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IFN-γ Prevents Adenosine Receptor (A2bR) Upregulation To Sustain the Macrophage Activation Response

Heather B. Cohen,*†,1 Amanda Ward,*† Kajal Hamidzadeh,*† Katya Ravid,‡ and David M. Mosser*†

The priming of macrophages with IFN-γ prior to TLR stimulation results in enhanced and prolonged inflammatory cytokine production. In this study, we demonstrate that, following TLR stimulation, macrophages upregulate the adenosine 2b receptor (A2bR) to enhance their sensitivity to immunosuppressive extracellular adenosine. This upregulation of A2bR leads to the induction of macrophages with an immunoregulatory phenotype and the downregulation of inflammation. IFN-γ priming of macrophages selectively prevents the induction of the A2bR in macrophages to mitigate sensitivity to adenosine and to prevent this regulatory transition. IFN-γ-mediated A2bR blockade leads to a prolonged production of TNF-α and IL-12 in response to TLR ligation. The pharmacologic inhibition or the genetic deletion of the A2bR results in a hyperinflammatory response to TLR ligation, similar to IFN-γ treatment of macrophages. Conversely, the overexpression of A2bR on macrophages blunts the IFN-γ effects and promotes the development of immunoregulatory macrophages. Thus, we propose a novel mechanism whereby IFN-γ contributes to host defense by desensitizing macrophages to the immunoregulatory effects of adenosine. This mechanism overcomes the transient nature of TLR activation, and prolongs the antimicrobial state of the classically activated macrophage. This study may offer promising new targets to improve the clinical outcome of inflammatory diseases in which macrophage activation is dysregulated. The Journal of Immunology, 2015, 195: 000–000.

IFN-γ represents a key component in chronic inflammatory environments (2). Activated NK cells, invariant NKT, and Th1 cells secrete IFN-γ, which primes macrophages to increase their production of inflammatory cytokines and mediators (6). In this way, IFN-γ plays vital roles in cell-mediated immunity and host defense against intracellular pathogens (7). However, macrophages exhibiting an “IFN signature” are observed in rheumatoid arthritis, multiple sclerosis, and many other autoimmune diseases, indicating that IFN-γ can have a pathogenic role during chronic macrophage activation (8, 9). Although the ability of IFN-γ to enhance the inflammatory potential of TLR-activated macrophages is a well-known phenomenon, how IFN-γ affects the intrinsic regulation of macrophage activation remains to be determined. In this study, we investigated the effect of IFN-γ on TLR-activated macrophages, and we reveal that IFN-γ sustains inflammatory macrophage activation by attenuating their sensitivity to extracellular adenosine. Extracellular adenosine can signal through four different adenosine receptors, designated A1, A3, A2a, and A2b (10). The relative expression of these receptors varies with cell type, anatomic location, and activation state (11). We demonstrate that IFN-γ treatment selectively blocks the induction of the adenosine 2b receptor (A2bR). We also show that IFN-γ inhibition of TLR-induced A2bR prevents completion of an important negative feedback loop, thus rendering macrophages hyperinflammatory. Finally, we demonstrate that overexpression of A2bR on macrophages rescues their ability to respond to adenosine. Thus, we reveal the therapeutic potential of targeting macrophage-specific A2bR to reduce chronic inflammation.

Materials and Methods

Mice and macrophage isolation

C57BL/6 mice were purchased from the Charles River Laboratories. These studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. Bone marrow–derived macrophages were prepared from 6–8-wk-old female C57BL/6, A2ar−/− (Jackson Labs), A2br−/− (12), or Stat1−/− (Taconic) mice and their
FIGURE 1. IFN-γ prolongs the macrophage activation state. (A and C) Cytokine production by LPS-stimulated unprimed (black squares, solid line) or IFN-γ–primed (gray circles, solid line) BMDMs over time. (A) TNF-α and (C) IL-12/23p40 was detected with ELISA. The means ± SEM of three independent experiments are shown. (B and D) Unprimed (black squares, solid line) or IFN-γ–primed (gray circles, solid line) BMDMs were stimulated with LPS and then washed with PBS (break in y-axis) and replaced with LPS-free media and monitored over the next 1–6 h for (B) TNF-α and (D) IL-12/23p40. (E) BMDMs were stimulated with LPS alone or in the presence of increasing doses of POM-1. Eight hours after stimulation, cytokine production was assessed with ELISA for TNF-α (black bars), IL-12/23p40 (dark gray bars), and IL-10 (light gray bars). The means ± SEM of three experiments are shown. (F–I) Unprimed BMDMs (black bars) and IFN-γ–primed BMDMs (gray bars) were stimulated with LPS for 4 h. (F) Il-10, (G) Arg1, (H) Il-33, and (I) Hmox-1 mRNA levels were determined with qPCR. The means ± SEM of at least three independent experiments are shown. (J) WT (black bars) or STAT1−/− BMDMs (striped bars) were left unprimed or primed with IFN-γ and then stimulated with LPS. TNF-α (left) and IL-12/23p40 (right) were measured in the supernatants 8 h after stimulation. Protein was measured by ELISA. The means ± SD of triplicates are shown. **p < 0.01, ***p < 0.001.
respective littermate controls, as described previously (13) and differen-
tiated in 29% L929 (LC14) conditioned media unless noted otherwise.
RAW264.7 cells (TIB-71) were obtained from American Type Culture
Collection (Manassas, VA).

Reagents
ATP and adenosine were purchased from Sigma-Aldrich (St. Louis, MO).
Recombinant mouse IFN-γ was purchased from R&D Systems (Minne-
apolis, MN) and reconstituted according to manufacturer’s directions.
Ultra Pure LPS Escherichia coli K12, Pam3CSK4, heat-killed Listeria
monocytogenes, polyinosinic-polyribopylic acid [Poly(I:C)] low m.w. and
high m.w. ST-FLA, and FSL-1 were purchased from InvivoGen (San
Diego, CA), MRSl754, CGS21680, N-ethyl carboxamidoadenosine (NECA),
and BAY 60-6583 were purchased from Tocris Bioscience (R&D Systems,
Minneapolis, MN).

Stimulation conditions
All in vitro experiments were performed at 1–2 × 10⁶ cells/ml in tissue
culture–treated plates. Mouse macrophage studies were performed in
DMEM/F12+GlutaMax (Life Technologies, Grand Island, NY) supple-
mented with 10% FBS, 1% penicillin–streptomycin, and 1% glutamine
(Life Technologies) unless otherwise indicated. For all experiments,
macrophages were primed for 16 h with 100 U/ml IFN-γ followed by the
addition of 10 ng/ml Ultra-Pure LPS with or without 10⁸ cells/ml heat-
killed L. monocytogenes, 0.5 µg/ml Pam3CSK4, 0.5 µg/ml Poly(I:C) low
m.w. and high m.w., 0.5 µg/ml ST-FLA, or 0.5 µg/ml FSL-1 with or with-
out 10 µM ATP or adenosine unless indicated otherwise.

Gene expression analysis
Total RNA was isolated by using TriZol (Invitrogen, Life Technologies)
and converted to cDNA using the ThermoScript Kit (Invitrogen, Life
Technologies) according to the manufacturer’s protocol. Quantitative PCR
(qPCR) analysis was performed using a LightCycler 480 Real-Time PCR
System (Roche Diagnostics Corporation-Roche Applied Science; Indian-
apolis, IN) and GoTaq qPCR Master Mix (Promega, Madison, WI). Primer
pairs used to amplify specific gene products are listed in Supplemental
Table I. Relative expression levels were calculated using the ΔΔCt method
(14). Gapdh was used as the housekeeping gene for normalization.

Flow cytometry Abs and analysis
Anti-mouse CD16/32 was purchased from AnaSpec (Freemont, CA). Anti-
mouse CD39-AF647 (24DMS1) was purchased from eBioscience (San
Diego, CA). Anti-mouse F4/80-pacific blue (BM8) was purchased from
BioLegend (San Diego, CA). Anti-mouse CD11b-FITC (M1/70) was
purchased from BD Biosciences. Anti-mouse TNFα-APC
(MP6-XT22) was purchased from BD Biosciences. Cell fixation and

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** IFN-γ blocks ATP-induced immunosuppression downstream of eATP hydrolysis. (A and B) Unprimed (black bars) and IFN-γ primed (gray bars) BMDMs were stimulated with LPS in the absence or presence of 10 µM ATP for 8 h. (A) TNF-α and (B) IL-12/23p40 production was measured by ELISA. The means ± SEM of three independent experiments are shown. (C and D) Unprimed (black bars) and IFN-γ–primed (gray bars) BMDMs were stimulated with LPS in the absence or presence of 10 µM adenosine for 8 h. (C) TNF-α and (D) IL-12/23p40 production was measured by ELISA. The means ± SEM of three independent experiments are shown. (E–G) Unprimed BMDMs (black bars) and IFN-γ–primed BMDMs (gray bars) were stimulated with LPS and adenosine (Ado) for 4 h, and mRNA levels were determined with qPCR. The means ± SEM of at least three independent experiments are shown. **p < 0.01, ***p < 0.001.
permeabilization for intracellular staining was performed using the BD Cytofix/Cytoperm fixation/permeabilization kit according to the manufacturer’s protocol. Samples were analyzed with a BD FACSCanto (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**Secreted cytokine detection**

Cytokine production was determined with sandwich ELISA using the following capture and biotinylated detection Ab pairs: anti-mouse TNF-purified (G281-2626), TNF-biotin (MP6-XT3), IL-12/23p40–purified (C15.6), IL-12/23p40–biotin (C17.8), IL-10–purified (JES5-2A5), IL-10–biotin (JES5-16E3).

**ATP hydrolysis assay**

Extracellular ATP (eATP) hydrolysis was detected using the ATPlite Luminescence ATP Detection Assay System (PerkinElmer, Waltham, MA) according to the manufacturer’s protocol but omitting the cell-lysis step (3).

**Inorganic phosphate release assay**

Adenine nucleotide hydrolysis was detected using the BIOMOL Green Reagent (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s protocol.

**Macrophage transfection**

RAW264.7 cells were transfected by nucleofection using the Amaxa Mouse Macrophage Nucleofector Kit (Lonza Cologne, Cologne, Germany) according to manufacturer’s protocol. Briefly, 1 × 10⁶ macrophages were resuspended in 100 µl of Nucleofector solution (Lonza Cologne) and nucleofected using the Nucleofector I Device and program Y-01. Cells were transfected with 2 µg pmaxGFP (Lonza Cologne) or with pEYPF-N1-A2bR plasmid (Addgene, Cambridge, MA). The CMV promoter drives the expression of both cDNAs. Prewarmed media (500 µl) was immediately added to the cuvette and cells transferred to tissue culture plates. Cells were allowed to recover for 24 h, and media was replaced prior to stimulation. For intracellular staining experiments, cells were stimulated for 4 h in the presence of Golgi-Plug per the manufacturer’s protocol (BD Biosciences).

**Statistical analysis**

Data analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA) and analyzed using the Student t test. The statistical differences between groups, with the p values, are indicated in the figures.

**Results**

**IFN-γ sustains inflammatory macrophage responses**

Priming bone marrow–derived murine macrophages with IFN-γ prior to TLR stimulation with LPS results in a heightened proinflammatory response, characterized by the secretion of high levels of TNF-α (Fig. 1A, 1B) and IL-12/23p40 (Fig. 1C, 1D). IFN-γ increased not only the magnitude but also the duration of inflammatory cytokine production by TLR-activated macrophages (Fig. 1B, 1D). To demonstrate this, unprimed or IFN-γ–primed macrophages were stimulated with LPS, and the LPS was removed after 2 h. The macrophages were recultured in fresh LPS-free media, and cytokine production was assessed 1–6 h later. Unprimed macrophages quickly stopped producing TNF-α (Fig. 1B) and IL-12/23p40 (Fig. 1D) following the removal of the activating stimuli. In contrast, IFN-γ–primed macrophages

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**FIGURE 3.** LPS-induced ATP release and hydrolysis is intact in the presence of IFN-γ. (A) Levels of ATP released from LPS-stimulated unprimed (black line) and IFN-γ primed (gray line) RAW264.7 cells were measured over time. The means ± SEM of two independent experiments are shown. (B) Flow cytometry analysis of CD39 surface expression (black histograms) on unprimed (left) and IFN-γ primed (right) BMDMs compared with isotype controls (gray histograms). Data are representative of three independent experiments. (C) The hydrolysis of 500 µM ATP by unprimed BMDMs (black squares, solid line) over time compared with IFN-γ–primed BMDMs (gray circles, solid line). Percent of hydrolysis was compared with levels of ATP detected in the absence of cells and determined by the ATPlite assay. The means ± SEM from three independent experiments are shown.
continued to secrete TNF-α and IL-12/23p40 with little reduction in the amounts secreted after the LPS had been removed from macrophage cultures. This prolonged cytokine production by macrophages following IFN-γ priming was similar to a phenotype we recently observed when macrophages lacking CD39 (an ectonucleoside triphosphate diphosphohydrolase) were stimulated with TLR ligands (3). Treating macrophages with POM-1, a CD39 inhibitor, resulted in a dose-dependent increase in the production of TNF and IL-12 and a decrease in IL-10 production (Fig. 1E), analogous to what we observed following IFN-γ priming.

In the absence of IFN-γ priming, TLR-stimulated macrophages gradually transition from an inflammatory (M1) phenotype into an anti-inflammatory, macrophage capable of regulating innate immune responses. These macrophages upregulate a number of immunoregulatory transcripts not expressed by M1 macrophages (3, 15). The priming of macrophages with IFN-γ prior to TLR stimulation prevents the production of regulatory transcripts that accompany this transition to regulatory macrophages. IFN-γ–primed macrophages show reduced levels of transcripts for Il-10, Arg1, Il-33, and Hmox-1 (Fig. 1F–I). IFN-γ is well known to signal through STAT1 (16). To determine whether STAT1-dependent signaling was necessary for enhanced cytokine production, macrophages from Stat1-deficient mice were examined and compared with wild type (WT) mice. Priming with IFN-γ failed to prolong inflammatory cytokine responses and did not result in an increase in TNF-α or IL-12/23p40 (Fig. 1J) in Stat1-deficient mice. Collectively, these results suggest that IFN-γ delays the normal transition that macrophages make from an inflammatory TLR-stimulated (M1) macrophage to an anti-inflammatory regulatory macrophage.

**ATP hydrolysis is intact in IFN-γ–primed macrophages**

In view of the above findings, we hypothesized that IFN-γ priming might inhibit a component of the macrophage purinergic autorregulatory mechanism we described recently (3). To determine whether IFN-γ prevented macrophage responses to ATP, macro-

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**FIGURE 4.** IFN-γ preferentially inhibits LPS-induced A2bR expression in macrophages. (A) mRNA expression of A1r (dotted bars), A2ar (horizontally striped bars), A2br (diagonally striped bars), and A3r (solid black bars) in unprimed and IFN-γ–primed BMDMs stimulated for 4 h. The means ± SEM of three independent experiments are shown. (B and D) Unprimed BMDMs (black bars) and IFN-γ–primed BMDMs (gray bars) were stimulated with the indicated TLR agonists for 4 h. (B) A2bR and (D) A2aR mRNA levels were determined by qPCR. The means ± SEM of at least three independent determinations are shown. (C) Unprimed BMDMs (black bars) and IFN-γ–primed BMDMs (gray bars) were stimulated with LPS over time. A2bR mRNA levels were determined by qPCR. The means ± SEM of two independent experiments are shown. (D) Unprimed BMDMs (black bars) and IFN-γ primed BMDMs (gray bars) were stimulated with the indicated TLR agonists for 4 h. A2bR mRNA levels were determined by qPCR. The means ± SEM of at least three independent determinations are shown. (E) WT BMDMs (black bars) or STAT1−/− BMDMs (striped bars) were left unprimed or IFN-γ primed, and then stimulated with LPS for 4 h. A2bR mRNA levels were determined by qPCR. The means ± SEM of at least three independent determinations are shown. *p < 0.05, **p < 0.01, ***p < 0.001.
phages were stimulated with LPS in the presence or absence of exogenous ATP, and cytokine production was measured the following day. ATP (10 μM) significantly reduced the amount of TNF-α (Fig. 2A) and IL-12/23p40 (Fig. 2B) secreted by macrophages stimulated with LPS alone. IFN-γ priming of macrophages negated the immunosuppressive effect of ATP. These cells continued to secrete high levels of TNF-α (Fig. 2A) and IL-12/23p40 (Fig. 2B) despite the presence of ATP. Similar studies were performed to determine whether IFN-γ renders macrophages less sensitive to extracellular adenosine. Macrophages were stimulated with LPS in the presence or absence of adenosine (10 μM), and LPS-induced TNF-α (Fig. 2C) and IL-12/23p40 (Fig. 2D) production by macrophages was measured. Similar to extracellular ATP, the attenuation of inflammatory cytokine secretion by adenosine was decreased by priming cells with IFN-γ. We previously demonstrated that the addition of adenosine to resting macrophages resulted in the rapid induction of several transcripts specific to regulatory macrophages. Priming macrophages with IFN-γ prevented this induction. Transcripts for IL-10 (Fig. 2E), IL-33 (Fig. 2F), and Arg1 (Fig. 2G) remained low in IFN-γ–primed macrophages, indicating that under these conditions the cells failed to transition to an immunoregulatory activation state.

The release of ATP and its hydrolysis to bioactive adenosine was then examined in IFN-γ–primed macrophages. Only negligible amounts of ATP were detected in the supernatants of primary macrophages following LPS stimulation, because these cells express high levels of CD39, which rapidly hydrolyzes ATP to AMP (3). Therefore, we performed this analysis on RAW264.7 cells that express low levels of CD39 (3). RAW264.7 cells released ATP following TLR stimulation, and the amount of ATP released was essentially unchanged by priming with IFN-γ (Fig. 3A). Next, the level of CD39 expressed on the surface of primary macrophages exposed to LPS alone was compared with expression on cells following IFN-γ priming. CD39 remained highly expressed by

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**FIGURE 5.** Specific antagonism of the A2bR recapitulates IFN-γ–mediated adenosine insensitivity. (A and B) Unprimed (black bars) and MRS1754–primed (gray striped bars) BMDMs were stimulated with LPS in the absence or presence of 10 μM adenosine for 8 h. (A) TNF-α and (B) IL-12/23p40 production was measured by ELISA. The means ± SEM from at least three independent experiments are shown. (C) Unprimed (black squares, solid line), IFN-γ–primed (gray circles, solid line), and MRS1754–primed (10 μM; gray open squares, dotted line) BMDMs were stimulated with LPS in the presence or absence of adenosine at various concentrations. Eight hours after stimulation, TNF-α levels were detected by ELISA. The percent of TNF-α produced is based on TNF-α levels produced by LPS alone for each condition. The means ± SEM from at least three independent experiments are shown. (D and E) Unprimed (black bars) and IFN-γ–primed (gray bars) BMDMs were stimulated with LPS in the absence or presence of 1 nM of the A2bR-specific agonist (D) NECA or (E) BAY 60-6583. TNF-α production was measured 8 h after stimulation by ELISA. Percent of TNF-α produced is based on TNF-α levels produced by LPS alone for each condition. The means ± SEM from three independent experiments are shown. **p < 0.01, ***p < 0.001.
macrophages regardless of culture conditions and was actually slightly upregulated by IFN-γ (Fig. 3B). We next examined the effect of IFN-γ on ecto-ATPase activity and observed similar rates of eATP hydrolysis between unprimed and IFN-γ primed macrophages (Fig. 3C). These data indicate that the ability of TLR-stimulated macrophages to release and hydrolyze eATP remains intact in the presence of IFN-γ. Together, these data suggest that the inability of IFN-γ–primed macrophages to transition readily to an anti-inflammatory (regulatory) state occurs downstream of eATP hydrolysis and is caused by a decreased ability of these macrophages to respond to adenosine.

**IFN-γ inhibits LPS-induced A2bR expression in macrophages**

Adenosine receptor expression on LPS-stimulated macrophages was examined in the presence or absence of IFN-γ priming. In unprimed macrophages, LPS dramatically upregulated A2bR transcript levels relative to naive unstimulated macrophages (Fig. 4A). This upregulation was specific to A2bR transcripts, because transcripts for the other three adenosine receptors were not significantly increased in response to LPS stimulation (Fig. 4A). This phenomenon was also observed in macrophages stimulated with a variety of other TLR agonists, including Pam3CSK4, HKLM, Poly(I:C), and bacterial flagellin, suggesting that the induction of A2bR in macrophages is not specific to TLR4 signaling (Fig. 4B).

The induction of the A2bR by LPS stimulation was severely inhibited by priming cells with IFN-γ (Fig. 4A). IFN-γ specifically inhibited A2bR expression on inflammatory macrophages and did not affect expression of the other adenosine receptors. Transcripts for A2bR were not simply delayed by IFN-γ priming, but inhibited for the entire 8-h observation period (Fig. 4C). In contrast to A2bR, the expression of A2aR transcripts encoding the A2aR were not decreased after IFN-γ priming, regardless of the stimulation (Fig. 4D). Adenosine receptor expression on LPS-stimulated macrophages after priming with IFN-γ was examined in WT macrophages and compared with macrophages lacking Stat1. The treatment of Stat1-deficient macrophages with IFN-γ did not result in a significant decrease in A2bR expression, whereas A2bR receptor expression on WT macrophages was substantially diminished (Fig. 4E). Thus, IFN-γ signals through STAT1 to inhibit the expression of A2bR selectively in macrophages exposed to TLR4 stimuli. These data suggest that IFN-γ may interfere with macrophage purinergic autoregulation by decreasing the sensitivity of macrophages to extracellular adenosine.

**IFN-γ inhibits A2bR-dependent immunosuppression**

We hypothesized that IFN-γ inhibits the induction of A2bR to maintain an inflammatory activation state. To address the functional significance of A2bR during macrophage activation, macrophages were treated with the A2bR-specific antagonist MRS1754 (17) and then exposed to LPS in the presence or absence of adenosine. Unprimed macrophages were highly sensitive to adenosine, and they exhibited marked reduction in LPS-induced TNF-α (Fig. 5A, black bars) and IL-12/23p40 production (Fig. 5B, black bars). The effect of adenosine was completely dependent on the A2bR, because macrophages treated with MRS1754 demonstrated no significant difference in TNF-α or IL-12/23p40 levels regardless of adenosine being present in their extracellular milieu (Fig. 5A, B, striped bars). Thus, A2bR antagonism by MRS1754 abrogates adenosine responsiveness, to levels similar to those observed after IFN-γ treatment. The sensitivity of macrophages to adenosine was directly measured by exposing unprimed or IFN-γ–primed macrophages to increasing doses of adenosine. On unprimed cells, adenosine suppressed LPS-induced TNF-α in a dose-dependent manner, indicating that macrophages are sensitive to low levels of extracellular adenosine (Fig. 5C). Macrophages treated with MRS1754 or primed with IFN-γ were less sensitive to adenosine, decreasing cytokine secretion by less than 30% in the presence of 10 μM adenosine (Fig. 5C), indicating that IFN-γ impairs adenosine-mediated immunosuppression via A2bR signaling blockade.

To examine the role of A2bR-dependent signaling in modulating cytokine production by macrophages, cells were exposed to the A2bR-specific agonist BAY 60-6583 and the A2-type adenosine receptor selective agonist NECA. TNF-α was measured. Unprimed macrophages stimulated with LPS and either of the A2bR agonists secreted 70–80% less TNF-α relative to macrophages exposed to LPS alone (Fig. 5D, E). Macrophages treated with IFN-γ how-

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**FIGURE 6.** IFN-γ inhibits A2bR-dependent immunosuppression in macrophages. (A and B) Unprimed (black bars) and IFN-γ–primed (gray bars) WT (A) or A2bR<sup>−/−</sup> (B) BMDMs were stimulated with LPS in the absence or presence of 10 μM adenosine for 8 h. TNF-α production was measured by ELISA. The means ± SEM from at least three independent experiments are shown. (C and D) Unprimed (black bars) and IFN-γ–primed (gray bars) WT (C) or A2aR<sup>−/−</sup> (D) BMDMs were stimulated with LPS in the absence or presence of 10 μM adenosine (Ado) for 8 h. TNF-α production was measured by ELISA. The means ± SEM from three independent experiments are shown. *p < 0.05, ***p < 0.001.
ever, largely retained (70%) their ability to secrete TNF-α, even in the presence of NECA (Fig. 5D) or BAY 60-6583 (Fig. 5E).

The ability of adenosine to attenuate TNF-α production in bone marrow–derived macrophages (BMDMs) from A2br-deficient mice was examined. Unprimed macrophages from WT mice were sensitive to 10 μM adenosine, decreasing TNF-α by ~50% (Fig. 6A, black bars). Unprimed A2br-deficient BMDMs, in contrast, were not responsive to adenosine. Stimulation of these cells with LPS in the presence of adenosine resulted in TNF-α production that was comparable to that from stimulation in the absence of adenosine (Fig. 6B, black bars). As controls, unprimed A2ar-deficient BMDMs resembled their WT littermate counterparts (Fig. 6C, black bars) and were sensitive to adenosine-mediated suppression of TNF-α production (Fig. 6D, black bars). Priming macrophages with IFN-γ decreased A2bR expression on WT macrophages and decreased adenosine sensitivity. Thus, after IFN-γ priming, WT macrophages resembled A2br-deficient cells, and TNF-α production returned to levels comparable to LPS stimulation alone (Fig. 6, gray bars). Together, these data indicate that IFN-γ hinders the induction of the A2bR, the major adenosine receptor responsible for regulating inflammatory cytokine production in TLR-stimulated macrophages.

A2br overexpression permits the development of immunosuppressive macrophages in the presence of IFN-γ.

TNF-α production was measured with intracellular staining and flow cytometry in RAW264.7 cells in which the A2bR was overexpressed. Resting, nonstimulated cells produced minimal TNF-α, regardless of their transfection status (Fig. 7A, left two panels). Priming cells with IFN-γ and stimulating them with LPS resulted in high TNF-α production. IFN-γ–primed cells that were transfected with control plasmid failed to respond to exogenous adenosine and continued to make high levels of TNF-α (Fig. 7A, right upper panel). In contrast, IFN-γ–primed macrophages
transfected with pA2bR were more sensitive to adenosine. The transfected cells (EYFP+) produced lower levels of TNFα in response to exogenous adenosine, whereas the EYFP− nontransfected cells continued to make comparable amounts of TNF-α (Fig. 7A, bottom right panel). The degree of TNF-α attenuation correlated with the level of A2bR expression. Macrophages overexpressing the A2bR secreted, on average, 40–50% less TNF-α compared with macrophage transfected with the GFP containing plasmid alone (Fig. 7B, 7C). These results demonstrate that the overexpression of A2bR in macrophages improves their ability to sense extracellular adenosine and to suppress inflammatory cytokine production. These data reveal that IFN-γ interferes with the autoregulation of macrophages after TLR activation by specifically attenuating A2bR-mediated responses, resulting in prolonged inflammatory responses.

Discussion

Although macrophage activation is a necessary component of host defense, if left uncontrolled it can lead to tissue damage, organ failure, and even death. Prolonged macrophage activation has been associated with an extensive list of chronic inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis, and a variety of macrophage activation syndromes (9, 18). However, the molecular mechanisms governing the regulation of macrophage activation under these pathologic conditions remains incompletely understood. In this study, we reveal a novel mechanism wherein IFN-γ interferes with the macrophage’s intrinsic ability to control its activation state, and demonstrate that IFN-γ actively desensitizes macrophages to immunosuppressive adenosine, thereby sustaining inflammatory responses.

This work demonstrates that IFN-γ inhibits a recently described purinergic autoregulatory loop that is initiated upon TLR ligation of macrophages. We previously demonstrated that macrophages secrete ATP and rapidly convert ATP to adenosine to autoregulate their activation state (3). That work suggested that TLR stimulation of macrophages was a transient event, followed by a transition to an immunoregulatory phenotype. The deletion or inhibition of the ecto-ATPase, CD39, prevented this transition and rendered macrophages hyperinflammatory because of an inability to convert ATP into adenosine. In this study, we reveal that this autoregulatory loop depends on the adenosine A2bR and that IFN-γ blocks the transition to an immunosuppressive state by preventing A2bR signaling.

Extracellular adenosine can signal through four receptors, designated A1, A3, A2a, and A2b (19). All adenosine receptors are G protein–coupled receptors; however, only the A2aR and A2bR are coupled to GoS, thus inducing the generation of intracellular cAMP upon stimulation (20, 21). We have previously demonstrated that the accumulation of intracellular cAMP can drive the development of anti-inflammatory macrophages (22). Because of the lower affinity of the A2bR to adenosine compared with the A2aR (23), it has also been proposed that the A2bR may be preferentially used by cells during pathophysiologic diseases when extracellular adenosine levels increase 10–100-fold above homeostatic conditions. Indeed, our work demonstrating the selective upregulation of the A2bR upon TLR stimulation is consistent with other reports showing that inflammatory TNF-α and hypoxia can induce the A2bR (24–26). Thus, these data collectively support a role for A2bR in mediating a negative feedback loop to prevent overactive inflammation.

The mechanisms by which the A2bR is regulated at the transcriptional level in macrophages remain poorly understood. Our current study shows that IFN-γ inhibits the development of regulatory macrophages after TLR activation by inhibiting A2bR upregulation following TLR ligation. Previous studies in other cell types have pointed to a link between IFN-γ and adenosine signaling (27); however, the molecular mechanism governing IFN-γ–mediated A2bR suppression in macrophages remains elusive. It has been shown, for example, that IFN-γ downregulates A2bR signaling in intestinal epithelial cells, leading to an inhibition of CREB phosphorylation and protein kinase A activation. It has also been shown that smooth muscle cells can signal through the A2bR to induce the downregulation of IFN-γ–induced major histocompatibility class II expression (27). A2bR signaling appears to promote anti-inflammatory macrophages; however, other studies have associated A2bR with inflammatory roles in disease models such as asthma, thus demonstrating diverse and perhaps tissue-specific roles for A2bR in the regulation of inflammation (28).

It is interesting that we observed increased A2br levels in macrophages exposed to TLR ligands or in response to exogenous purinergic nucleotides (data not shown). This observation suggests that adenosine signaling itself may positively regulate the expression of the A2bR. Indeed, putative CREB, IFN-γ–activated sequence (GAS), and specificity protein 1 (Sp1) binding sites can be found in close proximity to the transcriptional start site of A2br. Both CREB and Sp1 have been shown to play roles in the induction of many regulatory macrophage-associated genes, including HB-EGF and IL-10 (22, 29). Other potential mechanisms of A2bR regulation are not excluded (30). For example, in smooth muscle cells, TNF-α induced by LPS stimulation upregulates the A2br gene promoter, suggesting an effect via NF-κB sites within this gene promoter (25). It is not clear however, how IFN-γ inhibits A2br gene expression induced by TLR. Regardless of the transcriptional mechanism of control, it is interesting to speculate that the A2br may be included as a novel genetic marker of immunoregulatory macrophages.

This work points to the therapeutic potential of manipulating macrophage A2bR signaling to influence inflammation. Strategies to overexpress A2bR on macrophages may represent a novel approach to treat chronic inflammatory diseases, whereas A2bR downregulation may enhance host defense to intracellular infections. It will be valuable to assess the effects of macrophage-A2bR expression in vivo. High TNF-α and IFN-γ levels often accompany chronic inflammatory disorders (31). Moreover, extracellular adenosine levels have been demonstrated to increase 100-fold during chronic inflammatory conditions, such as rheumatoid arthritis (32). Thus, our results showing that CD39 activity is intact in the presence of IFN-γ are consistent with these observations. In this study, we show that IFN-γ potentiates the release of these inflammatory cytokines from macrophages by blocking induction of the anti-inflammatory receptor, A2bR. We demonstrate that preserving or overexpressing the A2bR signaling in macrophages may reverse the inflammatory phenotype of macrophages exposed to IFN-γ. Thus, targeting A2bRonts on macrophages may enhance our current arsenal of treatment options for chronic inflammatory diseases.

Disclosures

D.M.M. is the chief executive officer of a company that is developing therapeutics to mitigate macrophage inflammatory responses. The other authors have no financial conflicts of interest.

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