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*J Immunol* 2015; 195:1436-1448; Prepublished online 6 July 2015;
doi: 10.4049/jimmunol.1402039
http://www.jimmunol.org/content/195/4/1436
Adenosine Modulates NR4A Orphan Nuclear Receptors To Attenuate Hyperinflammatory Responses in Monocytic Cells

Daniel Crean,*† Eoin P. Cummins,† Bojlul Bahar,* Helen Mohan,* Jason P. McMorrow,* and Evelyn P. Murphy*†

Adenosine receptor–mediated regulation of monocyte/macrophage inflammatory responses is critical in the maintenance of tissue homeostasis. In this study, we reveal that adenosine potently modulates the expression of NR4A1, 2, and 3 orphan nuclear receptors in myeloid cells, and this modulation is primarily through the adenosine A2a receptor subtype. We demonstrate that A2a receptor activation of NR4A1-3 receptor synthesis is further enhanced in TLR4-stimulated monocytes. After TLR4 stimulation, NR4A receptor–depleted monocyte/macrophage cells display significantly altered expression of cell-surface markers and produce increased inflammatory cytokine and chemokine secretion rendering the cells an enhanced proinflammatory phenotype. Exposure of TLR4 or TNF-α–stimulated monocytes to adenosine analogs directs changes in the expression of MIP-3α and IL-23p19, with NR4A2 depletion leading to significantly enhanced expression of these factors. Furthermore, we establish that nuclear levels of NF-κB/p65 are increased in TLR/adenosine-stimulated NR4A2-depleted cells. We show that, after TLR/adenosine receptor stimulation, NR4A2 depletion promotes significant binding of NF-κB/p65 to a κB consensus binding motif within the MIP-3α proximal promoter leading to increased protein secretion, confirming a pivotal role for NF-κB activity in controlling cellular responses and gene expression outcomes in response to these mediators. Thus, these data demonstrate that during an inflammatory response, adenosine modulation of NR4A receptor activity acts to limit NF-κB–mediated effects and that loss of NR4A2 expression leads to enhanced NF-κB activity and hyperinflammatory responses in myeloid cells. The Journal of Immunology, 2015, 195: 1436–1448.

Adenosine is an endogenous purine nucleoside, which elicits profound anti-inflammatory or proinflammatory responses dependent on the local cellular environment and the presence or absence of inflammatory milieu. The accumulation of adenosine occurs through an array of processes including intracellular and extracellular generation, and release from necrotic cells, endothelial cells, and infiltrating neutrophils at sites of cellular stress/inflammation (1). Once produced, adenosine performs its actions through one or more of its four cell membrane G protein–coupled adenosine receptors A1, A2a, A2b, and A3 (2–4). These receptors bear varied affinities for adenosine, with A1, A2a, and A3 exhibiting high affinity, whereas A2b displays low affinity for the nucleoside (1). Furthermore, expression levels of each receptor can fluctuate because of cell type, cell differentiation point, stage of inflammation, and inflammatory mediators present (5).

Monocyte and monocyte-derived macrophage cells orchestrate inflammatory responses by a variety of means, including, but not limited to, secreting numerous cytokine and chemokine mediators, which recruit additional myeloid and other immune cells such as T cells to the site of inflammation (5, 6). Human monocytes express all four adenosine receptors, and it is appreciated that the dominant receptor in these cells is the A2a subtype, which is potently and selectively induced in response to inflammatory mediators such as LPS (7, 8). Thus, adenosine receptor activation can alter monocyte/macrophage cytokine and chemokine production and ultimately shape inflammatory outcome (5).

Selective activation of the A2a receptor in macrophage cells attenuates LPS-driven proinflammatory mediators in vitro and in vivo including TNF-α (7–9). However, it is also established that A2b receptors may also contribute to adenosine-dependent TNF-α suppression (5). Adenosine has further been shown to alter chemokine receptor expression on macrophage cells, thereby affecting their migration ability (10). Proinflammatory and anti-inflammatory responses elicited by monocyte/macrophage cells are equally important, and it is the appropriate regulation or fine-tuning of these responses that brings about inflammatory resolution (6, 11). Hence the combination of malleable immune cells with dynamic adenosine receptor expression levels demonstrates a high degree of cellular plasticity and highlights the central role adenosine receptor–mediated responses play in controlling tissue homeostasis.

The NR4A1-3 orphan nuclear receptors are a subfamily of early response regulators that have emerged as key regulators of inflammatory processes required in inflammatory disease initiation and progression, controlling the magnitude of the inflammatory response (11–17). The NR4A family member, NR4A1, is pivotal in monocyte cell differentiation, polarization, and T cell homeostasis (15–18). NR4A1-deficient monocytes are polarized toward a more proinflammatory phenotype displaying enhanced TLR signaling with increased NF-κB signaling and increased expression of MHC class II differentiation surface marker (16). The second family member, NR4A2, acts as a critical receptor in controlling a feedback mechanism limiting/modulating NF-κB activity in microglia and astrocytes.
during chronic inflammatory events (13). Thus, adenosine and NR4A receptors are known to control differentiation and inflammatory processes in cells of myeloid origin. We and others have shown that adenosine receptor stimulation can potently alter expression of NR4A family members in human synovioicyte, endothelial, mast, and monocyte cells (19–21). However, the transcriptional controls directed by NR4A receptors downstream of adenosine signaling in myeloid cells remain unknown and are the object of this study.

In this article, we demonstrate for the first time, to our knowledge, that in monocytic cells, adenosine potently upregulates NR4A1, 2, and 3 gene and protein expression, primarily through the A2a receptor. NR4A2 depletion significantly alters expression of classical (M1) proinflammatory phenotypic surface markers, and NR4A2/3 depletion significantly alters this expression pattern. Addition of 5′-ethylcarboxamidoadenosine (NECA), a stable adenosine analog, significantly attenuates IFN-γ + LPS-induced reduction of CD80, whereas reducing CCR7 expression. NR4A2 or NR4A3 depletion does not effect this NECA-mediated reduction in CCR7 or CD80 expression. However, NECA potently augments the expression of mediators including MIP-3α and IL-23p19, in primary and immortalized human monocytic cells primed using LPS or TNF-α. In NR4A2-depleted cells, NECA-dependent modulation of these mediators is further enhanced. We establish that NECA-induced NR4A2 activity can act as a feedback mechanism to regulate NF-κB signaling/activity and subsequent expression of target genes, MIP-3α and IL-23p19. Finally, we show that, after adenosine receptor stimulation, NR4A2 depletion promotes significantly enhanced binding of NF-κB/p65 to a κB binding motif on the MIP-3α promoter leading to increased protein secretion. Taken together, this supports the observation that NR4A2 activity is central to maintaining an appropriate feedback mechanism via NF-κB. Thus, NR4A2 functions as an important regulator of adenosine signaling by modulating NF-κB activity leading to subsequent changes in expression of several genes involved in controlling immune cell homeostasis.

Materials and Methods

Cell culture and treatments

Human monomeric THP-1 and murine macrophage RAW 264.7 cells obtained from American Type Culture Collection (ATCC TIB-202 and TIB-71) were cultured in RPMI 1640–GlutaMax media (Life Technologies) and DMEM 6546 (Sigma-Aldrich), respectively, and supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stable knockdown cells were maintained in an additional 5 μg/ml puromycin dihydrochloride (Sigma-Aldrich) (21). THP-1 cells were differentiated using 20 ng/ml PMA (Sigma-Aldrich) for 48 h before manipulation.

Cells primed with TNF-α (R&D Systems) or LPS (Sigma-Aldrich) for 18 h followed by addition of adenosine receptor agonists for 2–4 h. Appropriate controls include TNF-α or LPS treatment for 18 h followed by an additional 2–4 h with or without agonists (total 20–22 h; Tocris). NF-κB pathway inhibition was performed using pretreatment for 1 h with 10 μM BAY 11-7082 (NF-κB; Merck). Adenosine receptor–specific antagonists were used by pretreating for 30 min with specific antagonists (Tocris) toward each adenosine receptor (A1, A2a, A2b, and A3) at a final concentration of 0.5 μM.

Primary cell isolation

Peripheral venous blood was collected from healthy volunteers at St. Vincent’s University Hospital, Dublin, Ireland. Institutional Review Board approval was obtained from the local Ethics Committee at St. Vincent’s University Hospital, and written, informed consent was obtained from all volunteers. Twenty milliliters blood was collected into vacuum tubes (Becton Dickinson), inverted slowly, and allowed to stand for 15 min at room temperature. Blood was layered slowly onto polymorph prep solution (1:1) and centrifuged at 500 × g at 20°C for 35 min with the brakes off. The mononuclear layer was removed and mixed with equal volumes of 0.45% NaCl and centrifuged at 400 × g at 20°C for 10 min. Supernatant was discarded and pellet was resuspended in 1 ml ice-cold water and mixed by inverting gently for 1 min followed by the addition of 12 ml 1.8% NaCl and subsequent centrifugation at 300 × g at 20°C for 5 min. Cells were resuspended in media, counted, and seeded at a density of 2.5 × 10^6 cells/ml for total RNA isolation.

Real-time quantitative RT-PCR

RNA was extracted from cells using the column-based E.Z.N.A total RNA extraction kit (Omega Bio-Tek, Norcross, GA) followed by cDNA synthesis as previously reported (22). Real-time quantitative RT-PCR was performed using Sybr Green master mix (Applied Biosystems) and an ABI 7300 thermocycler (Applied Biosystems). Primer pair sequences used were human GAPDH (forward: 5′-CCCTCCATCGTTCTCTGA-3′, reverse: 5′-CCCCATGGTTCTGAGGCG-3′); IL-1β (forward: 5′-GACCCATCAGGTGGTGAGTTCTGAC-3′, reverse: 5′-GCACTACCACTCATGTGCGG-3′); TNF-α (forward: 5′-CCAAGGCCTTTGCAGGAAAC-3′, reverse: 5′-GCGATGCTTCTCAGGGCTG-3′); CCL2 (forward: 5′-ATTTCAGTCTCAAGGCACTAC-3′, reverse: 5′-AAATCTGGCCTGACATG-3′); GUSB (forward: 5′-TTTGGACGAGATTCTTCTCTC-3′, reverse: 5′-GGTCTGCTTCCTTGCG-3′); IL-10 (forward: 5′-GCAATGGCTCTAGCTAAGG-3′, reverse: 5′-TTGCTGCTAGGAGGCACTG-3′); MIP-1α (forward: 5′-AAATCTGGCCTGACATG-3′, reverse: 5′-GGTCTGCTTCCTTGCG-3′); IL-23 p19 (forward: 5′-AAGGAGCTTCCGTCTTCT-3′, reverse: 5′-TACACCATGCTTGGTCTG-3′); and 18S (forward: 5′-CTGAGGATGCTGTCTG-3′, reverse: 5′-CCGGTGTATGCGGAGTCT-3′). Relative expression/abundance levels of target genes were determined using qBase plus software (Biogazelle; Ghent University, Ghent, Belgium) with GAPDH as a reference target. Results are expressed as fold over untreated control (F.O.C.).

PCR array

For gene expression profiling, an in-house PCR array was performed consisting of 82 inflammatory genes and 4 reference genes (GAPDH, ACTB, HPRT1, and GUSB) using pooled mRNA from 3 separate experiments (pooled n = 3). cDNA synthesis was performed with 500 ng total mRNA using the RT-First Strand Kit (Qiagen, Crawley, U.K.) according to the manufacturer’s instructions. Real-time quantitative RT-PCR was performed containing 1 μl cDNA (after 1:5 dilution), 9 μl water, and 10 μl RT SYBR Green/ROX qPCR master mix (SABiosciences, Qiagen) in a 7300 Real Time PCRSystem (Applied Biosystems). Using a Web-based PCR array data analysis tool (SABiosciences, Qiagen), we normalized the cycle threshold values of the target genes against the four reference genes, and the relative abundance of gene transcripts is expressed as log 2^ΔCT.

Chromatin immunoprecipitation assay

A total of 7.5 × 10^5 THP-1 cells was seeded in a 100-mm cell culture dish to a volume of 10 ml after specific treatments. Chromatin immunoprecipitation assay (ChIP) assay was performed using the EZ-ChIP-chromatin immunoprecipitation kit (Millipore) as described in the manufacturer’s instructions. In brief, cells were fixed by addition of formaldehyde (1% final concentration) for 10 min to cross-link DNA and proteins. Fixation was suspended by addition of a glycine solution. Cells were pelleted at 1200 rpm, washed, and lysed using an SDS buffer (containing protease inhibitor mixture) before chromatin sonication. Twenty micrometres sheared chromatin was precipitated overnight at 4°C with rotation using 5 μg p65 Ab (sc-72; Santa Cruz) and isotype-matched control IgG (sc-2027; Santa Cruz), and immunocomplexes were collected using protein G agarose. After washing, protein DNA complexes were eluted and cross-links were reversed followed by DNA purification and analysis using end-point PCR for the NF-κB binding motif located at position −79/93 to the transcription start site (TSS). Primers used for the detection of the NF-κB site have been previously described: for: 5′-TTGAGGAAGAAGCAGGAATTTT-3′, rev: 5′-GTACACAGAGGGCGTGGTCTG-3′ (23). Samples were separated on a 1% agarose gel to visualize the expected 100-bp product. Relative band quantification was assessed using LI-COR Image Studio Lite Version 3.1.

Stable lentiviral knockdown

Stable knockdown of human NR4A2 and human NR4A3 was achieved with the transduction of short hairpin RNA (shRNA) using a Mission Lentiviral packaging mix as per instructions (Sigma-Aldrich) using THP-1 as
previously described (21). For controls, cells were transduced with scramble shRNA (Sigma-Aldrich). In brief, 150 μl THP-1 cells from a stock concentration of 2.5 × 10^6 cells/ml was pipetted into a single well of a 96-well plate and incubated overnight to yield 70% confluence. Hexadimethrine bromide was added to a final concentration of 8 μg/ml followed by the addition of 10 μl lentiviral particles and incubation for 48 h at 37°C. Puromycin was added to the cells (5 μg/ml) and media changed every 3–4 d until resistant colonies were identified. Western blotting and mRNA analysis were used to measure NR4A2/3 expression.

Western blotting

Nuclear lysates. Suspension cells were pelleted by centrifugation at 1200 rpm followed by resuspension in into 250 μl ice-cold hypotonic buffer (10 mM HEPES-NaOH buffer, pH 7.9, containing 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, and protease inhibitor mixture [Sigma-Aldrich, Poole, Dorset, U.K.]). Adherent cells were scraped directly into hypotonic buffer, placed on ice for 15 min, and lysed by the addition of 1/5 (v/v) Nonidet P-40. Samples were then spun at 12,000 × g for 1 min. Pellets were resuspended in high-salt buffer (20 mM HEPES-NaOH buffer pH 7.9 containing 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% [v/v] glycerol, and 0.5 mM PMSF) and agitated vigorously for 30 min at 4°C followed by centrifugation at 15,000 × g for 10 min at 4°C. Supernatant was removed and stored at −20°C as nuclear lysate fraction.

Whole cell lysates. Suspension cells were pelleted by centrifugation at 1200 rpm followed by resuspension into 100 μl radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% [v/v] glycerol, and 0.5 mM PMSF and agitated vigorously for 30 min at 4°C followed by centrifugation at 15,000 × g for 15 min. Supernatant was removed and stored at −20°C as whole cell lysate fraction.

Protein expression levels were measured by Western blot analysis using specific Abs for NR4A2 and NR4A3 (R&D Systems); NF-κB1 p105/p50, p65, and Lamin A/C (Cell Signaling); and TATA box-binding protein (Abcam). Denisometric analysis included for Western blot data were determined using LI-COR Image Studio Lite version 3.1. In brief, intensity of proteins of interest (NR4A1, NR4A2, NR4A3, p65, and p50) was quantified using LI-COR program relative to loading control protein (Tata binding protein [TBP] or β-tubulin). Values are displayed as raw arbitrary values.

Flow cytometry

After specific treatment, cells were pelleted at 1200 rpm and resuspended to a concentration of 1 × 10^6 cells/ml using Opti-MEM (Life Technologies). Fluorescently conjugated Abs (1:200) were then added to 200 μl cells and incubated on ice for 30 min in the dark, alongside unstained isotype IgG control cells. Fluorescently conjugated Abs included CD86 (Bvd 80 conjugated), CD80 (Alexa Fluor 700 conjugated), HMC class II-HLA-DR (allophycocyanin-H7 conjugated), and CCR7 (PE conjugated) supplied by BD Biosciences and used at concentrations as recommended by manufacturer’s instructions. Poststaining cells were washed using 1 ml PBS containing 1% FCS and 1% sodium azide followed by centrifugation at 1200 rpm. Supernatant was removed and cell pellet was washed and centrifuged again as described earlier. Supernatant was removed and cells were then resuspended in 500 μl PBS containing 0.1% sodium azide. Unstained cells (containing isotype-matched control IgG) were analyzed using the Cyan ADP Analyzer (Beckman Coulter) and gated to identify specific staining from fluorochromes. Unstained cells were confined within gate A as illustrated in pictograms within Figs. 3 and 4. Fluorochrome-conjugated Abs incubated cells were then analyzed on the same Cyan ADP Analyzer (Beckman Coulter) and percentage cells positive for staining was obtained (positive staining is determined by cells migrating toward gate B, C, or D, indicating increased fluorescence obtained by Ab-specific staining within a given treatment).

ELISA

Media from THP-1–treated cells were centrifuged briefly to remove cell debris, and supernatant was used for ELISA analysis. MIP-3α protein was measured using a human ELISA kit from RayBiotech as per manufacturer’s instructions.

Promoter studies and analysis

The Genomatix software suite program MatInspector (Genomatix Software GmbH) was used to analyze the human MIP-3α promoter 1000 bp upstream of the TSS. Two stringency tests were applied as per Genomatix software instructions to select binding sites that displayed the best probability as “real” binding motifs for transcription factors (core similarity of 1.0 and matrix similarity > 0.80). The first test is the core similarity test, and the maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the given sequence. The second test is the matrix similarity test and the score must be >0.80.
concentrations ranging from 1 nM to 100 nM (Fig. 2A). The specific A2b agonist BAY-60-6583 at 100 nM (Ki values on individual adenosine receptors A2b [3–10 nM] and A3 receptors [38 nM]) and A2a receptors 2-CI-IB-MECA (Ki values on individual receptors A3 [1.4 nM], A1 [220 nM], A2a [5360 nM], and A2b [10,000 nM], respectively) displayed no significant increases in NR4A1, 2, or 3 mRNA at all concentrations examined (100, 10, or 1 nM; data not shown).

To further investigate which adenosine receptor(s) are responsible for mediating NR4A1-3 modulation in undifferentiated THP-1 cells, we used selective antagonists toward each receptor. THP-1 cells were pretreated for 30 min with 0.5 μM of each antagonist followed by treatment with NECA, and changes in NR4A3 mRNA, the most potent NECA modulated NR4A family member in these cells, was measured. We observed significant increases (5- to 15-fold) in NR4A3 gene expression when A1, A2b, and A3 receptors were antagonized followed by exposure to NECA for 2 h. In contrast, treatment with the A2b-specific agonist BAY-60-6583 (1 μM) after LPS priming had no effect on NR4A family members (Fig. 2C, 2D). Induction of NR4A1-3 gene expression was also observed at lower concentrations (100 nM) of the specific A2a agonist, whereas no changes in mRNA expression levels were measured using 100 nM A2b agonist, BAY-60-6583 (Supplemental Fig. 1C). In LPS-primed cells, A1- and A3-specific agonists displayed no alteration in NR4A3 mRNA levels at all concentrations tested (1 nM, 10 nM, 100 nM, and 1 μM; data not shown). In LPS-primed cells, NECA and CGS-2160 treatment results in significant, albeit modest, induction of NR4A3 protein levels with minimal effects on NR4A1 levels (Fig. 2D). In contrast, NR4A2 protein levels are significantly and markedly induced, suggesting that NR4A2 may be the major adenosine-regulated member of the NR4A subfamily in these cells (Fig. 2D).

Cell-surface adenosine receptor expression levels significantly impact on a cell’s ability to respond to adenosine receptor ligands (5). We therefore measured adenosine receptor expression during LPS

FIGURE 1. Adenosine-altered NR4A genes in myeloid cells are concentration and time dependent. (A) THP-1 cells differentiated using 20 ng/ml PMA, and (B) undifferentiated THP-1 cells were exposed to varying concentrations of NECA (a pan adenosine receptor agonist) for 2 h. (B, Right panel) Undifferentiated THP-1 cells were exposed to 20 μM NECA for 30 min and 4 h followed by preparation of nuclear lysates, and subsequent Western blot analysis was performed for both NR4A2 and loading control TBP. (C) Human primary PBMCs were exposed to 20 μM NECA for 1, 2, and 3 h, and (B) murine raw macrophage cells 264.7 were exposed to 1 μM NECA for 2 h. (A–D) RNA was isolated and RT-PCR was performed to assess levels of NR4A1, 2, and 3, and control gene GAPDH. Densitometric analysis included for Western blot data were determined using LI-COR Image Studio Lite version 3.1 and displayed in arbitrary values above relevant treatments (n = 3). Data are expressed as F.O.C ± SEM or representative Western blots for n = minimum of 3 individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared with untreated control (Un).
priming and PMA differentiation before adenosine exposure. The A2a receptor is the dominant receptor subtype expressed on these cells during LPS treatment and displays significant mRNA increases within 2 h (7.83 ± 3.07-fold) to 8 h (5.07 ± 0.63-fold; Supplemental Fig. 1D). Adenosine receptor subtypes A1, A2b, and A3 mRNA levels remained unchanged upon LPS exposure (data not shown). The selective modulation of receptor subtype A2a mRNA levels observed in THP-1 cells in response to LPS parallels studies using primary isolated human and murine macrophage cells (7, 8, 32). Significantly, mRNA expression of all four adenosine receptors was increased at 24 h during PMA treatment (Supplemental Fig. 1E), although within these cells, our agonist data suggest that adenosine regulation of NR4A1-3 is primarily A2a receptor driven.

NR4A2 and NR4A3 regulate macrophage inflammatory gene expression and differentiation

To address whether NR4A receptor activity contributes to regulatory macrophage induction by LPS/TLR4 activation, we measured proinflammatory mediator production and surface markers of differentiation in NR4A2- and NR4A3-depleted THP-1 cells. TLR4 stimulation using LPS promotes a significant induction of NR4A2 and NR4A3 protein levels, and this regulation is absent in cells transduced with specific NR4A2 and NR4A3 shRNA (Fig. 3A). TLR4-induced inflammatory macrophage responses resulted in the rapid and potent secretion of TNF-α protein over time (Fig. 3B). LPS-stimulated cells also produce significantly increased levels of MCP-1 protein within 24 h, confirming TLR4-induced inflammatory responses in these cells (Fig. 3C). NR4A2 and NR4A3 depletion significantly potentiate LPS-induced TNF-α and MCP-1 (Fig. 3B, 3C).

To confirm the involvement of the NF-κB pathway in macrophage activation after TLR4 stimulation, we tested NF-κB inhibitor BAY 11-7082 (NF-κBi), and results indicate that pretreatment for 1 h with this inhibitor significantly reduced LPS-induced cytokine and chemokine mRNA changes (Fig. 3D). We further demonstrate that LPS-induced NR4As in macrophage cells are NF-κB dependent, which is consistent with previous studies (Supplemental Fig. 1F) (33). Taken together, these data confirm that, after LPS stimulation, NF-κB regulates levels of NR4A expression, which acts as a feedback mechanism to limit excessive proinflammatory cytokine and chemokine production.

We next determined the phenotypic changes occurring in scrambled and NR4A-depleted THP-1 cells treated with LPS. Using flowcytometric analysis, we measured a panel of four classical M1 surface markers of differentiation (CCR7, MHC class II [HLA-DR], CD86, and CD80). LPS-treated control cells displayed significant induction of MHC class II (HLA-DR), CD80, and enhanced CCR7 (p = 0.058) and CD86 (p = 0.072) surface marker expression (Fig. 3E). LPS treatment of NR4A2-depleted cells demonstrated significantly enhanced expression of CCR7 and CD80 compared with

**FIGURE 2.** Adenosine alters NR4A genes in myeloid cells primarily through A2a receptor. (A) THP-1 cells differentiated using 20 ng/ml PMA were exposed to 100, 10, and 1 nM CGS-21680 (A2a adenosine receptor–specific agonist) and BAY-60-6583 (A2b adenosine receptor–specific agonist) for 2 h. (B) Human primary PBMCs were exposed to 1 μM NECA for 2 h, 1 μM CGS-21680, and 1 μM BAY-60-6583 for 2 h. (C) Undifferentiated THP-1 cells were primed by exposure to 1 μg/ml LPS for 18 h followed by treatment with 1 μM NECA, 1 μM CGS-21680, and 1 μM bay-60-6583 for a further 2 h. (A–C) RNA was isolated and RT-PCR was performed to assess levels of NR4A1, 2, 3, and control gene GAPDH. (D) Undifferentiated THP-1 cells were primed by exposure to 1 μg/ml LPS for 18 h followed by treatment with 1 μM NECA, 1 μM CGS-21680, and 1 μM BAY-60-6583 for 4 h. Nuclear lysates were prepared and Western blot analysis was performed for NR4A1/2/3 and loading control TBP. Densitometric analysis included for Western blot data were determined using LI-COR Image Studio Lite version 3.1 and displayed in arbitrary values alongside relevant treatments (n = 3). Data are expressed as F.O.C ± SEM or representative Western blots for n = minimum of 3 individual experiments. *p < 0.05, ***p < 0.001, treatments compared with untreated control (Un); #p < 0.05, ###p < 0.001, treatments compared displayed here using a bar attachment.
NR4A depletion alters TLR4-mediated proinflammatory responses and differentiation in monocytes. (A) Undifferentiated THP-1 cells transduced using shRNA directed against scrambled nontarget control, NR4A2, or NR4A3 were subsequently treated with 1 μg/ml LPS for 4 h. Whole cell lysates were prepared followed by Western blot analysis for NR4A2, NR4A3, and loading control β-tubulin. (B) Undifferentiated shRNA transduced THP-1 cells were exposed to 1 μg/ml LPS followed by removal of media at 0, 3, 6, 8, and 24 h and subsequent ELISA analysis for secreted TNF-α. (C) Undifferentiated shRNA transduced THP-1 cells were exposed to 100 ng/ml LPS followed by removal of media at 24 h and subsequent ELISA analysis for secreted MCP-1. (D) Undifferentiated THP-1 cells were pretreated with 10 μM NF-κB inhibitor (NF-κBi) followed by the addition of 1 μg/ml LPS for 2 h. RNA was isolated and RT-PCR analysis was performed for TNF-α, MCP-1, and control gene GAPDH. (E) Undifferentiated shRNA transduced THP-1 cells were exposed to 100 ng/ml LPS for 24 h. After 24 h, THP-1 cells were stained using fluorescein-conjugated Abs toward CCR7, MHC class II (HLA-DR), CD86, and CD80, and assessed by flow cytometry as described in Materials and Methods. Representative histograms showing flow-cytometry staining are included alongside flow data. Gates A = negative staining, gates B+C = CCR7/CD86+ staining, and gates C+D = MHC class II (HLA-DR)/CD80+ staining. Densitometric analysis included for Western blot data were determined using LI-COR Image Studio Lite version 3.1 and displayed in arbitrary values above relevant treatments (n = 3). Data are expressed as F.O.C% cells positive for fluorescein-conjugated Abs ± SEM for n = minimum of 3 individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001, treatments compared with untreated control (Un); #p < 0.05, ##p < 0.01, ###p < 0.001, treatments compared displayed here using a bar attachment.
with NECA for a further 2 h (pooled n = 3). Gene expression analysis revealed that 30 genes in the array were shown to be LPS modulated (>2.0-fold), and 13 of these modulated genes were subsequently altered >1.5-fold (potentiated or repressed) with the addition of NECA (Supplemental Table I).

Fig. 5A shows a “selection” of genes modulated after the addition of NECA to LPS-primed PBMCs. We observe decreased LPS-induced TNF-α mRNA levels as a result of NECA stimulation, which is consistent with previous studies (1). We also show that NECA has the capacity to further enhance LPS-stimulated IL-6 gene expression, consistent with responses in peritoneal macrophage cells (35). Screening reveals that NECA treatment of LPS-primed monocytes results in the modulation of genes involved in immune cell chemotaxis (CCR1 and CCR4) and T cell activation (IL-23p19; Fig. 5A). In this study, we show using primary PBMCs that NECA can diminish significantly endogenous CCR1 and CCR4 mRNA expression (Supplemental Fig. 2A). Further confirmation of gene expression changes measured using the array was verified using isolated primary PBMCs; we verify that NECA significantly attenuates LPS-induced TNF-α expression (Fig. 5B) and potently augments LPS-induced IL-23p19 mRNA levels, compared with NECA stimulation alone (Fig. 5C).

To determine whether adenosine responses can alter gene expression profiles primed with a distinct proinflammatory mediator, we exposed primary PBMCs to TNF-α (5 ng/ml) and subsequently cotreated with NECA for 2 h. In contrast with LPS responses, TNF-α had minimal effect on IL-23p19 mRNA levels and the addition of NECA failed to alter IL-23p19 in TNF-α–primed primary PBMCs (Fig. 5C). We next monitored MIP-3α mRNA expression levels, an important mediator of immune cell chemotaxis, and our results indicate that NECA alone induces MIP-3α gene expression, albeit modestly; however, NECA cotreatment dramatically augments MIP-3α in TNF-α–primed PBMCs, whereas displaying minimal effects on robust LPS-dependent responses seen in these cells (Fig. 5D). Taken together, these data suggest that the context and potency of the cellular environment may influence adenosine receptor–mediated regulation of gene expression.

NECA effects on LPS-primed THP-1 cells were next monitored, and results verify a similar pattern of changes in gene expression of TNF-α (Fig. 5B), IL-23p19 (Fig. 5C, 5E), CCR1, and CCR4 (data not shown) as measured in PBMCs after LPS/NECA priming.

Using more selective adenosine receptor agonists, we tested the A2a agonist CGS-21680 (1 μM) and the A2b agonist BAY-60-6583 (1 μM) for their ability to alter expression levels of IL-23p19 and MIP-3α in THP-1 cells primed with LPS. The pattern and magnitude

**FIGURE 4.** NR4As are pivotal for appropriate M1 phenotype differentiation. Undifferentiated scrambled control, NR4A2-, and NR4A3-depleted THP-1 cells were exposed to 100 ng/ml LPS + 10 ng/ml IFN-γ (M1) for 24 h. After 24 h, THP-1 cells were stained using fluorescently conjugated Abs toward CCR7, MHC class II (HLA-DR), CD86, and CD80, and assessed by flow cytometry as described in Materials and Methods. Gate A = negative staining, Gates B+C = CCR7/CD86+ staining, and gates C+D = MHC class II (HLA-DR)/CD80+ staining. Representative pictograms showing flow-cytometry staining are included. Data are expressed as % cells positive for fluorescently conjugated Abs ± SEM for n = minimum of 3 individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001, treatments compared with untreated control (Un); #p < 0.05, ##p < 0.01, ###p < 0.001, treatments compared displayed here using a bar attachment.
of induction after CGS-21680 treatment is equivalent to NECA, whereas BAY-60-6583 had no effect on gene targets (1 M; Fig. 5E). NECA or CGS-21680 treatment of THP-1 cells alone showed no significant effects on IL-23p19 and MIP-3α gene expression levels (data not shown). Changes in IL-23p40 expression after adenosine receptor stimulation of control or NR4A-depleted cells primed with LPS were not observed (data not shown).

To further elucidate the downstream mediators involved in NECA-dependent modulation of IL-23p19 and MIP-3α mRNA, we monitored expression levels in THP-1 cells in response to TNF-α (0.1, 1.0, 5.0, and 10 ng/ml; Supplemental Fig. 2B, 2C). Pretreatment of TNF-α–primed cells with NF-κBi (10 μM) before NECA addition indicates that NF-κB activity is involved in mediating these NECA responses, as evident by the significant inhibitory effects after inhibition of this protein complex (NF-κBi) (Supplemental Fig. 2B, 2C).

NR4A depletion disrupts NF-κB/p65 nuclear accumulation leading to altered adenosine regulation of MIP-3α and IL-23p19 expression

In myeloid cells, adenosine A2 receptor stimulation alone or after LPS priming potently upregulates NR4A1-3 gene expression leading to robust changes in NR4A2 protein expression levels (Fig. 2). To ascertain the potential involvement of NR4A2 and NR4A3 in mediating adenosine regulation of gene expression changes, we assessed stably depleted NR4A2 and NR4A3 cells. Consistent with previous studies (1), NECA significantly attenuates LPS-induced TNF-α mRNA (Fig. 5B, Supplemental Fig. 2D). The involvement of NR4A receptors in mediating NECA effects on TNF-α expression was measured. Consistent with changes in TNF-α protein production and secretion (Fig. 3B), NR4A2- and NR4A3-depleted cells display significantly enhanced TNF-α mRNA levels during LPS exposure. However, NECA-dependent reduction of LPS-driven TNF-α is maintained in NR4A2 (1.8-fold) and NR4A3 (2.2-fold)-depleted cells (Supplemental Fig. 3B, 3C). The extent of NR4A2 and NR4A3 depletion is evident after NECA–LPS exposure in scrambled cells compared with stably NR4A4-depleted cells (Fig. 6A, Supplemental Fig. 3A, respectively).

In THP-1 cells, primed with TNF-α or LPS, NR4A2 depletion significantly potentiates NECA-induced IL-23p19 and MIP-3α mRNA levels compared with control cells (Fig. 6B, 6C, respectively) leading to increased secretion of MIP-3α (Fig. 6D). NR4A3 depletion does not lead to this effect, and levels of IL-23p19 and MIP-3α mRNA remain relatively unchanged (Supplemental Fig. 3B, 3C). These data suggest that NECA-induced NR4A2 activity may specifically act as a feedback mechanism to limit expression of downstream targets including IL-23p19 and MIP-3α.
To further examine the effects of NR4A depletion on expression levels and nuclear accumulation of NR4A2 and NF-κB family members, we primed THP-1 control (sh-scrambled) and NR4A2-depleted cells with LPS overnight followed by exposure to NECA over 30 min and 4 h, and changes in protein levels were measured (Fig. 6E). In control cells, LPS and NECA cotreatment led to time-dependent increases in nuclear NR4A2 protein levels; this induction was absent in shNR4A2 RNA-transduced cells. With LPS alone and NECA cotreatment of NR4A2-depleted cells, we observed increased p65 protein nuclear accumulation over control cells. LPS treatment also induces significant increases in p50 protein levels; however, no differences in nuclear p50 expression were observed between NR4A2-depleted and control cells, and p50 levels remain unaffected by the addition of NECA (Fig. 6E). Comparative effects on p65 and p50 protein nuclear accumulation were observed in NR4A3-depleted THP-1 cells after LPS treatment (Supplemental Fig. 3D), albeit comparable downstream effects on NF-κB-regulated target gene expression were not measured in NR4A3-depleted cells (Supplemental Fig. 3B, 3C) compared with that of NR4A2-depleted cells (Fig. 6B, 6C).

Furthermore, to determine the effects of NR4A2 depletion on p65 binding to an endogenous κB site, we performed ChIP analysis and primers designed to amplify a specific region within the proximal human MIP-3α promoter (-93/-79). This promoter region contains a κB site previously reported as a binding site for p65 (Supplemental Fig. 3E) (23). In NR4A2-depleted cells, a modest increase in basal p65 binding to the κB site is observed; however, upon LPS stimulation followed by NECA cotreatment, we observe a marked increase in p65 binding to the κB site that is not measured in control cells (Fig. 6F). Importantly, these changes in p65 promoter binding are consistent with the significant production and secretion of the MIP-3α protein levels in NR4A2-depleted cells after LPS/NECA exposure (Fig. 6C), with levels of MIP-3α increased significantly from 482 ± 17.8 to 747 ± 41.7 pg/ml. Importantly, although single LPS treatment can induce robust changes in MIP-3α-secreted protein levels, such significant differences were not measured between control and NR4A2-depleted cells treated with LPS alone compared with LPS/NECA cotreatment. Data discussed throughout have been summarized in Fig. 7.

Discussion

The endogenous purine nucleoside adenosine, produced at sites of cellular stress, is involved in both physiological and pathological processes including several diseases characterized by chronic inflammation such as rheumatoid arthritis, chronic obstructive pulmonary disease, inflammatory bowel disease, and ischemia-reperfusion injury (1–5, 36). Monocyte and monocyte-derived macrophage cells represent pivotal players involved in orchestrating inflammatory events through the production of cytokines, chemokines, growth factors, and angiogenic factors that regulate inflammation, angiogenesis, and granulation tissue formation. Importantly, the loss of controlling macrophage activation responses during an inflammatory response can result in injury leading to loss of tissue function (6). It has recently been established that engagement of adenosine receptors on macrophage cells can control the balance between TLR-induced inflammatory and regulatory macrophage differentiation (37). However, what remains to be elucidated are the cell-specific signal transduction events and the transcriptional mediators that act to promote adenosine receptor-mediated outcomes during these responses. In this study, we demonstrate that adenosine controls the nuclear orphan receptor NR4A1-3 subfamily gene expression and activity in myeloid cells. We further demonstrate that during an inflammatory response, adenosine modulation of NR4A2 receptor activity acts to limit NF-κB–mediated effects on gene transcription, and that loss of NR4A2 expression leads to enhanced NF-κB activity and hyperinflammatory responses in myeloid cells (Fig. 7).

Regulation of the inflammatory response is controlled by alternatively activated macrophages, with classically activated (M1) macrophages producing a wide variety of proinflammatory cytokines and chemokines. By contrast, alternatively activated (M2) macrophages participate in anti-inflammatory responses and promote inflammatory resolution and tissue repair (6). Significantly, synergistic interactions between TLR/MyD88 and adenosine A2A receptor signaling have emerged in the transition of macrophages to the M2 phenotype in vivo (38). In this study, we reveal that adenosine modulation of NR4A receptors can be controlled through the adenosine A2a receptor subtype and that A2a receptor activation of NR4A1-3 receptor synthesis is further enhanced in TLR-stimulated macrophages. Macrophage cell phenotype is not only characterized by its secretory capacity of cytokines and chemokines but also expression of surface markers including CD86, CD80, CCR7, and MHC class II (HLA-DR) for M1 and CD163, CD206, and mannose receptor for M2 characterization. Using a prototypical model for M1 macrophage differentiation (IFN-γ + LPS), we reveal adenosine significantly reduced M1-driven CD80 expression whereas attenuating CCR7 surface expression. In dendritic cells, adenosine has been shown to enhance LPS-driven CD80, CD86, CD54, MHC class I, and MHC class II, further revealing cell type and differentiation factor specificity regarding adenosine effects on cell differentiation marker expression (39). Depletion of NR4A2 or NR4A3 does not alter this adenosine-driven attenuation of surface marker expression. Intriguingly, we show that NR4A2 depletion significantly potentiates M1-driven CCR7 and CD86, whereas attenuating MHC class II (HLA-DR) expression. NR4A3 depletion, however, although having no effect on M1-driven CCR7 or CD86 expression, significantly attenuates MHC class II (HLA-DR) expression. Recent studies have shown NR4A1 depletion enhances MHC class II (HLA-DR) surface expression in peritoneal macrophages isolated from ApoE−/− mice fed a western diet (16). In this article, we reveal NR4A2 and NR4A3 may play opposing roles in MHC class II (HLA-DR) expression. However, specific factors driving differentiation may play an important role, as evident by enhanced MHC class II (HLA-DR) surface expression in NR4A2-depleted cells differentiated using LPS alone in the absence of IFN-γ. These data expand recent reports that identify NR4A receptors as effector molecules of TLR and cytokine signaling in human and murine macrophage cells and as important regulators of cellular differentiation (15–18, 40).

Treatment of macrophages with LPS, cytokines, or oxidized lipids triggers the transcriptional induction of all three NR4A receptors, and functional studies primarily demonstrate anti-inflammatory and reparative functions for NR4A1 receptors in these cells (16, 41). The role of NR4A1 receptors as transcriptional regulators of macrophage phenotypic differentiation has recently been explored and verifies that depletion of NR4A1 in monocytes and macrophages results in enhanced TLR-mediated signaling and polarization of macrophages toward a proinflammatory M1 phenotype (16). Our data extend these observations as we observe that depletion of additional family members NR4A2 and NR4A3 in THP-1 cells results in altered macrophage activation. We reveal an enhanced proinflammatory phenotype resulting in increased cytokine and chemokine production in both NR4A2- and NR4A3-depleted cells, whereas each family member displays distinct alterations in the expression of cell-surface differentiation markers.

In the presence of TLR agonists such as endotoxin (LPS), adenosine strongly and synergistically upregulates the expression of the angiogenic growth factor, VEGF, by macrophages, thus acting as an angiogenic switch leading to physiological angiogenesis, inflammatory resolution, and tissue repair (5). Importantly, adenosine A2A receptors
have been shown to regulate the processes involved in healing, including the transition of macrophages to the regulatory phase. Macrophages from mice lacking A2A receptors express enhanced inflammatory genes including TNF-\(\alpha\) in response to TLR agonists (42) but fail to undergo the angiogenic switch in response to A2A receptor agonists (38, 43). Furthermore, bone marrow–derived macrophage cells lacking the surface enzyme CD39, whose activity is needed to control endogenous adenosine production, are unable to transition to a regulatory state, and cells are rendered hyperinflammatory in response to TLR agonists (37). Interestingly, this study suggests that in response to LPS, adenosine attenuates inflammatory cytokine production via A2bR signaling.

**FIGURE 6.** NR4A2 is required for appropriate adenosine-mediated NF-κB modulation of T cell regulatory genes MIP-3\(\alpha\) and IL-23p19. (A) Undifferentiated THP-1 cells transduced using shRNA directed against scrambled nontarget control (Sc) and NR4A2 (A2) were subsequently treated with 1 \(\mu\)g/ml LPS for 18 h followed by addition of 20 \(\mu\)M NECA for a further 4 h. Whole cell lysates were prepared followed by Western blot analysis for NR4A2 and loading control β-tubulin. (B and C) Undifferentiated Sc and A2 THP-1 cells were exposed to 5 ng/ml TNF-\(\alpha\) or 1 \(\mu\)g/ml LPS for 18 h followed by treatment with 20 \(\mu\)M NECA for 2 h and appropriate controls TNF-\(\alpha\)/LPS/NECA alone. RNA was isolated and RT-PCR was performed to assess levels of IL-23p19, MIP-3\(\alpha\), and control gene GAPDH. (D) Undifferentiated Sc and A2 THP-1 cells were exposed to 1 \(\mu\)g/ml LPS for 18 h followed by treatment with 20 \(\mu\)M NECA for a further 8 h and appropriate controls LPS/NECA alone; media were collected and analyzed for secreted MIP-3\(\alpha\) protein using ELISA. (E) Undifferentiated Sc and A2 THP-1 cells were treated with 1 \(\mu\)g/ml LPS for 18 h followed by treatment with 20 \(\mu\)M NECA for 1 h. ChIP assay was then performed using p65 Ab pull-down, and end-point PCR was run for the κB binding site at position -93/-79 bp upstream of the TSS along with igg control and input. Data are expressed as representative end-point PCR image (n = 2 individual experiments), and relative quantification of bands was calculated from n = 2 using LI-COR Image Studio Lite version 3.1 and displayed in raw arbitrary values above relevant treatments. Controls included were appropriate: NECA alone (2/8 h) or LPS/TNF-\(\alpha\) alone (18 h overnight + 2/4/8 h additional time). Densitometric analysis included for Western blot data were determined using LI-COR Image Studio Lite version 3.1 and displayed in arbitrary values above relevant treatments (n = 3). Data are expressed as F.O.C ± SEM or representative Western blots for n = minimum of 3 individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001, treatments compared with untreated control (Un); #p < 0.05, ###p < 0.001, treatments compared displayed here using a bar attachment.
Notwithstanding the specific A2 receptor involved, these studies collectively suggest that switching monocytes/macrophages to a regulatory phenotype may be a prerequisite for adenosine-dependent resolution of inflammation. We have identified the NR4A subfamily as mediators of adenosine signaling in myeloid cells. Our findings indicate that in cells of myeloid origin, adenosine potently induces NR4A gene and protein expression primarily through the A2a receptor. Within PMA-differentiated monocyte cells, concentrations (1.0 and 10 nM) of the A2a agonist CGS-21680 leads to enhanced NR4A expression. A1 (2-chloro-N6-cyclopentyladenosine) and A3 (2-CI-IB-MECA) receptor–specific agonists, at all concentrations examined, displayed no effect on NR4A gene expression, thus excluding a role for A1 or A3 receptors in modulating NR4A expression in this cell type (data not shown). Of note, however, modest increases in NR4A expression are observed with the A2b BAY-60-6583–specific agonist at 100 nM concentrations. From established Ki values, this A2b receptor agonist concentration is specific for the A2b receptor (31); therefore, we cannot exclude A2b receptor involvement in modulating NR4A expression under these cell conditions. In LPS-primed THP-1 cells, modulation of NR4A expression cannot be measured in response to A1- and A3-specific receptor agonists at concentrations tested (data not shown). Furthermore, under these stimulated conditions, the specific A2b receptor agonist BAY-60-6583 displayed no change in NR4A mRNA levels, whereas the specific A2a receptor agonist CGS-21680 leads to significantly enhanced expression. The involvement of the A2a receptor in mediating adenosine effects on NR4A gene expression is consistent with a recent study where mice deficient in A2a receptor (A2aR2/2) exhibit prolonged peritonitis after i.p. thioglycollate injection (44). Further characterization after the isolation of A2aR2/2 thioglycollate-elicited peritoneal macrophages (A2aR2/2 thio-pMacs) reveals that these cells have lost their ability to upregulate the expression of NR4A1, confirming the essential role of the A2a receptor in this process.

As ligand-independent and constitutively active receptors, activity of the NR4A1-3 transcription factors is tightly controlled at the level of expression, nuclear localization, coactivator/corepressor recruitment, and posttranslational modifications (11, 14). Enhanced NR4A activity is not only controlled by NF-κB activity but NR4A receptors have been shown to modulate NF-κB functions downstream of LPS/TLR4 and TNF-α signaling in a dynamic fashion, either repressing or enhancing NF-κB target gene expression in monocyte/macrophage cells (13, 40, 45, 46). In microglial cells, NR4A2 is required for clearance of ligand-independent and constitutively active receptors, activity of the NR4A1-3 transcription factors is tightly controlled at the level of expression, nuclear localization, coactivator/corepressor recruitment, and posttranslational modifications (11, 14). Enhanced NR4A activity is not only controlled by NF-κB activity but NR4A receptors have been shown to modulate NF-κB functions downstream of LPS/TLR4 and TNF-α signaling in a dynamic fashion, either repressing or enhancing NF-κB target gene expression in monocyte/macrophage cells (13, 40, 45, 46). In microglial cells, NR4A2 is required for clearance of...
of NF-κB from target gene promoter during TLR4-mediated inflammatory events in vitro and in vivo (13). These NR4A2-dependent effects lead to the fine-tuning of inflammatory responses after NR4A2 docking with NF-κB/p65 subunits on target genes, promoting the recruitment of the CoREST corepressor complex and subsequent removal of NF-κB/p65 and transcriptional repression of several TLR-induced proinflammatory genes (13). We show that nuclear levels of NF-κB/p65 are enhanced in TLR/adenosine-stimulated, NR4A2-depleted macrophage cells. We further establish that, after LIR/adenosine receptor stimulation, NR4A2 depletion results in significant binding of NF-κB/p65 to a κB consensus binding motif within the MIP-3α proximal promoter leading to increased gene transcription and protein secretion. Collectively, these data suggest that adenosine may modulate the inflammatory response of monocyte/macrophage cells, in part by increasing NR4A2 expression/activity, which in turn acts to prevent excessive NF-κB activation leading to the attenuation of inflammation. Importantly, we reveal adenosine receptor-mediated attenuation of TNF-α during TLR4 stimulation is maintained in NR4A2-depleted cells, suggesting a greater level of complexity regarding the role of NR4As in specific adenosine-modulated target genes.

Critical to uncovering the contribution NR4A1-3 receptors play in guiding inflammatory responses and inflammatory outcome is the study of distinct models of inflammation-associated disease. A recent report uncovers NR4A1 receptor participation in a murine model of acute myocardial infarction during distinct phases of monocyte/macrophage phenotypic differentiation (41). Monocyte/macrophage NR4A1 activity is required for two critical phases of acute myocardial infarction, which are essential for tissue remodeling and healing. NR4A1 promotes and controls the early monocyte pro-inflammatory responses, which is required for the initiation of inflammatory resolution and subsequent monocyte-derived, macrophage-dependent reparative/healing phase. Similar molecular mechanisms are used in controlling immune homeostasis in LPS-induced sepsis to permit NR4A1-mediated control of hyper-inflammatory responses (47). From such studies it is becoming evident that the local environment influences the differentiation stage/function of immune cells, thus determining the cell-specific contribution NR4A receptors play in guiding the magnitude of inflammatory responses and outcomes (41). Within this study, we report a previously unidentified role of NR4A2 in limiting adenosine-mediated NF-κB/p65 hyperinflammatory responses in TLR4/NF-κB–primed cells. We observe adenosine receptor-mediated attenuation of LPS-induced TNF-α, and the magnitude of this effect is maintained in NR4A2/3-depleted cells where TNF-α production and secretion are significantly elevated. Furthermore, we identify that adenosine receptor stimulation of TLR4–primed monocyte cells promotes expression of MIP-3α and IL-23p19, with levels of MIP-3α protein significantly enhanced in NR4A2-depleted cells, coinciding with enhanced p65 binding to the proximal MIP-3α promoter. MIP-3α and IL-23p19 are pivotal mediators produced by activated macrophages and involved in dendritic cell, B cell, and T cell (T regulatory cell [Treg] and Th17) recruitment and activation (48). Interestingly, adenosine has been shown to promote Treg development and inhibit proinflammatory Th17 responses through A2a receptor signaling (49, 50). More recent studies endorse the observation that adenosine-dependent Treg recruitment, via A2B5 engagement, helps to limit inflammatory responses in endotoxin-induced pulmonary inflammation (51). Notably, under physiological and pathological conditions, NR4A2 controls Treg induction and suppression of aberrant production of Th1 cytokines (17, 18).

The ability of NR4A1-3 receptors to integrate and limit inflammatory signaling and promote tissue repair makes these receptors potential targets for therapeutic intervention. Although NR4A1-3 activity does not appear to be regulated by endogenous ligands, pharmacological modulation of NR4A4 activity can be achieved with agents including the antineoplastic agent, 6-mercaptopurine, and a series of 1,1-bis(3-indolyl)-1-(p-substituted phenyl) methanes identified as potential NR4A1/2 agonists (52–54). Recent studies reveal that in microglial cells, 1,1-bis(3-indolyl)-1-(p-substituted phenyl) methanes can increase NR4A2 nuclear localization, stabilization of Co-REST and NCoR2 complexes, and subsequent inhibition of NF-κB/p65 signaling (55). In addition, altering the expression level of these constitutively active receptors may be a feasible approach to modulate NR4A target genes in a cell-specific manner. The in vivo therapeutic potential of targeting NR4A/NF-κB interactions to control immune homeostasis has recently been uncovered (47). A chemical compound n-pentyl 2-[3,5-dihydroxy-2-(1-nanoyl)]-phenylacetate targets the ligand-binding domain of NR4A1 and prevents phosphorylation of NR4A1 by TLR-activated p38a. Loss of NR4A1 phosphorylation restores and maintains NR4A1 suppression of a hyperinflammatory response through NR4A1 inhibition of NF-κB/p65 binding to κB sites within promoter regions of target genes. Additional in vivo studies will determine whether the clinical benefits of adenosine on limiting macrophage activation and promoting inflammatory resolution may be mediated through molecular cross-talk with the NR4A orphan nuclear receptor subfamily. In this study, we reveal that selective A2aR agonists act as potent inducers of NR4A expression and activity in myeloid cells leading to contained inflammatory responses, thus elucidating a new regulatory pathway that could be targeted to treat diseases in which monocyte/macrophage hyperactivation plays a principal role.

Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental figure 1: a.) THP-1 cells differentiated using 20ng/ml PMA and undifferentiated THP-1 cells were exposed to 100nM and 10nM NECA for 2hrs. b.) Undifferentiated THP-1 cells were exposed to 0.5μM A1 (DPCPX), A2a (ZM 241385), A2b (PSB1115) and A3 (MRS 1220) receptor specific antagonists for 30 minutes prior to exposure with 20μM NECA for 2hrs. c.) Undifferentiated THP-1 cells were primed by exposure to 1μg/ml LPS for 18hrs followed by treatment with 100nM CGS-21680 and 100nM BAY-60-6583 for a further 2hrs. d.) Undifferentiated THP-1 cells were exposed to 1μg/ml LPS for 2, 8 and 18hrs. e.) THP-1 cells were differentiated using 20ng/ml PMA for 0hr, 2hrs, 24hrs and 48hrs. f.) Undifferentiated THP-1 cells were exposed to LPS (1μg/ml) for 2hrs with or without pre-treatment with 10μM NF-κB inhibitor BAY 11-7082 (NF-kBi) for 1hr, or NF-kBi alone for duration of experiment. a-f.) RNA was isolated at time points indicated and RT-PCR was performed to assess levels of NR4A1/2/3 (a, b, c and f), Adenosine receptors A1, A2a, A2b, A3 (d-e) and control gene GAPDH. Data are expressed as fold over untreated control (F.O.C) ± SEM for n = minimum of 3 individual experiments. *p< 0.05, **p< 0.01, ***p< 0.001 treatments compared to untreated control (Un).
Supplemental figure 2: a.) Human primary PBMC’s were exposed to 20μM NECA for 2hr. b,c.) Undifferentiated THP-1 cells were exposed to varying concentrations of TNFα (0 – 10ng/ml) for 18hrs followed by treatment with 20μM NECA or pretreated with 10μM NF-κB inhibitor BAY 11-7082 (NF-κBi) for 1hr post TNFα (5ng/ml) 18hr treatment and subsequent exposure to 20μM NECA for 2hrs. d.) Undifferentiated THP-1 cells were exposed to 1μg/ml LPS for 18hrs followed by treatment with 20μM NECA for a further 2hrs. RNA was isolated and RT-PCR was performed to assess levels of CCR1, CCR4 (a) IL-23p19, MIP-3α (b,c), TNFα (d) and control gene GAPDH. Data are expressed as fold over untreated control (F.O.C) ± SEM for n = minimum of 3 individual experiments. Appropriate controls were included were appropriate: NECA alone (2hrs) or LPS/TNFα alone (18hrs overnight + 2hrs additional time, total 20hrs). Values above bar attachments in part d refer to fold change between LPS and LPS + NECA. ***p< 0.001 treatments compared to untreated control (Un). #p< 0.05, ###p< 0.001 treatments compared displayed here using a bar attachment.
Supplemental figure 3: 
a) Undifferentiated THP-1 cells transduced using shRNA directed against scrambled non target control (Sc) and NR4A3 (A3) were subsequently treated with 1μg/ml LPS for 18hrs followed by addition of 20μM NECA for a further 4hrs. Whole cell lysates were prepared followed by western blot analysis for NR4A3 and loading control β-tubulin. 
b,c) Undifferentiated Sc and A3 THP-1 cells were exposed to 5ng/ml TNFα and 1μg/ml LPS for 18hrs followed by treatment with 20μM NECA for 2hrs alongside appropriate TNFα, LPS and NECA only controls. RNA was isolated and RT-PCR was performed to assess levels of MIP-3α, IL-23p19 and control gene GAPDH. 
d) Undifferentiated Sc and A3 THP-1 cells were exposed to 1μg/ml LPS for 18hrs followed by treatment with 20μM NECA for a further 30 minutes and 4hrs along with LPS only appropriate controls. Nuclear lysates were prepared and western blot analysis was performed for NR4A2, p65, p50 and loading control. 

Forward primer: aasp:aagaactttcctcgaggtttttatgtcactctaggg
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5'UTR: ttccttcgacccttcatgcctcctcctaatatgaga
TSS: tgcgatcagatagcagcc
control TATA binding protein (TBP). e.) Schematic detailing the κB site/binding motif (underlined and uppercase lettering) within the MIP-3α promoter, analysed using the genomatix software suite program MatInspector for 1000bp upstream of transcription start site (TSS) (shown here is 200bp upstream of TSS). Highlighted in red are the end point PCR primers used to amplify the κB site/binding motif. Data are expressed as fold over untreated control (F.O.C) ± SEM or representative western blots for n = minimum of 3 individual experiments. Densitometric analysis included for western blot data was determined using LI-COR® Image Studio Lite version 3.1 and displayed in arbitrary values above relevant treatments (n=3). Appropriate controls were included were appropriate: NECA alone (2/4hrs) or LPS / TNFα alone (18hrs overnight + 30 minutes/2/4hrs additional time). **p< 0.01, ***p< 0.001 treatments compared to untreated control (Un). *p< 0.05, ###p< 0.001 treatments compared displayed here using a bar attachment.
**Supplemental Table 1: Gene expression analysis.** Human primary PBMC’s were primed by exposure to 1μg/ml LPS overnight (18hrs) (LPS) followed by treatment with 20μM NECA for 2hrs (LPS + NECA). Total RNA was isolated (pooled n=3) and a PCR array (consisting of 82 target and 4 reference genes) was performed. 3 columns depict LPS, LPS + NECA fold over untreated control (F.O.C) and LPS + NECA fold over LPS (LPS + NECA V’s LPS).

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