4 experiments. *$P > 0.01$ but < 0.05; **$P > 0.001$ but < 0.01; ns indicates $P > 0.05$ (not significant).

to inhibit macrophage growth (**Fig. 3A**). Ado treatment resulted in an effect similar to that of extracellular ATP application (Fig. 3A). Bone marrow precursors and mature macrophages were able to degrade extracellular ATP (Fig. 3B), suggesting that the addition of ATP during macrophage differentiation could primarily be mediating its effect via its degradation into ADP and Ado. We then analyzed the expression of all the different purine receptors during macrophage differentiation. Of the expressed receptors, the expression of purine P2X and P2Y receptors increased over the period of differentiation (Fig. 3C and D). A_{2A} and A_{2B} Ado receptor expression slightly decreased during macrophage differentiation (Fig. 3C and D). We found no expression of A_1 and A_3 Ado receptors (data not shown), and very little expression for P2X2, P2X3, P2X5, P2X6, and $P2Y_1$ receptors at any time point in macrophage differentiation that was examined (Fig. 3D). Similar to ATP treatment, macrophages differentiated with Ado were found to be mature when assayed by surface markers (**Fig. 4A**). Next, we found that Ado treatment did not increase cell death or alter the cell cycle during macrophage differentiation (Fig. 4B and Supplemental Fig. 1A). Bone marrow precursors treated with the highly potent and general Ado receptor agonist NECA, the highly selective A_{2B} receptor agonist Bay-60-6583, or the A_{2A} receptor agonist PSB077 reduced macrophage growth during differentiation (Fig. 4C), suggesting that signaling through Ado receptors compensates among A_{2A} or A_{2B} receptors and blocks macrophage differentiation. However, the presence of KT5720, an inhibitor of PKA, which is activated by cAMP downstream of A_2 receptor stimulation, did not affect the arrest of macrophage growth by Ado (Supplemental Fig. 1B). The use of specific A_{2A} or A_{2B} receptor antagonists directly inhibited macrophage growth (Supplemental Fig. 1C), which has already been reported for immune cells as a receptor-independent effect [34]. To further investigate whether the degradation of ATP to Ado was responsible for inhibiting macrophage growth, we used ADA and the general Ado receptor antagonist theophylline in the presence of ATP. Both ADA and theophylline were able to significantly revert the inhibition exerted by ATP on macrophage growth (Fig. 4D). However, this inhibition was not total, suggesting that ATP could also be acting on other purinergic receptors to arrest macrophage growth. This was further confirmed using the nonhydrolyzable ATP analog AMP-PCP, which inhibited macrophage growth during differentiation at a similar rate as Ado and ATP (Fig. 4E). Taken together, a combination of Ado and P2 receptors could signal to arrest macrophage growth.

Macrophage differentiation in the presence of Ado results in an M2-like phenotype

Recently, it has been demonstrated that Ado is able to enhance macrophage polarization toward M2 when incubated with IL-4 or IL-13 [28]. We found that macrophages differentiated in the presence of Ado (Supplemental Fig. 1D shows an experimental diagram) presented an upregulation of the M2-related genes *Chi3l3* (which encodes for the lectin Ym1), *Arg1,* and *Mrc1* but not *Retnla* (which encodes for FIZZ1; **Fig. 5A**). Expression of the proinflammatory genes associated with the M1 type of macrophages (*Il1b, Tnfa,* and *Il6*) was not affected when macrophages were differentiated in the presence of Ado (Fig. 5A). The exception to this was *Nos2* (the gene encoding for the inducible form of NOS, iNOS), the expression of which tended to increase when macrophages were differentiated with Ado (Fig. 5A). Ado treatment during differentiation did not change the expression of Ado receptors A_{2A} (*Adora2a*) or A_{2B} (*Adora2b*; Fig. 5B). From all the genes examined, we found that the expression of the M2 marker gene *Chi3l3* quickly decreased during macrophage growth (Fig. 5C), and its expression level inversely correlated with the number of cells during differentiation (Fig. 5C and D).

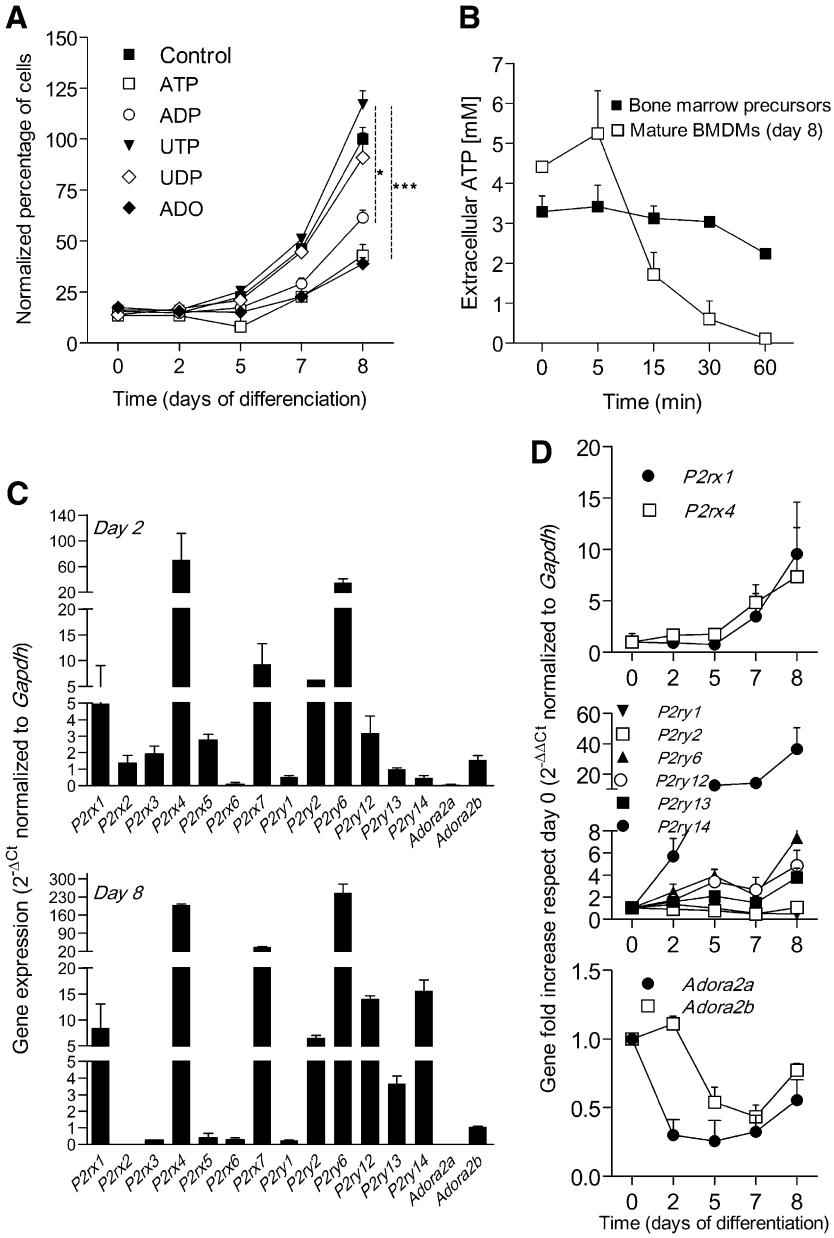

Figure 3. Effect of different extracellular nucleotides on macrophage growth during M-CSF differentiation. (A) Growth of bone marrow precursors measured by MTT assay during 8 d of differentiation with M-CSF and different extracellular nucleotides, as indicated (all at 0.1 mM). D 0 correspond to the day the bone marrow was isolated from the mice and plated. Control corresponds to macrophages not treated with nucleotides. Data presented as mean ± SEM of 3 independent experiments. (B) Degradation of extracellular ATP by bone marrow precursors (d 0, black squares) and mature BMDMs (d 8, white squares). Data presented as mean ± SEM of 3 independent experiments. (C, D) Quantitative PCR expression of different purine receptor genes during macrophage differentiation with M-CSF. Data presented as mean ± SEM of 3 independent experiments. $*P > 0.01$ but < 0.05; $***P < 0.001$.

Macrophages differentiated in the presence of Ado (Supplemental Fig. 1E shows an experimental diagram) were able to further increase the expression of M2-related gene markers after IL-4 treatment and presented with higher expression compared with macrophages differentiated with M-CSF alone (**Fig. 6**). In contrast, macrophages differentiated in the presence of Ado presented with decreased expression of the proinflammatory genes *Il1b, Il6,* and *Nos2* when polarized to M1 with LPS and IFN-γ, suggesting that Ado preferentially conditions macrophages to polarize to the M2 rather than the M1 phenotype.

Recently, it was reported that Ado is able to increase arginase activity during M2 macrophage polarization by IL-4 and IL-13 [28]. Using the experimental approach shown in Supplemental Fig. 1F, we confirmed that Ado increased the *Arg1* expression induced by IL-4 and also validated that Ado and IL-4 treatment on mature macrophages was able to increase the expression of the M2-related gene *Chi3l3* (**Fig. 6**). However, Ado did not change the expression of the M2 gene *Retnla* or *Mrc1* after IL-4 treatment (Fig. 6). In contrast, Ado decreased the expression of proinflammatory genes *Tnfa, Il6,* and *Nos2* in macrophages polarized to M1 by LPS and IFN-γ stimulation (Fig. 6) but increased the expression of the *Il1b* gene (Fig. 6). These results suggest that Ado affect the expression of a specific set of M1- and M2-related genes when the macrophage polarizes.

LPS arrests macrophage growth and induces the expression of *Chi3l3*

Finally, we compared the macrophage growth inhibition induced by LPS [35, 36], a classic M1-polarizing PAMP, with the effect of Ado, an M2-promoting molecule. We found that LPS

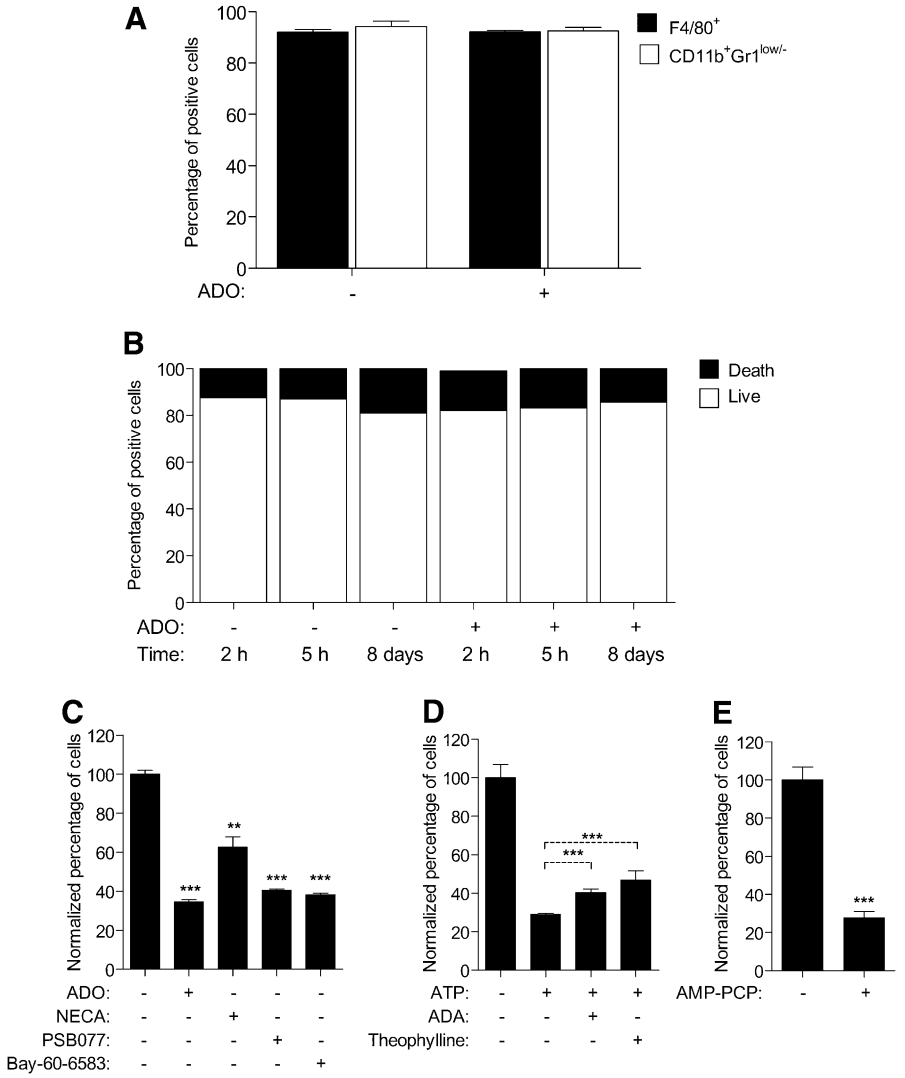

Figure 4. Macrophage growth inhibition by adenosine during M-CSF differentiation. (A) Percentage of F4/80$^+$ or CD11b$^+$Gr1$^{low/-}$ macrophages detected by flow cytometry after 8 d of differentiation with M-CSF in the presence or absence of Ado (1 mM), as indicated. Data presented as mean ± SEM of 3 independent experiments. (B) Percentage of viable and dead cells measured by Live/Dead Fixable Dead Cell Staining kit at different times of differentiation as in (A). Data presented as average of 5 experiments. (C) Growth of bone marrow precursors measured by MTT assay after 8 d of differentiation with M-CSF supplemented with Ado (0.1 mM), NECA (0.1 mM), PSB077 (selective A_{2A} agonist; 1 μM), or Bay-60-6583 (selective A_{2B} agonist, 1 μM). Data presented as mean ± SEM of 5 independent experiments. (D, E) Growth of bone marrow precursors measured as explained in (C), in the presence of AMP-PCP (nonhydrolyzable ATP analog, 0.3 mM; (E) or a combination of ATP (1 mM) and ADA (10 U/ml; (D) or theophylline (nonselective adenosine receptor antagonist, 50 μM; (D). Data presented as mean ± SEM of 4 independent experiments. $^{**}P > 0.001$ but < 0.01; $^{***}P < 0.001$.

arrested macrophage growth to a lesser extent than did Ado (**Fig. 7A**), and the combination of Ado together with LPS was not able to decrease the number of macrophages further than LPS treatment alone (Fig. 7A). As expected, macrophages that were differentiated in the presence of LPS had an increase in the expression of the proinflammatory genes *Il1b, Tnfa, Il6,* and *Nos2* (Fig. 7B). Surprisingly, it also increased the expression of the M2-related genes *Chi3l3* and *Arg1*; however, the expression of both *Mrc1* and *Retnla* was decreased (Fig. 7B). LPS delayed the decrease of *Chi3l3* expression found during macrophage differentiation (Fig. 7C). We then found that the effect of Ado was synergistic with LPS in increasing *Chi3l3* expression (Fig. 7D), but this combination of activators did not affect the expression of any of the other M1 or M2 marker genes analyzed (data not shown). The presence of recombinant Chi3l3 (Ym1) during macrophage differentiation exerted a significant inhibition of macrophage growth, but it was somewhat less than the Ado effect (Fig. 7E).

In summary, we found the expression of the M2-related gene *Chi3l3* (which encodes for Ym1) decreased during macrophage differentiation from bone marrow precursors. When macrophage growth was inhibited during differentiation by Ado (toward M2 polarization) or LPS (toward M1 polarization), the expression of *Chi3l3* was increased. Therefore, Ym1 emerges as a novel marker for macrophage growth inhibition and for the M2 polarization phenotype of mouse macrophages.

DISCUSSION

Most reports studying the effects of ATP and Ado on myeloid cell biology have been conducted on mature macrophages. However, in this study, we examined how purinergic signaling affects macrophage differentiation from bone marrow precursors. We found that both ATP and Ado are able to decrease the number of differentiated macrophages, resulting in fewer mature macrophages. However, macrophages maturated in the presence of Ado presented with upregulation of some M2 markers, including the lectin Ym1 (encoded by the *Chi3l3* gene).

Macrophages are a highly plastic cell type; they adapt to different environments and produce cytokines to regulate the

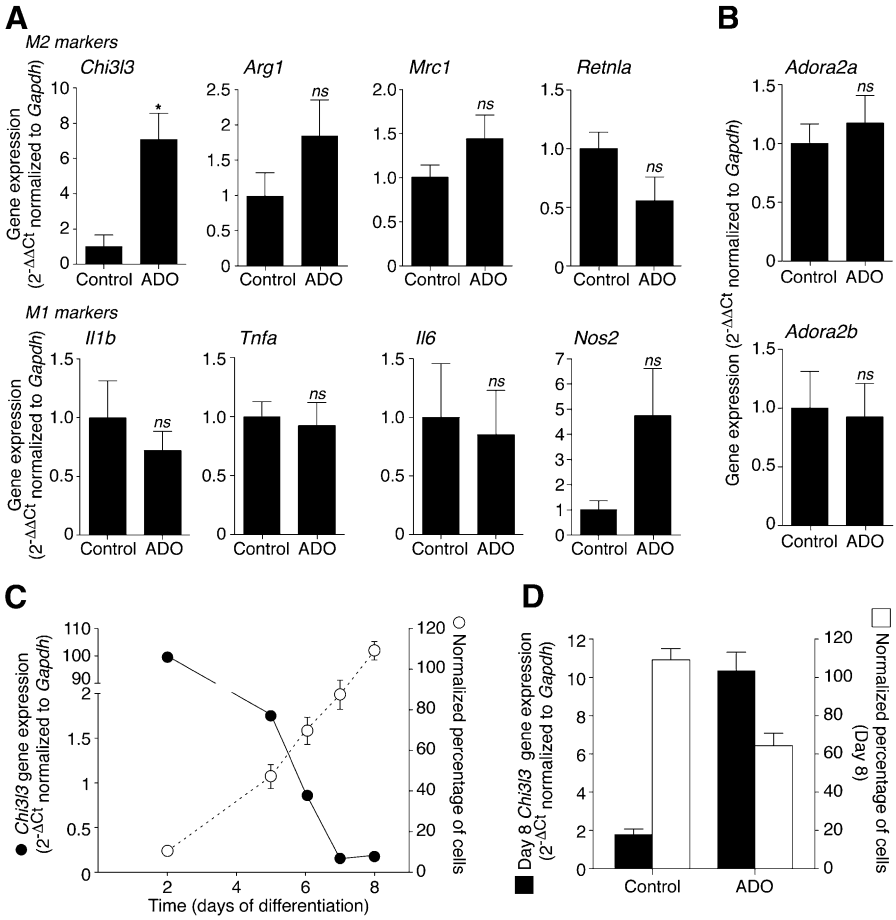

Figure 5. Macrophages differentiated with adenosine result in M2-like polarization. M2 and M1 markers (A) and adenosine receptor (B) gene expression analyzed by quantitative PCR (qPCR) after macrophage differentiation with M-CSF and Ado (0.1 mM), as indicated. Data presented as mean ± SEM of 3 independent experiments. (C) *Chi3l3* gene expression measured by qPCR and percentage of cells determined by MTT assay during macrophage differentiation with M-CSF. Data presented as mean ± SEM of 3 independent experiments. (D) *Chi3l3* gene expression analyzed by qPCR and percentage of cells measured by MTT assay after macrophage differentiation with M-CSF alone (control) or supplemented with Ado (0.1 mM), as indicated. Data presented as mean ± SEM of 3 independent experiments. An experimental approach diagram for the experiments shown in (A) and (B) is shown in Supplemental Fig. 1D. $*P > 0.01$ but < 0.05; ns indicates $P > 0.05$ (not significant).

inflammatory response during both initiation (via M1 macrophage polarization) and resolution (via M2 macrophage polarization) [1, 2]. Ado is able to synergistically increase *Arg1* gene expression when added to mature macrophages, in combination with IL-4 or IL-10, thereby enhancing the M2 macrophage phenotype [28, 37]. Our findings are in agreement with these studies, and we have confirmed that Ado is able to increase the expression of *Arg1* in mature macrophages treated with IL-4. In addition, we found that Ado is able to upregulate *Chi3l3* (Ym1) but not other M2-related genes, such as *Mrc1* (MRC1) or *Retnla* (FIZZ1). In contrast, similar to other studies [38], Ado inhibited the expression of the proinflammatory gene *Tnfa*, but, surprisingly, in our study, we found that *Il1b* gene expression is upregulated by Ado treatment. These results suggest that Ado promotes a characteristic phenotype of macrophage and question the broad anti-inflammatory or M2-promoting effect of Ado arising from studies that measured just one gene as a general marker of the M1 or M2 phenotype [28].

M-CSF macrophage differentiation from mouse bone marrow precursors leads to a resting mature macrophage that can then be polarized toward M1 or M2, depending on the type of priming signal that challenges those mature macrophages [17, 39]. However, this model could be different when bone marrow precursors integrate different signals to increase or decrease the production of macrophages, such as when differentiation occurs within an environment containing extracellular nucleotides (e.g., in the case of total body irradiation for bone marrow transplantation) [40, 41]. Our study found that both ATP and Ado are able to arrest macrophage growth from bone marrow precursors; however, we ruled out the possible effect of ATP activating P2X7R and inducing cell death. We also found that macrophages differentiated in the presence of ATP or Ado were mature macrophages. Moreover, our data found that ATP is quickly degraded by bone marrow precursors and the resulting Ado could explain the inhibition of macrophage proliferation. The use of the selective A_{2A} receptor agonist PSB077 inhibited macrophage proliferation with the same potency as the A_{2B} receptor agonist Bay-60-6583, indicating that Ado signals through both receptors. This is in contrast to a previous study that identified A_{2B} receptor signaling through cAMP production and subsequent $p27^{kip-1}$ expression as inhibiting macrophage proliferation in a PKA-dependent manner [42]. Moreover, in our study, the inhibition of PKA had no effect on Ado arresting macrophage proliferation. Furthermore, the nonhydrolysable ATP analog (AMP-PCP) was able to inhibit macrophage proliferation at the same rate as Ado, and the metabolization of ATP-derived Ado to inosine, by Ado deaminase, or the blocking of Ado receptors with theophylline, achieved only a minimal recovery of the ATP effect on macrophage growth. Taken

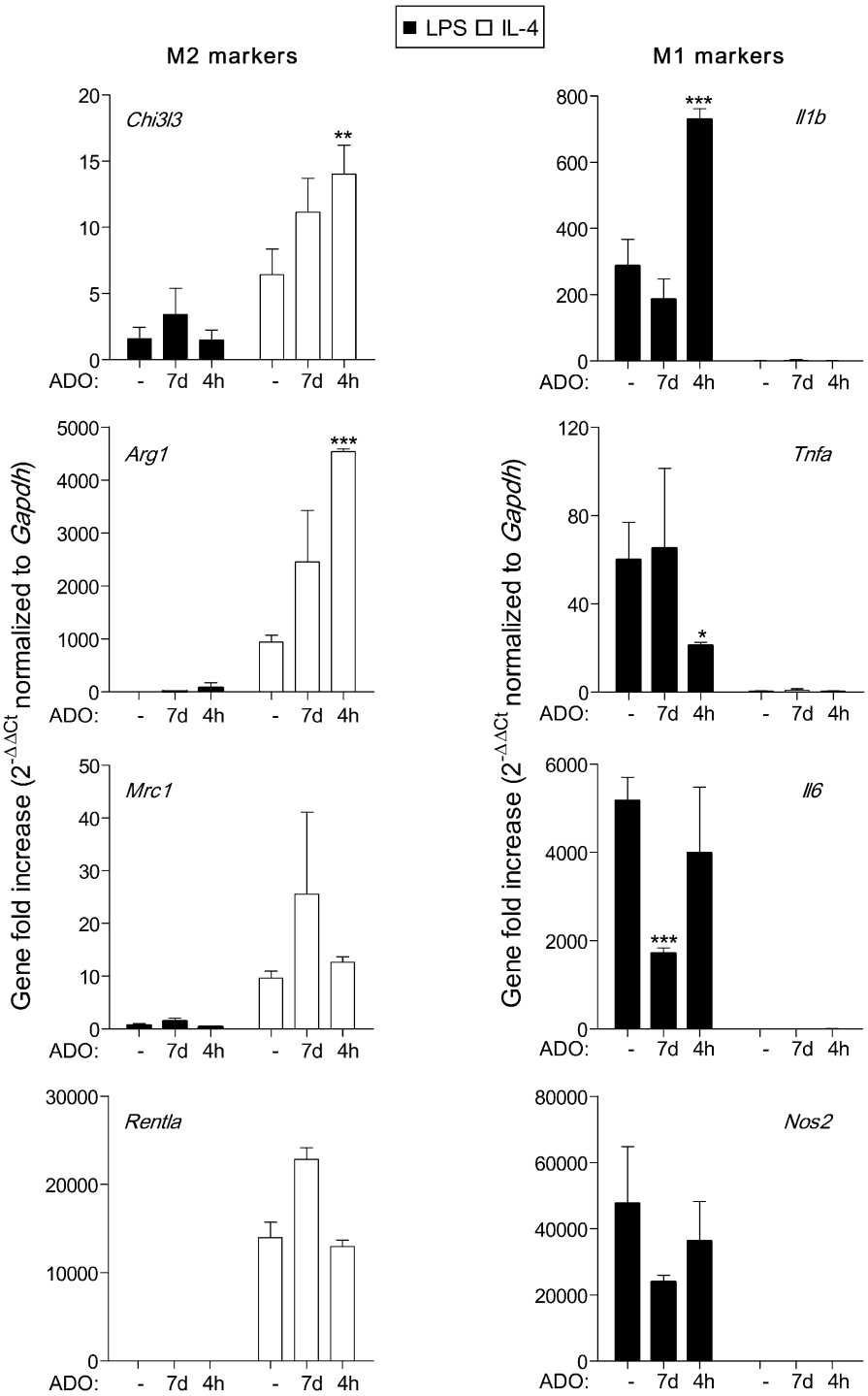

Figure 6. M1 and M2 macrophage polarization is affected by Ado. Gene expression analyzed by quantitative PCR for different M2 and M1 markers after macrophage differentiation with M-CSF in the absence (−) or presence of Ado (0.1 mM), as indicated during 7 d and subsequently polarized to M1 with LPS + IFN-γ (black bars) or to M2 with IL-4 (white bars). Alternatively, BMDMs matured with M-CSF alone were polarized during 4 h to M1 with LPS + INF-γ (black bars) or to M2 with IL-4 (white bars) in the presence of Ado (0.1 mM), as indicated (4 h). Data presented as mean ± SEM of 3 independent experiments. An experimental approach diagram for both experimental conditions is shown in Supplemental Fig. 1E (7-d condition) and 1F (4-h condition). $^{**}P > 0.001$ but < 0.01; $^{***}P < 0.001$.

together, we could not rule out signaling through other P2 receptors or alternative Ado receptors with similar downstream pathways of activation. P2Y and A_2 receptors are both G protein-coupled receptors that recognize ATP (and several other nucleotides, including ADP) or Ado, respectively [43]. Ado receptors work through either inhibition or stimulation of adenylyl-cyclase to decrease or increase intracellular cAMP levels. A_1 and A_3 receptors provoke an inhibitory regulative G-protein-mediated decrease in cAMP levels, and stimulative regulative G-protein receptors of the A_2 family increase cAMP concentrations [44]. Alternatively, A_{2B} could signal through the Gq protein, which activates phospholipase Cβ [45], leading downstream to the increase of cytosolic Ca^{2+}. Several P2Y receptors, such as P2Y1, -2, -4, and -14 follow similar activation pathways through the Gq protein, and these P2Y receptors are activated by either ATP or ADP [43]. However, we were unable to study whether

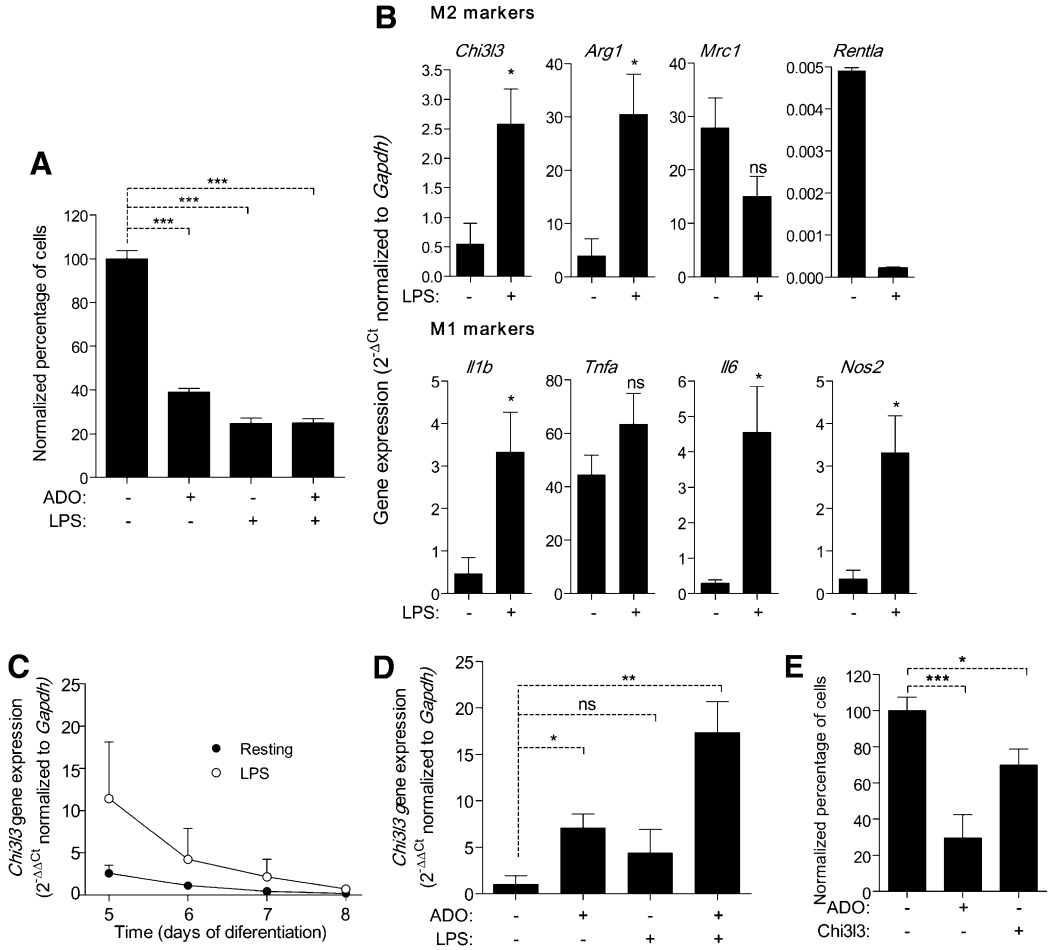

Figure 7. Effect of LPS on macrophage growth and *Chi3l3* expression. (A) Growth of bone marrow precursors measured by MTT assay after 8 d of differentiation with M-CSF and Ado (0.1 mM) or LPS (10 ng/ml), as indicated. Data presented as mean ± SEM of 3 independent experiments. (B) M2 and M1 gene expression analyzed by quantitative PCR (qPCR) after macrophage differentiation with M-CSF and LPS (10 ng/ml), as indicated. Data presented as mean ± SEM of 3 independent experiments. (C) *Chi3l3* gene expression measured by qPCR at d 5, 6, 7, and 8 of macrophage differentiation with M-CSF and LPS (10 ng/ml). Data presented as mean ± SEM of 3 independent experiments. (D) *Chi3l3* gene expression analyzed by qPCR after macrophage differentiation with C-MSF and Ado (0.1 mM) or LPS (10 ng/ml), as indicated. Data presented as mean ± SEM of 3 independent experiments. (E) Growth of bone marrow precursors measured by MTT assay during 8 d of differentiation in the presence of M-CSF and Ado (0.1 mM) or recombinant mouse Chi3l3 protein (Ym1, 500 ng/ml), as indicated. Data presented as mean ± SEM of 3 independent experiments. $*P > 0.01$ but < 0.05; $**P > 0.001$ but < 0.01; $***P < 0.001$; ns indicates $P > 0.05$ (not significant).

an increase in intracellular Ca^{2+} downstream purinergic signaling was involved in macrophage cell growth inhibition, because chelation of intracellular Ca^{2+} per se during the 8 d of differentiation had already blocked the growth of macrophages (data not shown). We studied gene expression for all P2Y receptors during the maturation of hematopoietic precursors to mature macrophages and found that *P2ry2*, *P2ry6,* and *P2ry12* are highly expressed after 2 d of maturation. All the examined purine P2Y receptors, with the exception of *P2ry1* and *P2ry2*, increased their expression during macrophage differentiation. The pharmacological approach could not establish one single purine receptor as being responsible for the arrest of macrophage growth, because different purine receptor antagonists were able to inhibit macrophage growth during differentiation when used in the absence of nucleotides, confirming a recent report showing that some specific Ado receptor antagonists blocked proliferation of immune cells through a receptor-independent effect [34]. Our data suggest that a combination of both A_{2A} and A_{2B} Ado receptors and also other P2 receptors, probably P2Y, signal to inhibit macrophage proliferation. From the different M1 and M2 markers analyzed in our study, macrophages differentiated in the presence of Ado resulted in the upregulation of *Chi3l3*, the gene that encodes for the lectin Ym1, a well-established marker of murine M2 macrophages [8, 17, 46]. Ym1 is a secretory protein synthesized by murine macrophages that binds to heparin and glucosamine [47], and its expression is strongly induced by IL-4 and IL-13 compared with the classic M1 stimulation of murine macrophages [8, 48]. Ym1 expression induced by IL-4 treatment involves the activation of the nuclear factor STAT6 [17].

Although Ym1 expression has been associated with M2 macrophages, it has also been identified both as a protein that

forms crystals in chronically inflamed lungs and associated with macrophage deregulation [49, 50]. In the present study, we found the expression of Ym1 was associated with the inhibition of macrophage proliferation and is induced, not only by the anti-inflammatory signal Ado, but also in response to the proinflammatory signal LPS, which similarly arrests M-CSF macrophage differentiation [35, 36]. The expression of the *Chi3l3* gene was inversely correlated to macrophage proliferation; it was expressed early by bone marrow precursors, with rapidly decreasing expression during macrophage differentiation. These results are in agreement with a study that reported transient expression of Ym1 in early myeloid precursor cells of hematopoietic tissues, initially in the yolk sac and subsequently in the fetal liver, spleen, and bone marrow [51]. We also found that the addition of recombinant Ym1 during macrophage maturation was able to exert a modest, but significant, inhibition of macrophage proliferation during differentiation. Therefore, the Ym1 lectin might be involved in both hematopoiesis and inflammation. It is tempting to speculate that Ym1 production could be involved in the inhibition of macrophage proliferation after purinergic signaling or LPS treatment.

AUTHORSHIP

M.B.-C. and A.B.-M. performed and analyzed experiments and helped with the materials and methods and figure legend writing. P.P. and A.B.-M. wrote the manuscript. P.P. conceived, designed, and supervised this study.

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DISCLOSURES

The authors declare no conflicts of interest.

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