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Protein Kinase A Inhibition of Macrophage Maturation Is Accompanied by an Increase in DNA Methylation of the Colony Stimulating Factor 1 Receptor Gene

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List of abbreviations: Colony stimulating factor (CSF), CSF-1 receptor (CSF1R), fms intronic regulatory element (FIRE), prostanglandin E_2 (PGE_2), protein kinase A (PKA), exchange protein activated by cAMP (Epac), DNA methyltransferase (DNMT), ten-eleven translocation (TET), chromatin immunoprecipitation (ChIP), vasodilator stimulated phosphoprotein (VASP), cAMP response element binding (CREB), interleukin (IL), tumor necrosis factor (TNF), lipopolysaccharide (LPS)

Abstract

Macrophage colony stimulating factor (CSF)-1 plays a critical role in the differentiation of mononuclear phagocytes from bone marrow precursors, and maturing monocytes and macrophages exhibit increased expression of the CSF-1 receptor, CSF1R. The expression of CSF1R is tightly regulated by transcription factors and epigenetic mechanisms. We previously showed that prostaglandin E_2 and subsequent activation of protein kinase A (PKA) inhibited CSF1R expression and macrophage maturation. Here, we examine the DNA methylation changes that occur at the *Csf1r* locus during macrophage maturation in the presence or absence of activated PKA. Murine bone marrow cells were matured to macrophages by incubating cells with CSF-1-containing conditioned medium for up to 6 days in the presence or absence of the PKA agonist 6-bnz-cAMP. DNA methylation of *Csf1r* promoter and enhancer regions was assayed by bisulfite pyrosequencing. DNA methylation of *Csf1r* decreased during normal macrophage maturation in concert with an increase in *Csf1r* mRNA expression. Treatment with the PKA agonist inhibited *Csf1r* mRNA and protein expression, and increased DNA methylation at the *Csf1r* promoter. This was associated with decreased binding of the transcription factor PU.1 to the *Csf1r* promoter. Treatment with the PKA agonist inhibited the responsiveness of macrophages to CSF-1. Levels of endogenous PKA activity decreased during normal macrophage

maturation, suggesting that attenuation of this signaling pathway contributes to the increase in CSF1R expression during macrophage maturation. Together, these results demonstrate that macrophage maturation is accompanied by *Csf1r* hypomethylation, and illustrates for the first time the ability of PKA to increase *Csf1r* DNA methylation.

Introduction

Circulating monocytes and tissue-recruited macrophages are key defenders of host defense [1] that differentiate from myeloid precursors in a highly regulated process [2]. Macrophage colony stimulating factor (CSF)-1 is an essential factor for monocyte/macrophage maturation [3]. Mice with a natural mutation of *Csf1* or with knockout of the *Csf1* gene exhibit substantial reductions in tissue-derived mononuclear phagocytes [4]. CSF-1 has furthermore been shown to increase macrophage proliferation, differentiation, and activation (reviewed in [5-7]). Impairment in CSF-1 expression is associated with a variety of diseases including osteoporosis [8], atherosclerosis [9], and Alzheimer's disease [10], demonstrating the critical importance of mature monocytes and macrophages in tissue homeostasis and disease prevention.

The actions of CSF-1 are mediated by its receptor, CSF1R (also known as CSF-1R, M-CSF-R, fms, CD115), whose expression is essential for mononuclear cell development and maturation [11]. CSF1R is expressed at low levels in hematopoietic stem cells [12, 13] and is selectively upregulated during monocytic differentiation [14]. Increased expression and activation of CSF1R not only drives the differentiation of myeloid progenitors into mature mononuclear phagocytes, but can even reprogram granulocytes, pre-B, and mature B lymphocytes into expressing markers of monocyte/macrophage lineage [15-17]. As a reflection of the critical importance of CSF1R, expression of CSF1R is regulated at both the transcriptional and post-transcriptional levels [18, 19]. Studies have shown that the transcription factor PU.1 occupies the *Csf1r* promoter even when *Csf1r* mRNA is expressed at low levels in hematopoietic stem cells [14, 20]. Committed monocytes and macrophages exhibit enhanced expression of *Csf1r* mRNA due to transcription factor binding of the fms intronic regulatory element (FIRE), and remodeling of chromatin including histone

acetylation and methylation [20]. In addition, other epigenetic changes including DNA methylation are also observed, especially in nonmyelopoietic cells such as fibroblasts and T cells, which are hypermethylated at the *Csf1r* locus [16]. Transcription of *Csf1r* mRNA is thus a consequence of a highly coordinated interplay of transcription factors and epigenetic modifications focused at the *Csf1r* promoter and enhancer regions.

Prostaglandin E_2 (PGE_2) is a pleiotropic lipid mediator that inhibits many macrophage functions including phagocytosis and bacterial killing [21]. Studies decades ago showed that PGE_2 [22, 23] and other cAMP elevating agents [24] also inhibit CSF-1-induced macrophage differentiation, proliferation, and expression of urokinase plasminogen activator, but the operative mechanism for this was unknown. We recently showed that PGE_2, via signaling through the E prostanoid 2 receptor with subsequent increase in cAMP and protein kinase A (PKA) activity, inhibited monocyte/macrophage maturation, which was associated with a decrease in CSF1R expression [25]. How CSF1R expression is inhibited by increased PKA activity is unknown. Since DNA methylation remains one of the most fundamental epigenetic mechanisms and has been shown to vary significantly within the *Csf1r* locus between different cell types [16], we investigated how DNA methylation of *Csf1r* changes during normal macrophage maturation, and how DNA methylation is affected by PKA signaling. We found that DNA methylation of *Csf1r* decreased during macrophage maturation, and that exogenous activation of PKA resulted in an increase in the DNA methylation at the *Csf1r* promoter, which was associated with a decrease in PU.1 transcription factor binding and *Csf1r* mRNA expression. Our studies thus identify an ability for PKA, one of the most fundamental canonical signaling molecules, to affect the DNA methylation of a key gene involved in monocyte/macrophage maturation.

Materials and Methods

Cell Culture and Maturation of BMDM

Murine macrophages were matured from bone marrow precursors *in vitro* with the addition of recombinant CSF-1, or conditioned medium from CSF-1-secreting L929 cells,

for up to 6 days [26, 27]. This well-established approach results in relatively homogenous primary macrophages that are not conditioned by a specific tissue microenvironment. Bone marrow cells were isolated under aseptic conditions by flushing the marrow cavities of femurs and tibiae of C57BL/6 mice purchased from Jackson Laboratory (Bar Harbor, ME). All animal studies were approved by University of Michigan Committee on the Use and Care of Animals. Bone marrow cells were cultured for up to 6 days in RPMI (Thermo Fisher Scientific, Waltham, MA) containing 30% conditioned medium from L929 cells as previously described [26, 27]. L929, a murine fibrosarcoma cell line that synthesizes CSF-1, was cultured to confluence in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) with 10% fetal bovine serum (Hyclone, Logan, UT) supplemented with 100 U/ml of penicillin/streptomycin. To examine the effects of PKA activation, bone marrow cells were treated with or without the selective PKA agonist 6-bnz-cAMP (500 µM, Axxora, Farmingdale, NY) at day 0 and again at day 3. In some experiments, cells were treated at day 0 with 8-pCPT-2'-*O*-Me-cAMP (500 µM, Axxora), a selective agonist for the alternative cAMP effector, exchange protein activated by cAMP (Epac), or with IBMX (250 µM, BIOMOL, Farmingdale, NY), an inhibitor of the cAMP-degradative enzyme phosphodiesterase.

For siRNA experiments, cells were treated with 50 nM of control siRNA or combinations of either DNA methyltransferase (DNMT)-3L siRNA alone, DNMT3L siRNA with DNMT3a siRNA (at 50 nM each), or DNMT3L siRNA with DNMT3b siRNA (at 50 nM each) (all from Qiagen, Valencia, CA) in OptiMEM (Thermo Fisher Scientific) at day 0 and again at day 1. In other experiments, cells were treated at day 0 and again at day 1 with 50 nM of control siRNA or siRNA against the ten-eleven translocation (TET) "demethylases" TET2 or TET3 (all from Dharmacon, Lafayette, CO). Because cells were treated in OptiMEM, 20 ng/ml of CSF-1 (PeproTech, Rocky Hill, NJ) was added to the medium to induce macrophage differentiation.

Flow cytometry

Cells were suspended in PBS with 2 mM EDTA and 0.5% FCS. Fc receptor–mediated and nonspecific antibody binding were blocked by the addition of excess CD16/CD32 (BD Biosciences, San Jose, CA). Staining was performed at 4°C in the dark for 15 minutes. The following monoclonal antibodies were used at appropriate dilutions for staining: CD11b, (BD Biosciences Pharmingen), CSF1R (BioLegend, San Diego, CA), and F4/80 (eBioscience, San Diego, CA). A FACSCalibur flow cytometer (BD Biosciences) was used for flow cytometric characterization of cell populations, and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

RT-PCR

RNA was isolated from cells using Trizol (Thermo Fisher Scientific), made into cDNA, and amplified by quantitative real-time PCR on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR-green Master Mix (Applied Biosystems) and primers against murine *Csf1r* and β-actin. The primers were as follows: for *Csf1r*: CGAGGGAGACTCCAGCTACA (forward) and AGAAGTCGAGACAGGCCTCA (reverse); for β-actin: CTGCCTGACGGCCAAGTC (forward) and CAAGAAGGAAGGCTGGAAAAGAG (reverse). Primers and probes for DNMT1 (Mm01151063_m1), DNMT3a (Mm00432881_m1), DNMT3b (Mm01240113_m1), DNMT3L (Mm00457635_m1), TET1 (Mm01169087_m1), TET2 (Mm00524395_m1), and TET3 (Mm00805756_m1) were obtained from Applied Biosystems. Relative gene expression was determined by the comparative C_T method ($\Delta\Delta C_T$) with β-actin used as a reference gene.

Bisulfite sequencing

DNA was isolated from cells using the Dneasy kit (Qiagen), and bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) as per manufacturers' instructions. After bisulfite conversion, regions of the murine *Csf1r* promoter, FIRE, and 1 kilobase pair upstream of FIRE (FIRE -1 kb) were amplified with the following primers: for *Csf1r* promoter: AAGGGGAAGAGGAGTTAGTG (forward) and CCCCTTTTCTCCCCTTACCAT (reverse); for FIRE -1 kb:

GATGAAGAATGGTTAGGATTAGGGTATTA (forward) and CCCTAAAAATCACAAAACACATCTTTAAAT (reverse); for FIRE: GATAATGGTTAGGAGGTTAGGGAAGTA (forward) and AACTCAAACCCCCTATCAAATC (reverse). All reverse primers were biotin labeled which allowed the amplified products to be isolated with sepharose beads, denatured, and annealed to sequencing primers for pyrosequencing on the Pyromark Q24 (Qiagen). The sequencing primers for *Csf1r* promoter were: AGAGGAGTTAGTGTAATAGATA, for FIRE -1 kb: AAGGGAAGGTAGTGA, and for FIRE: GGGAAGTAGAAGTGAGA.

Chromatin Immunoprecipitation (ChIP)

Bone marrow cells (1×10^7) were treated with or without the PKA agonist 6-bnz-cAMP for 3 days in L929 conditioned medium. To perform ChIP, cells were incubated for 5 min in 1% formaldehyde to crosslink DNA with protein. Nuclear material was isolated using nonionic detergent buffers, and chromatin was sheared to 200-500 bp fragments by 20 min of sonication using a Covaris sonicator (Covaris, Inc, Woburn, MA). Chromatin was incubated overnight with antibody to PU.1 (1:25, #2266 Cell Signaling, Danvers, MA) or isotype control. Chromatin was immunoprecipitated using the ActiveMotif ChIP-IT Express Kit (ActiveMotif, Carlsbad, CA) according to the manufacturer's protocol and reverse cross-linked. DNA was cleaned using the DNA cleanup kit (Qiagen), and isolated DNA was amplified by real-time PCR using primers targeting a CpG dense and a non-CpG dense region of the *Csf1r* promoter. The primer sequences for the non-CpG dense region (primer set 1) were CAGGAACAGACTTGAAGCGT (forward) and CCGCTGAACCAGGTCTTCTTA (reverse) and for the CpG dense region (primer set 2) were ATCCCCTGGAGGCTATGGAG (forward) and TCTTTGCAACACTCCCCCAG (reverse).

Immunoblotting

Cell lysates were collected in lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor (Roche) and phosphatase inhibitor (EMD Chemicals) cocktails. Proteins were resolved by SDS-PAGE, transferred

to nitrocellulose membranes, and immunoblotted with antibodies against the PKA-dependent phosphoproteins phosphorylated vasodilator stimulated phosphoprotein (VASP, 1:1000, Cell Signaling) or phosphorylated cAMP response element binding (CREB, 1:1000 Cell Signaling), and the loading control α-tubulin (1:1000, Sigma-Aldrich). Membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence reagent (GE Healthcare). Densitometry was performed on visualized bands using Image J from the National Institutes of Health (Bethesda, MD).

cAMP assay

Bone marrow cells were cultured in L929 supernatant for 6 days; cells were lysed in 0.1 M HCl and cAMP levels were assayed using a colorimetric kit from Enzo (Farmingdale, NY).

Interleukin (IL)-6, IL-10, and Tumor Necrosis Factor (TNF)-α ELISA

Bone marrow cells were cultured in L929 supernatant for 6 days in the presence or absence of 500 μM PKA agonist (6-bnz-cAMP). Nonadherent cells were washed, and adherent cells were re-plated at equal number for 1 h stimulation with 20 ng/ml of recombinant CSF-1 (Peprotech, Rocky Hill, NJ) followed by 24 h stimulation with 100 ng/ml of lipopolysaccharide (LPS) (Sigma, St Louis, MO). Cell supernatants were cleared of cells by centrifugation, and supernatant IL-6, IL-10, and TNF-α levels were assayed by ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis

Data were analyzed on GraphPad Prism 6.0 (GraphPad Prism Software, San Diego, CA) using ANOVA or Student's t-test, as appropriate, with $P < 0.05$ defined as statistically significant. Data are expressed as mean ± SE measurement.

Results

Maturation of macrophages was associated with a decrease in DNA methylation at the Csf1r promoter and FIRE

Transcription of *Csf1r* has been previously shown to occur at low levels in hematopoietic stem cells [14] and to be selectively upregulated in mature macrophages, with its upregulation mediated by transcription factor assembly at the promoter and FIRE enhancer region [20]. Alterations in histone modifications have been shown to contribute to the increased expression of CSF1R during macrophage maturation [20]. There are six CpG loci closely clustered within a 100 bp-region in the promoter/exon 2 region of *Csf1r*, and a relatively high density of CpG loci clustered within 200 bp-regions located in the FIRE enhancer as well as 1 kb upstream of FIRE (FIRE -1 kb) (Fig 1a). Although methylation-sensitive restriction enzymes were used to show on average that DNA methylation was low in the promoter and FIRE regions of both myeloid progenitors and mature macrophages [16], we used bisulfite sequencing to more sensitively quantitate the DNA methylation levels at each of the CpG loci within the promoter, FIRE, and FIRE -1 kb during normal macrophage maturation. DNA methylation levels decreased at each of the CpG sites within the promoter (Fig 1b) and FIRE -1 kb (Fig 1c) regions as macrophages matured over time. Certain CpG sites in the FIRE enhancer region also exhibited decreased methylation during macrophage maturation, although baseline levels of methylation were much lower in the FIRE versus FIRE -1 kb and promoter regions (Fig 1d).

Activation of PKA inhibited Csf1r mRNA expression and increased DNA methylation at the Csf1r promoter

We previously demonstrated that prostaglandin E_2 (PGE_2) signaled through PKA to inhibit macrophage maturation and that treatment of bone marrow cells at day 0 with a PKA agonist, 6-bnz-cAMP, resulted in decreased numbers of CSF1R-expressing $F4/80^{pos}$ $CD11b^{pos}$ mature macrophages [25]. To determine whether PKA operated at the transcriptional or post-transcriptional level to inhibit expression of CSF1R, bone marrow cells were treated at day 0 with the PKA agonist 6-bnz-cAMP, and CSF1R protein and mRNA levels were assayed at days 3 and 6 during macrophage maturation. As expected,

cell surface expression of CSF1R increased between days 3 and 6 during macrophage maturation, and expression was inhibited in the presence of a PKA agonist (Fig 2a). Examination of mRNA levels indicated that the increase in CSF1R protein expression during maturation and its inhibition with a PKA agonist were accompanied by corresponding changes in *Csf1r* mRNA (Fig 2b).

Treatment with the PKA agonist was associated with DNA hypermethylation of Csf1r promoter and decreased PU.1 binding

We next examined the effect of activating PKA on the DNA methylation of the *Csf1r* promoter, FIRE -1 kb, and FIRE regions. Treatment with the PKA agonist resulted in increased *Csf1r* DNA methylation, particularly within the promoter (Fig 3a). A mild increase in DNA methylation was also noted in the FIRE -1 kb region (Fig 3b), and no change in DNA methylation was observed at the FIRE enhancer region (Fig 3c). To determine whether the increase in DNA methylation was specific to the activation of PKA, bone marrow cells were treated with 8-pCPT-2'-*O*-Me-cAMP, a cAMP analog that specifically activates the alternative cAMP effector, the cAMP-activated guanine nucleotide exchange protein Epac, but not PKA. Just as we had previously shown that inhibition of macrophage maturation by cAMP was a specific consequence of the activation of PKA and not Epac [25], treatment with the Epac agonist did not increase the DNA methylation of *Csf1r* promoter as was observed with the PKA agonist (Fig 3d).

PU.1 is a critical transcription factor involved in monocyte/macrophage differentiation [28, 29] and it upregulates expression of CSF1R [30, 31]. We next sought to determine whether binding of PU.1 to the *Csf1r* promoter was associated with the changes in DNA methylation observed after treatment with a PKA agonist. Treatment of bone marrow cells with the PKA agonist did not significantly affect expression of PU.1 (data not shown). We next performed ChIP using an antibody specific to PU.1 and observed that there was decreased PU.1 binding to the *Csf1r* promoter in cells treated with the PKA agonist (Fig 4). The decrease in PU.1 binding was specific to the region where DNA hypermethylation was observed; by comparison, an upstream region without DNA

hypermethylation showed no difference in PU.1 binding between cells treated with or without PKA. These results demonstrate that activation of PKA leads to both DNA hypermethylation and decreased PU.1 binding at specific regions within the *Csf1r* promoter.

Macrophage maturation was associated with a decrease in endogenous PKA activity

PKA can be activated by a myriad of signals and, given the profound ability of an exogenous PKA agonist to inhibit macrophage maturation, we sought to determine whether normal macrophage maturation was associated with a decrease in endogenous levels of PKA activity. Phosphorylation of the PKA substrates VASP and CREB are commonly used readouts of intracellular PKA activity, and levels of phosphorylated VASP decreased over time during normal macrophage maturation (Fig 5a). A similar decrease in phosphorylated CREB was also observed (Fig 5b) suggesting that diminished activity of PKA accompanies normal macrophage maturation. PKA is activated by cAMP, and intracellular levels of cAMP were likewise lower at days 3 and 5 during macrophage maturation compared to day 0 (Fig 5c). Treatment of bone marrow cells with the phosphodiesterase inhibitor IBMX, which inhibits cAMP degradation, resulted in both higher levels of cAMP and decreased expression of CSF1R (Fig 5d and 5e). This finding inversely links cAMP levels to expression of CSF1R, indicating that the increased expression of CSF1R during macrophage maturation is attributable to the decrease in endogenous levels of cAMP and PKA activity.

Treatment of bone marrow cells with a PKA agonist inhibited macrophage maturation and cytokine generation

To determine the functional consequence of inhibiting macrophage maturation on generation of immunoregulatory cytokines – a property of mature macrophages – adherent bone marrow cells treated with or without 6-bnz-cAMP (500 μM) for 6 days were re-plated, washed and treated for 1 h with CSF-1 (20 ng/ml) followed by 24 h of LPS (100 ng/ml), and IL-6, IL-10, and TNF-α levels were assayed. Cells treated with the PKA agonist for 6 days demonstrated markedly reduced ability to synthesize IL-6, IL-10, and

TNF-α in response to CSF-1 in the absence or presence of LPS (Fig 6). As previously reported, treatment with the PKA agonist did not affect cell viability [25], suggesting that the impaired responsiveness to CSF-1 and LPS is a consequence of lower CSF1R expression and generally impaired bone marrow macrophage maturation, and not due to decreased cell numbers or apoptosis.

Expression of TET and DNMT proteins during macrophage maturation

DNMTs are responsible for the addition of methyl groups to DNA and consist of three catalytically active isoforms – DNMT1, -3a, and -3b – and DNMT3L, which is catalytically inactive, but facilitates the actions of DNMT3a and -3b. TET enzymes were recently discovered to add hydroxyl groups to methylated cytosine [32, 33], resulting in base-excision repair and active demethylation. Given that macrophage maturation is associated with a decrease in *Csf1r* methylation, and that methylation is increased when PKA is activated, we examined the expression of various TET and DNMT isoforms during macrophage maturation in the presence or absence of a PKA agonist. Concurrent with a decrease in *Csf1r* methylation, TET2 and TET3 expression increased during macrophage maturation, which was inhibited in the presence of a PKA agonist (Fig 7a-c). In parallel, expression of DNMT1, DNMT3b, and DNMT3L decreased during macrophage maturation, but only DNMT3L expression increased in the presence of the PKA agonist (Fig 7d-g).

Silencing TET and DNMT expression did not significantly alter Csf1r expression during macrophage maturation or during treatment with a PKA agonist

Since TET2 and TET3 expression increased during macrophage maturation and their actions are associated with demethylation, we sought to determine whether silencing TET2 or TET3 would result in persistent hypermethylation of *Csf1r* and decreased *Csf1r* mRNA levels during maturation. Bone marrow cells were treated with control, TET2, or TET3 siRNA at both day 0 and day 1, and levels of *Csf1r* mRNA were determined at day 3. Neither silencing of TET2 nor TET3 inhibited the increase of *Csf1r* expression at day 3 of maturation (Fig 8a-b).

DNMT3L was the only DNMT isoform whose expression decreased with time as early as 3 days and also increased in the presence of a PKA agonist, making it the best candidate DNMT whose dynamic alterations in expression might explain both the hypomethylation of *Csf1r* observed during macrophage maturation and the hypermethylation of *Csf1r* seen during treatment with a PKA agonist. We thus used siRNA to silence expression of DNMT3L to determine if this is sufficient to abolish the actions of the PKA agonist. Although we were able to achieve a robust knockdown of DNMT3L expression with siRNA (Fig 8c), the PKA agonist was still able to decrease expression of CSF1R, suggesting that the increase in DNMT3L by PKA is not responsible for *Csf1r* hypermethylation (Fig 8d). Since DNMT3L itself is enzymatically inactive and binds to DNMT3a and DNMT3b to stimulate their activity [34], we also examined levels of *Csf1r* mRNA when DNMT3a and DNMT3L, and when DNMT3b and DNMT3L were silenced together. Neither the combined silencing of DNMT3a and DNMT3L nor the combined silencing of DNMT3b and DNMT3L were sufficient to inhibit the ability of the PKA agonist to suppress *Csf1r* expression (data not shown).

Discussion

CSF1R is the major receptor for CSF-1, and upregulation of CSF1R is essential for monocyte/macrophage development and differentiation [11]. PGE_2 and other cAMP-elevating agents were described many years ago to inhibit CSF-1-mediated macrophage responses [22-24], and we recently showed that through PKA signaling, these mediators inhibit CSF1R expression and macrophage maturation [25]. Here, we show that activation of PKA led to an increase in the DNA methylation of the *Csf1r* gene at both the promoter and FIRE -1 kb regions. This was associated with a decrease in PU.1 binding to the gene promoter and a decrease in *Csf1r* mRNA levels. Macrophage maturation was accompanied by both a decrease in basal levels of *Csf1r* DNA methylation and a decrease in intrinsic PKA activity, suggesting that endogenous PKA activity in bone marrow progenitors play a role in suppressing expression of *Csf1r*. These results demonstrate how PKA signaling

may influence the dynamic changes in DNA methylation of key genes crucial to monocyte/macrophage differentiation.

PKA was one of the first-described canonical signaling kinases, and has since been shown to modulate a range of diverse cellular functions including proliferation, differentiation, and survival [35, 36], often through the activation of other downstream mediators and transcription factors such as CREB. Our results demonstrated that PKA is also capable of inducing DNA methylation, in this case, at the *Csf1r* gene locus. A previous study noted that PKA activation increased DNA methylation and histone methylation in embryonic stem cells [37]. Ours is the first to show that PKA activation affected DNA methylation in bone marrow cells.

The regulation of CSF1R expression is complex, and levels of *Csf1r* mRNA levels are dependent on the dynamic interaction of transcription factors, chromatin remodeling, and DNA methylation changes at the *Csf1r* promoter and enhancer regions [13, 18]. Transcription factors such as PU.1 have been identified to sit poised on the *Csf1r* promoter even in myeloid progenitors which express low levels of *Csf1r* mRNA [14], and through chromatin remodeling and transcription factor binding at the FIRE enhancer region, transcription of *Csf1r* is upregulated in mature macrophages [20]. These studies have suggested that DNA methylation plays a minor role in regulating *Csf1r* expression in hematopoietic stem cells as methylation levels at the *Csf1r* promoter and FIRE enhancer regions were low in early myeloid progenitors [16]. Our study, however, suggests that the DNA methylation levels of *Csf1r* in bone marrow cells are readily measurable and that DNA methylation levels in fact decrease over time with macrophage maturation. Heterogeneity in bone marrow cells and our use of a more sensitive, quantitative assay for DNA methylation at specific CpG loci may explain differences between our data and other studies.

Our results showed that decreased DNA methylation levels are associated with increased *Csf1r* mRNA expression during maturation and that hypermethylation in the presence of increased PKA activity is associated with its decreased expression. We

recognize that we cannot ascertain whether DNA methylation changes are responsible for *Csf1r* expression, or whether DNA methylation changes are merely a consequence of changes in transcription factors and chromatin modifications that regulate *Csf1r* expression. Treatment of cells with the PKA agonist inhibited PU.1 binding to the *Csf1r* promoter, and examination of other possible chromatin modifications induced by the PKA agonist was beyond the scope of our study. Expression of PU.1 was not significantly affected by treatment with the PKA agonist, although it has been reported that increased cAMP levels affects PU.1 transcription [38] and decreases PU.1-DNA binding in other experimental contexts [39-41]. Precedent for the ability of DNA methylation to regulate *Csf1r* expression, however, was best exemplified in fibroblasts, T cells, and leukemic cells that exhibited high levels of *Csf1r* methylation, which correlated strongly with inhibition of *Csf1r* expression [16, 42, 43].

We were unable to determine the relative importance of increased *Csf1r* methylation vs. inhibited demethylation in response to activation of PKA, as expression of various DNMT and TET enzymes all changed with time and with PKA treatment. TET2 and TET3, which participate in DNA hydroxymethylation and lead to demethylation [32, 33], were observed to increase with maturation, and decrease by the PKA agonist. Silencing of TET2 and TET3 during normal macrophage maturation, however, still resulted in an increase in *Csf1r* expression, suggesting that progressive hypomethylation of *Csf1r* cannot be explained purely by the increase in TET2 or TET3 during normal maturation. Notably, we were only able to effectively silence TET2 and TET3 by ~50%, and complete knockdown of these genes may be required to conclusively rule out a role for these enzymes in mediating *Csf1r* hypomethylation. In addition, TET2 and TET3 expression was not observed to increase until day 5, indicating that these enzymes may still have a role in later stages of macrophage maturation. Among the DNMT isoforms, only DNMT3L showed both a progressive loss of expression during maturation and an increase in expression with addition of the PKA agonist, suggesting its potential involvement in *Csf1r* demethylation during maturation and hypermethylation in the presence of active PKA. Targeted silencing of DNMT3L, however, did not affect *Csf1r* mRNA levels. Furthermore,

combined silencing of both DNMT3L and its interacting partners DNMT3a/DNMT3b also did not affect the ability of the PKA agonist to inhibit *Csf1r* expression. These data initially suggest that these enzymes are not required for the hypermethylation of *Csf1r* observed after the addition of a PKA agonist. However, an alternative hypothesis is that hypomethylation of *Csf1r* during normal maturation and hypermethylation in the presence of a PKA agonist may be the consequence of a more complex interplay between transcription factors and histone modifying enzymes that recruit DNMTs and TETs. PKA may interfere with PU.1 binding which may indirectly result in *Csf1r* hypermethylation. Furthermore, the simultaneous silencing of DNMT and overexpression of TET isoforms may be what is necessary to inhibit the hypermethylation of *Csf1r* by the PKA agonist. Further studies would also be needed to determine whether PKA induces global hyper- or hypomethylation of genes across the genome in macrophages and myeloid progenitors. Of note, we previously reported that PGE_2 increased expression of DNMT3a in fibroblasts, resulting in global changes to DNA methylation in these cells [44]. Changes in DNMT and TET isoform expression in myeloid progenitors may thus affect the DNA methylation of other genes that may be important to macrophage maturation.

We treated cells with a PKA agonist to examine the effects of PKA activity on *Csf1r* methylation, but we also observed that macrophage maturation is itself associated with a decrease in endogenous PKA activity. PKA is traditionally activated by cAMP, which can be increased by various mediators including PGE_2, which has been shown to inhibit a variety of macrophage functions including phagocytosis, bacterial killing, and cytokine generation [21, 45]. PGE_2 also inhibits macrophage differentiation [25] and hematopoiesis in general [46, 47]. Our results demonstrating that PKA activation alters the DNA methylation profile of a key gene in macrophage maturation may partly explain how PGE_2 inhibits hematopoiesis. However, we also previously showed that PGE_2 levels do not decline during macrophage maturation [25], so the decline in endogenous cAMP levels and PKA activity during normal maturation is likely to reflect changes in some cAMP-coupled receptor ligand other than PGE_2. Epac is another intracellular cAMP effector molecule, and a selective Epac agonist failed to alter *Csf1r* DNA methylation levels. It is

interesting to note that Epac expression has been reported to increase during monocyte/macrophage differentiation, and that Epac becomes functional in mature macrophages [48]. Taken together with our contrasting results showing decreased endogenous PKA activity during macrophage differentiation, these findings suggest that there may be a coordinated switch in the effector molecules engaged by cAMP – from PKA to Epac – during macrophage differentiation. Finally, it should be mentioned that we did not examine whether PGE_2 or PKA post-transcriptionally regulates CSF1R expression, and a recent study suggests that PGE_2 may activate CSF1R through phosphorylation [49].

Mononuclear cell maturation *in vitro* was accomplished herein through a well-established method described in the literature [26, 27]. Freshly obtained bone marrow cells were not pre-sorted in our studies and one might speculate that the effects of PKA activation on *Csf1r* methylation may be due to the expansion of hematopoietic stem cells or other nonmyeloid cell types rather than direct actions on mononuclear phagocytes. However, since adherence is characteristic of macrophages, wash steps and adherence purification during maturation ensured that macrophages were the predominant cell type in our studies. Although our studies were performed *in vitro*, we have previously shown that the ability of PKA to inhibit macrophage development is applicable *in vivo* in thioglycollate induced peritonitis [25], with important consequences as demonstrated in mouse models of infection and asthma [50]. Furthermore, the decrease in endogenous PKA activity observed during normal maturation indicates that mediators that regulate this signaling pathway are relevant for physiologic homeostasis.

CSF-1 activates many different macrophage functions, including phagocytosis, production of reactive oxygen species, chemotaxis, and microbial killing [51], and understanding regulation of CSF1R and its promoter DNA methylation has functional implications beyond differentiation. We observed that inhibition of CSF1R expression by PKA was associated with diminished cytokine expression in the presence of LPS. Other studies have furthermore demonstrated that loss of CSF1R expression may contribute to oncogenic transformation in leukemia [42, 52, 53]. In conclusion, we show that the progressive increase in CSF1R expression during monocyte/macrophage maturation is

accompanied by progressive demethylation at the *Csf1r* gene and that PKA inhibition of *Csf1r* mRNA expression correlated with DNA hypermethylation. PGE_2, which activates PKA, can inhibit the maturation of macrophages; we now show for the first time that this intracellular signaling pathway is accompanied by modulation of DNA methylation and DNA methylation machinery. These studies demonstrate how epigenetic levels change during hematopoiesis and their regulation by PKA, and in a broader context, provide an illustration of how intracellular signaling pathways are capable of regulating DNA methylation patterns during differentiation and development.

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Author contributions

ZZ and SKH designed the study, ZZ, AMS, and SKH performed the experiments, and ZZ, MPG, and SKH wrote the paper.

Conflicts of Interest

None

References

1. Gordon S, Taylor PR: **Monocyte and macrophage heterogeneity**. *Nat Rev Immunol* 2005, **5**(12):953-964.
2. Epelman S, Lavine KJ, Randolph GJ: **Origin and functions of tissue macrophages**. *Immunity* 2014, **41**(1):21-35.

3. Becker S, Warren MK, Haskill S: **Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures**. *Journal of immunology* 1987, **139**(11):3703-3709.
4. Dai XM, Zong XH, Sylvestre V, Stanley ER: **Incomplete restoration of colony-stimulating factor 1 (CSF-1) function in CSF-1-deficient Csf1op/Csf1op mice by transgenic expression of cell surface CSF-1**. *Blood* 2004, **103**(3):1114-1123.
5. Chitu V, Stanley ER: **Colony-stimulating factor-1 in immunity and inflammation**. *Current opinion in immunology* 2006, **18**(1):39-48.
6. Hamilton JA: **Colony-stimulating factors in inflammation and autoimmunity**. *Nature reviews Immunology* 2008, **8**(7):533-544.
7. Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y, Yeung YG: **Biology and action of colony--stimulating factor-1**. *Molecular reproduction and development* 1997, **46**(1):4-10.
8. Wei S, Dai XM, Stanley ER: **Transgenic expression of CSF-1 in CSF-1 receptor-expressing cells leads to macrophage activation, osteoporosis, and early death**. *Journal of leukocyte biology* 2006, **80**(6):1445-1453.
9. Di Gregoli K, Johnson JL: **Role of colony-stimulating factors in atherosclerosis**. *Current opinion in lipidology* 2012, **23**(5):412-421.
10. Flanagan AM, Lader CS: **Update on the biologic effects of macrophage colony-stimulating factor**. *Curr Opin Hematol* 1998, **5**(3):181-185.
11. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, Sylvestre V, Stanley ER: **Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects**. *Blood* 2002, **99**(1):111-120.
12. Hu M, Krause D, Greaves M, Sharkis S, Dexter M, Heyworth C, Enver T: **Multilineage gene expression precedes commitment in the hemopoietic system**. *Genes & development* 1997, **11**(6):774-785.
13. Tagoh H, Melnik S, Lefevre P, Chong S, Riggs AD, Bonifer C: **Dynamic reorganization of chromatin structure and selective DNA demethylation prior to stable enhancer complex formation during differentiation of primary hematopoietic cells in vitro**. *Blood* 2004, **103**(8):2950-2955.
14. Tagoh H, Himes R, Clarke D, Leenen PJ, Riggs AD, Hume D, Bonifer C: **Transcription factor complex formation and chromatin fine structure alterations at the murine c-**

fms (CSF-1 receptor) locus during maturation of myeloid precursor cells. *Genes & development* 2002, **16**(13):1721-1737.

15. Borzillo GV, Ashmun RA, Sherr CJ: **Macrophage lineage switching of murine early pre-B lymphoid cells expressing transduced fms genes**. *Molecular and cellular biology* 1990, **10**(6):2703-2714.
16. Tagoh H, Schebesta A, Lefevre P, Wilson N, Hume D, Busslinger M, Bonifer C: **Epigenetic silencing of the c-fms locus during B-lymphopoiesis occurs in discrete steps and is reversible**. *The EMBO journal* 2004, **23**(21):4275-4285.
17. Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, Cook AD, Himes SR, Hamilton JA, Hume DA: **Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1**. *Journal of leukocyte biology* 2007, **82**(1):111-123.
18. Bonifer C, Hume DA: **The transcriptional regulation of the Colony-Stimulating Factor 1 Receptor (csf1r) gene during hematopoiesis**. *Frontiers in bioscience : a journal and virtual library* 2008, **13**:549-560.
19. Chambers SK, Gilmore-Hebert M, Wang Y, Rodov S, Benz EJ, Jr., Kacinski BM: **Posttranscriptional regulation of colony-stimulating factor-1 (CSF-1) and CSF-1 receptor gene expression during inhibition of phorbol-ester-induced monocytic differentiation by dexamethasone and cyclosporin A: potential involvement of a destabilizing protein**. *Experimental hematology* 1993, **21**(10):1328-1334.
20. Krysinska H, Hoogenkamp M, Ingram R, Wilson N, Tagoh H, Laslo P, Singh H, Bonifer C: **A two-step, PU.1-dependent mechanism for developmentally regulated chromatin remodeling and transcription of the c-fms gene**. *Molecular and cellular biology* 2007, **27**(3):878-887.
21. Aronoff DM, Canetti C, Serezani CH, Luo M, Peters-Golden M: **Cutting edge: macrophage inhibition by cyclic AMP (cAMP): differential roles of protein kinase A and exchange protein directly activated by cAMP-1**. *Journal of immunology* 2005, **174**(2):595-599.
22. Hume DA, Gordon S: **The correlation between plasminogen activator activity and thymidine incorporation in mouse bone marrow-derived macrophages. Opposing**

actions of colony-stimulating factor, phorbol myristate acetate, dexamethasone and prostaglandin E. *Experimental cell research* 1984, **150**(2):347-355.

23. Hamilton JA, Vairo G, Knight KR, Cocks BG: **Activation and proliferation signals in murine macrophages. Biochemical signals controlling the regulation of macrophage urokinase-type plasminogen activator activity by colony-stimulating factors and other agents**. *Blood* 1991, **77**(3):616-627.
24. Vairo G, Argyriou S, Bordun AM, Whitty G, Hamilton JA: **Inhibition of the signaling pathways for macrophage proliferation by cyclic AMP. Lack of effect on early responses to colony stimulating factor-1**. *The Journal of biological chemistry* 1990, **265**(5):2692-2701.
25. Zaslona Z, Serezani CH, Okunishi K, Aronoff DM, Peters-Golden M: **Prostaglandin E2 restrains macrophage maturation via E prostanoid receptor 2/protein kinase A signaling**. *Blood* 2012, **119**(10):2358-2367.
26. Boltz-Nitulescu G, Wiltschke C, Holzinger C, Fellinger A, Scheiner O, Gessl A, Forster O: **Differentiation of rat bone marrow cells into macrophages under the influence of mouse L929 cell supernatant**. *Journal of leukocyte biology* 1987, **41**(1):83-91.
27. Stanley ER, Heard PM: **Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells**. *The Journal of biological chemistry* 1977, **252**(12):4305-4312.
28. DeKoter RP, Walsh JC, Singh H: **PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors**. *The EMBO journal* 1998, **17**(15):4456-4468.
29. Scott EW, Simon MC, Anastasi J, Singh H: **Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages**. *Science* 1994, **265**(5178):1573-1577.
30. Ross IL, Yue X, Ostrowski MC, Hume DA: **Interaction between PU.1 and another Ets family transcription factor promotes macrophage-specific Basal transcription initiation**. *The Journal of biological chemistry* 1998, **273**(12):6662-6669.
31. Yue X, Favot P, Dunn TL, Cassady AI, Hume DA: **Expression of mRNA encoding the macrophage colony-stimulating factor receptor (c-fms) is controlled by a constitutive promoter and tissue-specific transcription elongation**. *Molecular and cellular biology* 1993, **13**(6):3191-3201.

32. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y: **Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification**. *Nature* 2010, **466**(7310):1129-1133.
33. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L *et al*: **Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1**. *Science* 2009, **324**(5929):930-935.
34. Liao HF, Tai KY, Chen WS, Cheng LC, Ho HN, Lin SP: **Functions of DNA methyltransferase 3-like in germ cells and beyond**. *Biology of the cell / under the auspices of the European Cell Biology Organization* 2012, **104**(10):571-587.
35. Huang SK, Wettlaufer SH, Chung J, Peters-Golden M: **Prostaglandin E2 inhibits specific lung fibroblast functions via selective actions of PKA and Epac-1**. *American journal of respiratory cell and molecular biology* 2008, **39**(4):482-489.
36. Schmitt JM, Stork PJ: **PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1**. *Molecular cell* 2002, **9**(1):85-94.
37. Yamamizu K, Fujihara M, Tachibana M, Katayama S, Takahashi A, Hara E, Imai H, Shinkai Y, Yamashita JK: **Protein kinase A determines timing of early differentiation through epigenetic regulation with G9a**. *Cell stem cell* 2012, **10**(6):759-770.
38. Pongubala JM, Atchison ML: **Activating transcription factor 1 and cyclic AMP response element modulator can modulate the activity of the immunoglobulin kappa 3' enhancer**. *The Journal of biological chemistry* 1995, **270**(17):10304-10313.
39. Shackelford R, Adams DO, Johnson SP: **IFN-gamma and lipopolysaccharide induce DNA binding of transcription factor PU.1 in murine tissue macrophages**. *J Immunol* 1995, **154**(3):1374-1382.
40. Park SY, Lee SW, Baek SH, Lee CW, Lee WS, Rhim BY, Hong KW, Kim CD: **Suppression of PU.1-linked TLR4 expression by cilostazol with decrease of cytokine production in macrophages from patients with rheumatoid arthritis**. *Br J Pharmacol* 2013, **168**(6):1401-1411.
41. Saeki K, Saeki K, Yuo A: **Distinct involvement of cAMP-response element-dependent transcriptions in functional and morphological maturation during retinoid-mediated human myeloid differentiation**. *Journal of leukocyte biology* 2003, **73**(5):673-681.
42. Felgner J, Kreipe H, Heidorn K, Jaquet K, Zschunke F, Radzun HJ, Parwaresch MR: **Increased methylation of the c-fms protooncogene in acute myelomonocytic leukemias**.

Pathobiology : journal of immunopathology, molecular and cellular biology 1991, **59**(4):293-298.

43. Follows GA, Tagoh H, Richards SJ, Melnik S, Dickinson H, de Wynter E, Lefevre P, Morgan GJ, Bonifer C: **c-FMS chromatin structure and expression in normal and leukaemic myelopoiesis**. *Oncogene* 2005, **24**(22):3643-3651.
44. Huang SK, Scruggs AM, Donaghy J, McEachin RC, Fisher AS, Richardson BC, Peters-Golden M: **Prostaglandin E(2) increases fibroblast gene-specific and global DNA methylation via increased DNA methyltransferase expression**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2012, **26**(9):3703-3714.
45. Kim SH, Serezani CH, Okunishi K, Zaslona Z, Aronoff DM, Peters-Golden M: **Distinct protein kinase A anchoring proteins direct prostaglandin E2 modulation of Toll-like receptor signaling in alveolar macrophages**. *J Biol Chem* 2011, **286**(11):8875-8883.
46. Gentile P, Byer D, Pelus LM: **In vivo modulation of murine myelopoiesis following intravenous administration of prostaglandin E2**. *Blood* 1983, **62**(5):1100-1107.
47. Hoggatt J, Mohammad KS, Singh P, Hoggatt AF, Chitteti BR, Speth JM, Hu P, Poteat BA, Stilger KN, Ferraro F *et al*: **Differential stem- and progenitor-cell trafficking by prostaglandin E2**. *Nature* 2013, **495**(7441):365-369.
48. Bryn T, Mahic M, Enserink JM, Schwede F, Aandahl EM, Tasken K: **The cyclic AMP-Epac1-Rap1 pathway is dissociated from regulation of effector functions in monocytes but acquires immunoregulatory function in mature macrophages**. *J Immunol* 2006, **176**(12):7361-7370.
49. Digiacomo G, Ziche M, Dello Sbarba P, Donnini S, Rovida E: **Prostaglandin E2 transactivates the colony-stimulating factor-1 receptor and synergizes with colony-stimulating factor-1 in the induction of macrophage migration via the mitogen-activated protein kinase ERK1/2**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2015, **29**(6):2545-2554.
50. Zaslona Z, Przybranowski S, Wilke C, van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, Wilkinson JE, Moore BB, Peters-Golden M: **Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma**. *Journal of immunology* 2014, **193**(8):4245-4253.
51. Barreda DR, Hanington PC, Belosevic M: **Regulation of myeloid development and function by colony stimulating factors**. *Dev Comp Immunol* 2004, **28**(5):509-554.

52. Boultwood J, Rack K, Kelly S, Madden J, Sakaguchi AY, Wang LM, Oscier DG, Buckle VJ, Wainscoat JS: **Loss of both CSF1R (FMS) alleles in patients with myelodysplasia and a chromosome 5 deletion**. *Proc Natl Acad Sci U S A* 1991, **88**(14):6176-6180.

53. Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, Akashi K, Fiering S, Tenen DG: **Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1**. *Nat Genet* 2004, **36**(6):624-630.

Figure Legends

Figure 1. The DNA methylation of *Csf1r* is decreased during macrophage maturation. Cells from murine bone marrow were matured in L929 conditioned medium and DNA was collected from cells at days 0, 3, and 5. Bisulfite sequencing, as described in Methods, was performed to assess the DNA methylation of CpG sites within the *Csf1r* gene, as illustrated (a). DNA methylation of CpG sites were analyzed within the gene promoter (b), 1 kb upstream of fms intronic regulatory element (FIRE -1 kb) (c), and FIRE (d) regions (n=3). * $P < 0.05$.

Figure 2. Expression of *Csf1r* mRNA was decreased in bone marrow cells treated with a PKA agonist. Cells from murine bone marrow were matured in L929 conditioned medium for 3 or 6 days in the presence or absence of the PKA agonist 6-bnz-cAMP (500 μM). (a) CSF1R cell surface protein expression was assayed by flow cytometry; the mean fluorescence intensity from a representative experiment of three independent experiments is shown. (b) *Csf1r* mRNA levels were assayed by RT-PCR (n=3). * $P < 0.05$.

Figure 3. Treatment of BM cells with a PKA agonist was associated with an increase in *Csf1r* DNA methylation. (a-c) Cells from murine bone marrow were matured in L929 conditioned medium for 3 or 5 days in the presence or absence of the PKA agonist 6-bnz-cAMP (500 μM). The DNA methylation of CpG sites within the Csf1r promoter (a), FIRE

-1 kb (b), and FIRE (c) were assayed by pyrosequencing (n=3). * $P < 0.05$. (d) Bone marrow cells were treated with either the PKA agonist 6-bnz-cAMP (500 μM), the exchange protein activated by cAMP (Epac) agonist 8-pCPT-2'-*O*-Me-cAMP (500 μM), or vehicle control and DNA methylation within the *Csf1r* promoter was assayed. Shown is a representative result from two independent experiments.

Figure 4. Treatment of cells with the PKA agonist resulted in decreased PU.1 binding at the *Csf1r* promoter. Cells from murine bone marrow were matured in L929 conditioned medium for 3 days in the presence or absence of the PKA agonist 6-bnz-cAMP (500 μM). Chromatin was isolated and immunoprecipitated using an antibody to PU.1 or isotype control. Real-time PCR was performed using primers illustrated in (a), and the relative enrichment from cells treated with or without the PKA agonist is shown for primer set 1 (b) and primer set 2 (c) (n=3). * $P < 0.05$.

Figure 5. Macrophage maturation was associated with a decrease in endogenous cAMP levels and PKA activity. Cells from murine bone marrow were matured to macrophages in L929 conditioned medium over 5-6 days. Levels of phosphorylated vasodilator-stimulated phosphoprotein (VASP) (a) and phosphorylated cAMP responsive element binding (CREB) protein (b) were assayed by immunoblot. Representative blot (n=3) is shown. (c) Levels of cAMP were assayed from the lysates of maturing cells at days 0, 3, and 5. Results are normalized to control levels (n=3). Bone marrow cells were given IBMX (250 μM), an inhibitor of the cAMP-degradative enzyme phosphodiesterase, for 6 days and levels of cAMP (d) and CSF1R mRNA (e) were assayed (n=3). * $P < 0.05$.

Figure 6. Inhibition of macrophage maturation with a protein kinase A (PKA) agonist attenuated the generation of immunoregulatory cytokines in response to macrophage colony stimulating factor (CSF)-1 and lipopolysaccharide (LPS). Bone marrow derived monocytes/macrophages (BMDM) were matured in L929 conditioned medium for 5 days in the presence or absence of the PKA agonist 6-bnz-cAMP (500 μM). Cells were then re-plated and treated for 1 h +/- MCSF (20 ng/ml) and then subsequently for 24 h +/- LPS

(100ng/ml) and supernatants were assayed for levels of interleukin (IL)-6 (a), IL-10 (b), and tumor necrosis factor (TNF)-α (c) (n=3 for each experiment).

Figure 7. PKA altered expression of DNA methyltransferases (DNMTs) and Ten-eleven Translocation (TET) proteins during macrophage maturation. Bone marrow cells were treated with or without the PKA agonist 6-bnz-cAMP (500 μM) during macrophage maturation and levels of (a) TET1, (b) TET2, (c) TET3, (d) DNMT1, (e) DNMT3a, (f) DNMT3b, and (g) DNMT3L mRNA were assayed by RT-PCR. n=6, $P < 0.05$.

Figure 8. Silencing of TET2, TET3, or DNMT3L did not affect expression of *Csf1r* in the presence or absence of PKA. Bone marrow cells were treated with control siRNA or siRNA targeted against TET2 or TET3, as described in Methods, and levels of TET2 and TET3 mRNA (a) were assayed by RT-PCR (n=3). * $P < 0.05$ relative to control siRNA. ** $P < 0.01$ relative to control siRNA. (b) Levels of *Csf1r* mRNA were assayed by RT-PCR in cells treated with TET2 or TET3 siRNA (n=3). * $P < 0.05$ relative to control siRNA. NS, non-significant. (c-d) Bone marrow cells were treated with control siRNA or siRNA targeted against DNMT3L in the presence or absence of the PKA agonist 6-bnz-cAMP (500 μM), as described in Methods. Levels of DNMT3L mRNA (c, n=3, * $P < 0.05$ relative to control siRNA) and *Csf1r* (d, n=4, * $P < 0.05$ relative to day 0) were assayed by RT-PCR.