NF-κB Regulates PD-1 Expression in Macrophages

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Programmed cell death-1 (PD-1) is responsible for T cell exhaustion during chronic viral infections and is expressed on a variety of immune cells following activation. Despite its importance, the mechanisms that regulate PD-1 in cell types other than CD8 T cells are poorly defined. In this study, the molecular mechanisms for inducing PD-1 expression in CD4 T cells, macrophages, and B cells were explored. In CD4 T cells, PD-1 induction following TCR stimulation required NFAT, as the calcineurin/NFAT pathway inhibitor cyclosporin A was able to block PD-1 induction in a manner similar to that seen in CD8 T cells. In contrast, LPS but not PMA and ionomycin stimulation was able to induce PD-1 expression in macrophages in a manner insensitive to cyclosporin A–mediated inhibition. B cells could use both pathways, although the levels of PD-1 expression were highest with PMA and ionomycin. An NF-κB binding site located upstream of the gene in conserved region C was required for NF-κB–dependent PD-1 gene activation in macrophages. Chromatin immunoprecipitation showed NF-κB p65 binding to this region following stimulation of macrophages with LPS. PD-1 induction was associated with histone modifications characteristic of accessible chromatin; however, in contrast to CD8 T cells, conserved region B in macrophages did not lose CpG methylation upon stimulation and PD-1 expression. The linkage of TLR/NF-κB signaling to the induction of PD-1 suggests the possibility of an opportunistic advantage to microbial infections in manipulating immune inhibitory responses. The Journal of Immunology, 2015, 194: 4545–4554.
NF-κB REGULATES PD-1

Patients express substantially more PD-1 on the surface of blood monocytes compared with both aviremic HIV-infected individuals and healthy donors (29). When expressed on macrophages and monocytes, PD-1 expression correlates with increased IL-10 and decreased IL-12 levels in the blood of HIV-infected patients, which in turn limits T cell responses against the infection (29, 30). A variety of bacterial-derived TLR ligands, including LPS and CpG DNA, induce PD-1 expression on human macrophages (29), suggesting a role for TLR signaling pathways in regulating PD-1. Additionally, PD-1 expression in macrophages can be induced by multiple cytokines. IFN-α signaling through STAT1/2 heterodimers and an IFN-sensitive response element leads to increased PD-1 expression, as does treatment with TNF-α, IL-1β, or IL-6 (20, 22). However, cytokine-stimulated regulation of PD-1, particularly when signaling through STAT proteins or IFN response factors, does not correlate with the observed increases in PD-1 expression levels induced directly by TLR ligands in these cells nor does it adequately address modulation of PD-1 levels seen in vivo. Although no “exhausted”-like phenotype has been observed in macrophages expressing PD-1, the induced anti-inflammatory cytokine profile in PD-1–expressing macrophages has ramifications for the proper functioning of the immune system during infection.

TLR signaling is of vital importance in the early immune response prior to the engagement of the adaptive immune system and the corresponding cytokines secreted at that time. In response to cognate ligands, transcriptional signaling through TLRs is mediated by NF-κB (31). Although NF-κB activation can occur through multiple distinct pathways, this transcription factor has not been shown previously to be involved in PD-1 regulation. In this study, we have investigated the fundamental mechanisms that initiate PD-1 expression following cellular activation of B cells, CD4 T cells, and macrophages. Although each produced varied levels of induction, IgM crosslinking of B cells, TCR-mediated activation of CD4 T cells, or treatment with PMA and ionomycin (Io) resulted in PD-1 expression through pathways that were inhibited by cyclosporin A (CsA), implicating NFAT as the critical inducer/activator of PD-1 expression in these cell types. In contrast, following stimulation of macrophages with TLR ligands, PD-1 expression was induced by NF-κB. Furthermore, PD-1 expression in macrophages was unaffected by either engagement or disruption of the calcineurin pathway, making macrophages the first cell type found to regulate PD-1 independent of NFATc1. Following LPS stimulation, the NF-κB subunit p65 bound CR-C at a consensus site. p65 binding to CR-C was coupled with histone modifications associated with gene activation and increased accessibility of the region. Intriguingly, the CR-B region, which loses DNA methylation in a manner that follows PD-1 expression, contains cell line–specific MHC class II+ and MHC class II+ cells.

**Materials and Methods**

**Cell lines and mouse strains**

The murine macrophage line RAW264.7 and fibroblast cell line L929 were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Primary CD4 T cells and primary B cells were isolated from wild-type C57BL/6 mouse spleens using negative selection magnetic bead separation columns (Miltenyi Biotec, San Diego, CA) according to the manufacturer’s instructions. Primary bone marrow–derived macrophages (BMDMs) were prepared from femur bone marrow of C57BL/6 or MyD88<sup>−/−</sup> mice as previously described (32). Briefly, bone marrow cells were isolated from mouse femurs and cultured for 7 d in DMEM with 10% FBS, 100 U/ml penicillin/streptomycin, and 20% L-929 cell–conditioned media, which contains cell line–produced M-CSF. Purity of BMDMs was determined by flow cytometry for CD11b<sup>+</sup>, CD11c<sup>+</sup>, and MHC class II<sup>+</sup> and was >90% (Supplemental Fig. 1A). Where indicated, cells were treated with anti-CD3/CD28 activation beads (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol, 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO), 2 μM Io (Sigma-Aldrich), 2.5 μg/ml F(ab<sup>2</sup>)<sup>′</sup> anti-IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), 10 μg/ml LPS (Sigma-Aldrich), 10 μg/ml zymosan (InvivoGen, San Diego, CA), 5 μg/ml polyinosinic-polycytidylic acid [poly(I:C)] (low molecular weight; InvivoGen), 1 μg/ml CsA (Sigma-Aldrich), 8 μM helenalin (Santa Cruz Biotechnology, Santa Cruz, CA), or 3 μM BMS 345541 (Sigma-Aldrich) for the times indicated. All animal experiments were conducted in accordance with protocols approved by the Emory University Institutional Animal Care and Use Committee.

**Flow cytometry and Abs**

Cells were stained for flow cytometry at 4°C for 30 min in FACS buffer (PBS, 1% BSA, and 1 mM EDTA) plus appropriate Abs and fixed in 0.1% paraformaldehyde for at least 1 h. Magnetically sorted B cells were gated on CD19<sup>+</sup>B220<sup>+</sup> CD11b<sup>+</sup> and CD11c<sup>+</sup> events. Magnetically sorted CD4 T cells were gated on CD3<sup>+</sup>CD8<sup>+</sup> CD4<sup>+</sup> and CD11c<sup>+</sup> events. Primary BMDMs were gated on CD11b<sup>+</sup>CD11c<sup>+</sup> MHC class II<sup>+</sup> cells. RAW264.7 macrophages, primary BMDMs, T cells, and B cells were all gated with anti-PD-1 Ab for analysis. Abs were used CD4 PerCP-Cy5.5 (clone RM45, Tonbo Biosciences, San Diego, CA), CD8 FITC (clone 53-6.7, Tonbo Biosciences), CD19 FITC (clone 1D3, BD Pharmingen, San Diego, CA), B220 allophycocyanin (clone RA3-6B2, BD Pharmingen), CD11c allophycocyanin (clone M1/70, BD Pharmingen), and PD-1 PE (clone RMP1-30, BioLegend, San Diego, CA). Flow cytometry was performed on a BD LSR II and analyzed using FlowJo 9.6.4 software.

**mRNA quantification by quantitative RT-PCR**

Total RNA was isolated from cells using the RNaseasy kit (Qiagen, Germantown, MD). cDNA was prepared from 1 μg RNA using SuperScript II reverse transcriptase (Life Technologies). PD-1 mRNA levels were quantified in technical duplicates by real-time PCR. Primers used for real-time PCR are listed in Supplemental Table I. Quantitative RT-PCR experiments were performed from three independent RNA preparations. The data presented were normalized to 18S rRNA levels (33).

**Bisulfite sequencing**

The protocol for bisulfite sequencing was adapted from Youngblood et al. (25). Briefly, genomic DNA from BMDMs cultured in the presence or absence of LPS for 24 h was isolated and bisulfite converted using the EpiTect bisulfite kit (Qiagen). Bisulfite-converted DNA was amplified via PCR and cloned using the TOPO TA cloning kit (Invitrogen, San Diego, CA). Three independently purified plasmids were used and around eight colonies were selected for sequencing (Sanger sequencing). Bisulfite sequencing was performed using the M13 forward and reverse primers. The bisulfite-converted sequence was aligned to the MHC class II<sup>+</sup> gene. All transfections were performed at least three times.

**Cloning and luciferase reporter assays**

DNA sequences containing the Pdcld promoter, CR-B, and CR-C regions with mutated potential NF-κB binding sites were generated by PCR from existing plasmids and used as primers listed in Supplemental Table I. These sequences were cloned into a pGL3-Basic luciferase reporter vector (Promega, Madison, WI) using XhoI (New England Biolabs, Ipswitch, MA). All clones were verified by DNA sequencing. Cell transfections were performed using an Amaxa Nucleofector II instrument. RAW264.7 cells (2 × 10<sup>6</sup>) were transfected in 100 μl of a solution of 120 mM Na<sub>2</sub>CO<sub>3</sub> buffer (pH 7.2), 5 mM KCl, and 15 mM MgCl<sub>2</sub> with 200 ng pRL-TK Renilla luciferase gene plasmid and 1000 ng of the indicated firefly luciferase reporter plasmid. Transfected cell populations were split into multiple cultures for different treatments and allowed to rest in culture for 16 h. Cells were subsequently stimulated with LPS or helenalin for the times indicated. Luciferase activity of the reporter plasmids was quantified using the Dual-Glo luciferase assay system (Promega), and the firefly luciferase activity was normalized to luminescence of the constitutively active Renilla gene. All transfections were performed at least three times.
Data were plotted as mean plus SD and statistical significance was determined by ANOVA.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (24, 35). Chromatin was prepared from RAW264.7 cells (4 × 10⁷) treated with LPS for 4 h and crosslinked in 1% formaldehyde for 10 min. Chromatin was sonicated to an average length of 400–600 bp. Chromatin (5 μg) was immunoprecipitated with protein G beads using 0.5 μg Abs for control IgG (rabbit polyclonal Ab, EMD Millipore, Billerica, MA), NF-kB p65 (clone sc-372, Santa Cruz Biotechnology), histone 3 lysine 4 monomethylation (H3K4me1) (rabbit polyclonal Ab, EMD Millipore), H3 lysine 4 trimethylation (rabbit polyclonal Ab, EMD Millipore), or H3 lysine 27 acetylation (H3K27ac) (rabbit polyclonal Ab, EMD Millipore). Immunoprecipitates were then quantitated by quantitative real-time PCR and calculated as a percentage of input. Primer sets used in these assays are provided in Supplemental Table I. All ChIP assays were performed at least three times from independent experiments. The data were averaged and plotted as percentage of input chromatin.

**Statistical analyses**

Statistical significance was calculated using a Student t test in Microsoft Excel, a Fisher exact test, or a two-way ANOVA with repeated measures using a Bonferroni posttest calculated by GraphPad Prism 4.0 software. A p value <0.05 was considered statistically significant.

**Results**

**NFATc1 regulates PD-1 on B and T cells but not on macrophages**

Although the basic mechanisms of Pdcd1 gene induction in CD8 T cells has been explored, little is known about its regulation in CD4 T cells, B cells, or macrophages. A variety of CD4 T cells express PD-1, including effector cells upon initial activation and follicular helper T cells, which constitutively express PD-1 within germinal center reactions (28). Similar to T cells, B cells and macrophages also display a transient increase in PD-1 expression upon initial activation (4). To explore the general activation of PD-1 ex vivo, mouse splenic CD4 T cells and B cells were isolated and stimulated with PMA and Io, a process that directly activates cells through their Ag receptor pathways (36). Following a 48-h stimulation with PMA/Io or anti-CD3/CD28 beads, CD4 T cells showed a durable response with >50% of cells expressing PD-1 (Fig. 1A). B cells stimulated with PMA/Io for 24 h displayed a high level of PD-1 expression with >90% expressing PD-1 (Fig. 1B). Likewise, upon activation of the B cell Ag receptor by crosslinking with anti-IgM, ~40% of the cells became PD-1 positive, but this level was not as high as with PMA/Io (Fig. 1B). Thus, PD-1 expression can be induced ex vivo on both CD4 T and B cells through engagement of their Ag receptors or through processes that mimic receptor signaling and activation of the calcineurin pathway.

To determine whether the induction of PD-1 in CD4 T cells and B cells was mediated by processes similar to those of CD8 T cells, which use NFATc1 as the primary activator of PD-1 expression following TCR stimulation (19), the calcineurin pathway inhibitor CsA was used (Fig. 1). Prior to activation, NFATc1 is phosphorylated and resides in the cytoplasm. Upon activation, the phosphatase calcineurin dephosphorylates NFAT proteins, facilitating their translocation to the nucleus. CsA blocks the activity of calcineurin in this process and ultimately prevents the activation of NFAT (37, 38). PD-1 surface expression induced in primary murine CD4 T cells activated with anti-CD3/CD28 beads or PMA/Io

![FIGURE 1.](http://www.jimmunol.org/)
was completely blocked by pretreatment of cells with CsA (Fig. 1A). Similarly, PD-1 surface expression in B cells was inhibited fully by treatment with CsA irrespective of whether stimulation was through PMA/Io or anti-IgM crosslinking (Fig. 1B). Collectively, these data implicate the same NFAT pathway that modulates PD-1 expression in CD8 T cells acts as the primary driver of expression in both CD4 T cells and B cells in response to stimuli targeting their respective Ag receptors. In contrast, treatment of RAW267.4 cells (a murine macrophage cell line) with PMA/Io failed to induce PD-1 (Fig. 1C), indicating that the NFAT-mediated pathway is nonfunctional in inducing PD-1 in this macrophage cell line. This result was recapitulated in primary BMDMs (Fig. 1D), again showing that macrophages do not induce PD-1 through the PMA/Io-induced NFAT pathway.

TLR agonists are common activators of macrophages (39), and they have previously been correlated with increased PD-1 surface expression in peripheral blood monocytes (29). Treatment of primary CD4 T cells using LPS, a potent stimulator of TLR4, yielded no changes in expression of PD-1 (Fig. 2A), indicating that this pathway was not involved in PD-1 regulation in CD4 T cells. However, treatment of either B cells, RAW264.7 macrophage cells, or primary macrophages with the TLR4 ligand LPS resulted in a robust induction of PD-1 with nearly 90% of B cells and RAW264.7 cells (Fig. 2B, 2C) or 55% of primary BMDMs (Fig. 2D) responding. Pretreatment of either B cells, RAW267.4 cells, or primary macrophages with CsA prior to stimulation with LPS showed no effect in reducing PD-1 expression on the above cells (Fig. 2B–D), suggesting that PD-1 induction through this TLR pathway was independent of calcineurin signaling. Thus, distinct pathways may be used by different cell types in response to different classes of stimuli; TCR/BCR mediated through the NFATc1 pathway and TLR mediated through an independent mechanism.

To correlate surface protein levels of PD-1 with Pdcd1 gene transcription, Pdcd1 mRNA levels in B cells, CD4 T cells, and RAW264.7 cells following activation were compared with and without inhibition of the calcineurin/NFATc1 pathway with CsA. B cells induced by anti-IgM Ab or PMA/Io exhibited a 10- and 30-fold induction of Pdcd1 mRNA expression, which was inhibited by treatment with CsA (Fig. 3A, left). Similarly, CD4 T cells also failed to upregulate Pdcd1 mRNA in response to CD3/CD28 beads or PMA/Io stimulation following inhibition with CsA (Fig. 3A, middle), again indicating that both of these cell types require NFAT activation to induce Pdcd1 expression. As seen in surface protein expression, PMA/Io failed to induce Pdcd1 mRNA in RAW264.7 cells (Fig. 3A, right). As above, and correlating with protein expression, LPS induced Pdcd1 mRNA in B cells and RAW264.7 cells, but not in CD4 T cells (Fig. 3B). As above, CsA had no effect on LPS-activated Pdcd1 mRNA in RAW264.7 cells (Fig. 3B). These data demonstrate that LPS-mediated induction of Pdcd1 in macrophages occurs through a pathway that is independent of the NFAT/calcineurin pathway used by Ag receptors in lymphocytes. Given that macrophages modulate PD-1 mRNA and surface expression independently of the previously described NFAT-dependent mechanism, Pdcd1 gene regulation in macrophages was further examined.

**FIGURE 2.** LPS induces PD-1 in an NFAT-independent manner in B cells and macrophages. (A) Primary splenic CD4 T cells, (B) B cells, (C) RAW264.7 macrophages, or (D) BMDMs were treated for 24 h with LPS in the presence of absence of CsA as indicated. At 24 h, cells were stained and analyzed by flow cytometry. Representative histogram plots are shown (left) with fluorescence minus one (FMO) controls, and average frequency of PD-1+ cells across all samples is graphed with SD (right). Primary cell cultures of CD4 T cells, B cells, and BMDMs were prepared from independent preparations from three separate mice, and the data in RAW264.7 cells were collected from at least six independent splits of cell culture. Experiments in RAW264.7 cells were performed concurrently with those in Fig. 1C, and therefore untreated (No Tx Cont), CsA-only (No Tx CsA), and FMO controls are the same as in that figure. Statistical significance was determined by Student t test. * * p < 0.01, ** * p < 0.001.
NF-κB governs PD-1 induced by TLR ligands

TLR signaling in macrophages can result in the activation of multiple transcription factor pathways, including members of the NF-κB family of transcription factors such as p65/p50 hetero-dimers (31). To determine whether NF-κB plays a role in PD-1 regulation in macrophages, helenalin, a selective NF-κB inhibitor that works by alkylation of the DNA binding site of p65 (40, 41), was used to examine the dependence on p65 for induction of PD-1 expression in macrophages. Treatment of RAW264.7 cells with helenalin resulted in complete ablation of Pdcd1 mRNA induction by LPS (Fig. 4A, left). To verify these results, and rule out possibilities of off-target effects of treatment with helenalin, TLR-stimulated RAW264.7 cells were treated with BMS-345541, an alternative inhibitor that blocks p65 activity by neutralizing the function of the IκB kinase complex (42). Macrophages treated with this small molecule inhibitor also exhibited a complete loss of Pdcd1 mRNA induction triggered by LPS (Fig. 4A, right), confirming the findings with helenalin.

TLR2 and TLR3 signal through distinct pathways that are each shared with TLR4. TLR2 utilizes the MyD88 pathway (43), whereas TLR3 uses the TOLL/IL-1R domain–containing adapter inducing IFN-β (TRIF) pathway (31, 44). To identify relevant signaling pathways and to determine whether other TLRs could similarly induce Pdcd1 expression, RAW264.7 cells were activated with zymosan and poly(I:C) to stimulate TLRs 2 and 3, respectively. Whereas zymosan robustly induced PD-1 expression, poly(I:C) failed to modulate PD-1 mRNA levels. Moreover, both p65 inhibitors, helenalin and BMS-345541, fully blocked the zymosan-induced PD-1 expression (Fig. 4B).

To verify that experiments performed in a macrophage cell line reflect similar events in primary cells, BMDMs were treated with the TLR ligands zymosan, poly(I:C), and LPS. The effects of NF-κB inhibition by helenalin were also examined in these cells at 4 h after treatment. As observed in RAW264.7 cells, primary BMDMs showed an increase in PD-1 mRNA in response to zymosan and LPS that was fully blocked by helenalin-mediated inhibition of p65 (Fig. 4C, top). Surface expression of PD-1 following LPS stimulation was also examined on BMDMs. In this experiment, a 24 h time point was used. Because helenalin shows cytotoxicity at 24 h (but not at 4 h), BMS-345541, which is not toxic at 24 h, was used to inhibit NF-κB activity (Supplemental Fig. 2). As observed in RAW264.7 cells, induction of surface PD-1 in primary macrophages by LPS was fully blocked upon NF-κB inhibition (Fig. 4D).

Taken together, these data indicate that Pdcd1 expression triggered by TLR2 or TLR4 stimuli in macrophages, but not in the TRIF-dependent TLR3 activation pathway, acts through the p65 subunit of NF-κB and thus may be mediated through the MyD88 pathway. To test the involvement of MyD88 in activating the NF-κB pathway to induce Pdcd1, the same experiment was repeated in BMDMs derived from MyD88<sup>−/−</sup> knockout mice. In cells from these mice, zymosan-driven expression of PD-1 was not observed (Fig. 4C, bottom), consistent with an MyD88-dependent pathway for PD-1 induction. When treated with LPS, however, MyD88<sup>−/−</sup> BMDMs still induced PD-1 mRNA to approximately two-thirds of that observed in wild-type BMDMs (Fig. 4C). The decrease in PD-1 expression is consistent with the MyD88-dependent hypothesis. However, the residual expression that is seen may be due to MyD88/TRIF-independent activation of cells by LPS, as was recently reported in TLR4<sup>−/−</sup> mice in which LPS was still able to induce inflammatory responses through noncanonical pathways (45, 46).

cis elements in CR-C regulate LPS-triggered PD-1 expression

A recent ChIP sequencing (ChIP-seq) study performed in macrophages activated with the TLR4 agonist KLA examined the binding of the NF-κB subunit p65 across the macrophage genome (47). Notably, peaks of p65 occupancy appeared at multiple sites within the Pdcd1 gene following macrophage activation. The strongest peak in this region appeared within CR-C, with notable peaks located −3.7 and +17.1 kb from the transcription start site.
CR-C, the −3.7, and the +17.1 regions were previously shown to be responsible for NFATc1- and STAT3-mediated activation of Pdcd1 (21). To determine whether these regions continue to function in gene regulation in response to LPS signaling, luciferase reporter constructs containing the Pdcd1 promoter driving a firefly luciferase reporter gene in the pGL3 basic plasmid vector were used as described previously (21). In these plasmids, the Pdcd1 promoter was contiguous with CR-B (pPD-1 B), CR-B and CR-C (pPD-1 B/C), or with CR-B spliced together with the −3.7 (pPD-1 B/3.7) or +17.1 (pPD-1 B/17.1) regions, as illustrated in Fig. 5A. These reporter plasmids were cotransfected into RAW264.7 cells along with a Renilla control luciferase gene reporter, and cells were activated with LPS or without helenalin to assess the contribution of NF-κB p65 (Fig. 5B). The pPD-1 B/C plasmid responded to LPS treatment with increased activity, whereas the pPD-1 B, pPD-1 B/3.7, and pPD-1 B/17.1 plasmids failed to respond to LPS in these cells (Fig. 5B). However, the untreated, background levels of pPD-1 B/3.7 and pPD-1 B/17.1 showed significant expression of the reporter gene compared with the inactive pPD-1 B plasmid, suggesting that additional factors or mechanisms in these cells could contribute to a basal level of PD-1 expression through these elements in an LPS-independent manner. When cells were simultaneously cultured with LPS and helenalin to block NF-κB activity, the LPS-mediated induction of the reporter construct observed with the pPD-1 B/C plasmid was abrogated (Fig. 5B), indicating not only that the LPS-responsive DNA element resides solely within CR-C, but also that activity of this cis element requires NF-κB.

To locate the specific sequence and cis-acting DNA element where NF-κB is acting on PD-1, the LPS-responsive CR-C sequence, located in the pPD-1 B/C plasmid, was analyzed using the online JASPAR database (located at jaspar.genereg.net) to find predicted NF-κB p65/RELA binding sites based on established transcription factor binding site motifs. Two potential binding sites were highly predicted within the pPD-1 B/C plasmid and corresponded with sequences excluded from the unresponsive pPD-1 B plasmid. The highest predicted sequence 5′-GGGGATCCCC-3′ is located near the middle of the CR-C conserved region. A slightly lower predicted sequence, 5′-AGAATGCCCC-3′, resides at the proximal end of CR-C and correlates with the center of the p65 ChIP-seq peak (47). These two potential binding sites were scrambled to 5′-TGTAGCAATT-3′ or 5′-TCTAACCTCT-3′, respectively, sequences that were predicted to have no NF-κB p65 or other

**FIGURE 4.** NF-κB is necessary for PD-1 upregulation in macrophages. (A) Three independent populations of RAW264.7 cells were activated with LPS for 4 h, and NF-κB activation was blocked with helenalin (left) or BMS-345541 (BMS) (right). (B) RAW264.7 cells were activated with TLR2 and TLR3 agonists zymosan (top) or poly(I:C) (bottom), respectively, for 4 h. As indicated, some samples were cotreated with helenalin (left) or BMS 345541 (right) as above. For all panels in (A) and (B), independent populations of cell lines were activated in triplicate, and Pdcd1 mRNA was analyzed by RT-PCR, with results graphed as a percentage of 18S rRNA. (C) Primary BMDMs from wild-type (top) or MyD88−/− (bottom) mice were activated for 3 h with TLR ligands zymosan, poly(I:C), or LPS and cotreated with helenalin as indicated. mRNA levels were quantitated using real-time RT-PCR. Pdcd1 mRNA levels are shown as percentage of 18S rRNA. (D) Primary BMDMs from three wild-type mice were stimulated with LPS for 24 h in the presence or absence of BMS-345541. PD-1 surface expression was analyzed by flow cytometry, and characteristic histograms are shown on the left, graphed with fluorescence minus one (FMO). Frequency of PD-1+ cells is graphed on the right. Significance was determined by Student t test. *p < 0.05, **p < 0.01.
NF-κB p65 binds to the CR-C region

To prove that the LPS-responsive element in CR-C is in fact the site to which p65 binds and from which it can directly promote PD-1 expression in this system, and to validate the previous ChIP-seq data, a ChIP paired with quantitative PCR assay was performed. In addition to CR-C, the −3.7 and +17.1 regions associated with the two other major NF-κB peaks from the ChIP-seq dataset (47) in Pdcd1 were also examined even though these regions did not demonstrate LPS responsiveness on their own in the above luciferase assays. Using a p65-specific Ab, a nearly 10-fold increase in p65 binding was observed for CR-C but not −3.7 or +17.1 following LPS treatment of the macrophage cell line (Fig. 6A). A previously described site in the Sod2 gene with very high levels of p65 binding served as a positive control (47–49), displaying significant p65 occupancy only after LPS treatment (Fig. 6A). A negative control region located 8.5 kb upstream of the Pdcd1 promoter showed minimal levels of p65 binding both before and after LPS treatment (Fig. 6A). ChIP using a control IgG Ab showed no binding at any of these sites (Fig. 6A).

Despite the CR-C, −3.7, and +17.1 sites all showing enhancer activity in CD8 T cells (21), as well as being predicted as potential p65 binding sites by ChIP-seq, the luciferase assays and p65 ChIP showed no activity at the −3.7 and +17.1 sites. To determine whether these sites displayed histone modifications that are associated with regulatory or enhancer activity, a ChIP for H3K4 monomethylation and H3K27 acetylation was performed at CR-B, CR-C, −3.7, and +17.1. The results showed that only H3K27ac was found at CR-B and CR-C (Fig. 6B, left). This is consistent with the modification patterns observed in CD8 T cells, in which H3K27ac, but not H3K4me1, is seen at CR-B and CR-C (21). The

transcription factor binding potential. The scrambled sequences were then cloned into the pPD-1 B/C plasmid, subsequently termed pPD-1 B/C kB1 or pPD-1 B/C kB2, for the higher and lower predicted binding sites, respectively (Fig. 5A). Mutant plasmids, the wild-type pPD-1 B/C plasmid, or empty pGL3 basic vector were nucleofected into RAW264.7 cells and activated with and without LPS for 24 h. In this system, the mutant pPD-1 B/C kB2 vector showed a nearly complete loss of induction following LPS treatment (Fig. 5C). In contrast, both the wild-type and pPD-1 B/C kB1 constructs showed greatly increased reporter activity in response to LPS activation at a comparable level to each other (Fig. 5C). These data demonstrated that the kB2 site but not kB1 was the functional NF-κB site required for LPS-initiated Pdcd1 gene activation through CR-C. Additionally, the fact that the kB1 reporter vector did not change its expression level provided a mutated control for these experiments.

FIGURE 5. NF-κB binding sequence located in CR-C is necessary for PD-1 induction in macrophages. (A) Schematic of luciferase constructs based in the pGL3 basic luciferase reporter vector compared with the native murine gene locus. Constructs contain the PD-1 promoter and CR-B with either CR-C, −3.7, or +17.1 regulatory regions. An “S” signifies the location of the scrambled NF-κB consensus element in plasmids pPD-1 B/C kB1 and kB2. (B) RAW264.7 cells were nucleofected twice in triplicate with plasmids containing different regulatory regions, allowed to rest for 16 h, and stimulated for 4 h with LPS with and without helenalin (hel), after which firefly luciferase expression was quantitated. (C) RAW264.7 cells were nucleofected in two triplicates with wild-type and NF-κB plasmids. Cells were allowed to rest for 16 h and then stimulated for 24 h with LPS. Significance was determined by two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6. p65 binds to CR-C. ChIPs were performed using (A) anti-p65 Ab or (B) anti-histone modification Abs H3K4me1, H3 lysine 4 trimethylation (H3K4me3), and H3K27ac. Chromatin was prepared from two sets of three independently stimulated populations of RAW264.7 cells, treated with or without LPS for 3 h. A nonspecific, control IgG was used with each set of ChIPs to determine background levels of detection. The intronic enhancer from the Sod2 gene was used as a positive control for NF-κB binding (53), and cont represents a nonspecific sequence located ~8 kb from the Pdcd1 gene (24). Significance was determined by two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
CR-B does not demethylate to induce PD-1 expression

The above data indicate that the regulatory elements for Pdcd1 operate under a novel paradigm in macrophages. Epigenetic modifications, such as the methylation of cytosines in promoter and regulatory regions, correlate with the silencing of a locus and may contribute to the regulation in macrophages. In naïve CD8 T cells, sequences surrounding CR-B and CR-C are extensively methylated. Following activation of CD8 T cells, these sequences lose their DNA methylation and then regain it as the cells transition to late effector and memory phases of an immune response, in correlation with the transient gain and subsequent loss of PD-1 expression (25). To determine whether the same epigenetic dynamics exist in macrophages, the DNA methylation states of CpG sites in surrounding CR-B and CR-C were analyzed using bisulfite sequencing before and after LPS induction of primary BMDMs. In the uninduced state, BMDMs showed nearly complete methylation of the CpGs within CR-B that were queried. Contrary to the patterns observed in CD8 T cells, following LPS stimulation for 24 h the CR-B sequences exhibited no loss of methylation (Fig. 7) despite highly elevated expression of PD-1 on the cell surface (25). To determine whether the fundamental mechanisms that control PD-1 induction were similar across a variety of cells. The results suggest that there are at least two major pathways that induce PD-1. These pathways share common features. First, PD-1 expression is initiated by recognition of a cell-activating ligand, either Ag signaling to lymphoid cells using Ag-specific receptors or using pattern recognition receptors on macrophages. Second, both pathways use two major transcription factors of immune cells that respond to extracellular challenges: NFAT and NF-κB. Intriguingly, the pathways were distinct to the cell types in that CD8 T cells did not respond to LPS signaling or TNF treatment (data not shown) to induce PD-1 through NF-κB, and macrophages did not respond to NFAT activation by PMA/IO. This may ensure that only Ag-specific signals induce PD-1 on T cells. Notably, B cells, which may act as APCs and/or differentiate into functional plasma cells, were able to engage both the BCR-induced NFAT pathway, as well as the TLR-induced NF-κB pathway. In this respect, B cells demonstrate that some cells may engage multiple distinct PD-1 inducing pathways either discretely or simultaneously upon recognition of certain pathogens.

Although statistically less than wild-type BMDMs, MyD88-deficient BMDMs still induced PD-1 following LPS stimulation. As LPS induction of NF-κB can occur through both a MyD88-dependent and TRIF-dependent pathway (31, 45, 46), this observation suggests that both signaling pathways could be involved in PD-1 expression or that one can substitute for the other. The MyD88-independent pathway, utilizing TRIF as the signaling adapter, is not sufficient or the main pathway for PD-1 expression, as stimulation of TLR3, a receptor that signals exclusively through TRIF, did not lead to PD-1 induction. Conversely, PD-1 induction following stimulation of TLR2, which is exclusively dependent on MyD88, was fully blocked in the MyD88 knockout BMDMs. Thus, it is most likely that whereas TLR3-based TRIF activation does not induce PD-1, both pathways may be nonetheless operational in regulating PD-1 expression in these cells through stimulation of TLR4, and that there is the potential for other TLR ligands to stimulate PD-1 expression.

ChiP-seq datasets suggested that NF-κB was bound to several regions across the PD-1 gene. Among those regions were CR-C, −3.7, and +17.1 kb, three key regions for induction of Pdcd1 expression in CD8 T cells following TCR and STAT signaling. When tested by conventional ChiP, only CR-C, which was the strongest of the peaks in the ChiP-seq datasets, was found to bind NF-κB following LPS stimulation. One reason for the discrepancy is likely to be that the ChiP-seq study used disuccinimidyl glutarate as a second crosslinking agent. Disuccinimidyl glutarate fixes interactions occurring at a much greater distance than

![FIGURE 7. CR-B is not demethylated following activation whereas CR-C is initially unmethylated in macrophages. (A) BMDMs from three C57Bl/6 mice were grown and treated with or without LPS for 24 h. DNA from cells was bisulfite converted, PCR amplified, and cloned. Eight clones from each mouse were sequenced. Incomplete sequences were discarded. Clonal CpG DNA methylation is shown for sequences at CR-B (left) and CR-C (right) before and after LPS. Black circles represent methylated CpG sites; open circles represent unmethylated sites. Percentage of total clones methylated at each site is represented below (B). Differences in untreated and LPS-treated cells were not significant at both regions as determined by a Fischer exact test.](http://www.jimmunol.org/content/181/11/5625.full/4552.png)
formaldehyde (50). As the −3.7 and +17.1 regions interact with the Pdcd1 promoter during cell activation and PD-1 expression (21), the additional crosslinking may be revealing these chromatin structures rather than direct p65 binding.

The finding that NF-kB binds to CR-C continues to denote this region as the critical cis element for the induction of Pdcd1 expression, as it is the focal point of binding for at least two major transcriptional control pathways: the NFAT pathway in CD8 T cells and NF-kB in macrophages. This is recapitulated by the luciferase data. Similarly to what was seen in T cells, the CR-C region alone is sufficient to induce a reporter gene construct following stimulation of macrophages. In contrast, the −3.7 and +17.1 regions were insufficient without CR-C to induce significant expression, although the ability of these other two regions to augment CR-C activity was not tested.

The DNA methylation experiments provided a surprising addition to our understanding of the regulation of PD-1. In murine CD8 T cells, the CpG-rich regions designated as CR-C and CR-B are highly methylated in naive cells. As mentioned above, following activation, both regions lose methylation and this is coincident with PD-1 expression. At the late effector cell stage for CD8 T cells, PD-1 expression is lost and DNA methylation returns, with memory CD8 T cells showing similar patterns to the naive state. Because of this, it was assumed that Cpg methylation at CR-B and CR-C must be lost in order for PD-1 expression to occur in T cells. In stark contrast, BMDMs showed complete methylation of CR-B and nearly no initial methylation at CR-C. Following LPS stimulation and Pdcd1 expression, no changes in DNA methylation were observed at either CR-B or CR-C. These data suggest that unlike CD8 T cells (19), in macrophages, CR-B is not used for Pdcd1 expression and/or that DNA methylation serves to restrict the binding of factors that could modulate Pdcd1 expression in macrophages prior to activation. The lack of DNA methylation at CR-C in macrophages implies that the region is constitutively accessible for the binding of NF-kB and rapid activation of Pdcd1 transcription. The finding of histone H3K27 acetylation at 3- to 4-fold background levels at the CR-C supports an accessible chromatin configuration. LPS stimulation of BMDMs and induction of Pdcd1 was maximal at 4 h. In contrast, stimulation of CD8 T cells ex vivo through the TCR is not immediate with expression peaking at 24 h. The difference in chromatin accessibility and DNA methylation may account for the difference in the timing of Pdcd1 induction, as both NFAT and NF-kB are induced immediately upon stimulation of their pathways. Although the mechanism that specifically methylates CR-B and CR-C in naive CD8 T cells or causes demethylation of these regions upon T cell activation is unknown, its failure to fully engage in macrophages reinforces the concept that these cells regulate inhibitory receptors through unique pathways. Thus, these data show that demethylation of CR-B is not an absolute requirement for cellular expression of PD-1 in all cell types, although it may aid in or be necessary for durable expression, as is seen on exhausted T cells.

Chronic-phase immune system failure remains an important problem in medicine. It is now understood that PD-1 plays a critical role in inducing and maintaining CD8 T cell exhaustion. This function of PD-1 may be necessary on an evolutionary scale to help prevent or reduce autoimmunity (51, 52). However, despite numerous observations of PD-1 expression on other cell types, an exhausted phenotype has never been described in macrophages or dendritic cells, although in all of these cell types the function of PD-1 has been shown to be the same: slowing of effector functions and cell replication (26, 27, 29, 30). The importance of PD-1 expression on cells other than CD8 T cells is coming into light. As PD-1 is a critical mechanism for controlling both the early and chronic stages of immune responses, understanding regulation of this gene has important implications for many immunological processes, including those associated with chronic infections, transplantation, and cancers. The data presented in the present study demonstrate that the two major transcription factor activation pathways of immune cells (NFAT and NF-kB) are both critical components of the PD-1 system across multiple inflammatory cell types. The finding in this study of TLR-mediated induction of PD-1 opens up the intriguing possibility that bacteria or other microbes may take advantage of this pathway with the ultimate goal of inducing an immune inhibitory responses. Thus, understanding the molecular mechanisms that control PD-1 expression is critical to being able to target this molecule for therapeutic interventions or enhancements in a wide variety of inflammatory conditions.

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Disclosures
R.A. has patents and receives licensing fees related to the PD-1 pathway. The remaining authors have no financial conflicts of interest.

References
**Supplementary Figure S1:**

**A) BMDMs are greater than 90% pure after derivation.**
An aliquot of cells used in experiments utilizing bone-marrow derived macrophages were collected and stained for flow cytometry using antibodies specific to CD11b and CD11c to determine purity following derivation. A representative flow plot is shown. Bone marrow-derived macrophages are in the CD11c- CD11b+ quadrant.

**B) BMDM PD-1 expression increased in bisulfite-sequenced cells.**
Aliquots of BMDM cells that were treated +/- LPS for bisulfite sequencing were stained for flow cytometry for both macrophage purity (data not shown) as well as PD-1 expression. Histogram plots of PD-1 expression are shown for control (Cont-1, Cont-2, and Cont-3) and LPS-treated samples, representative of populations used for bisulfite sequencing. Mean fluorescence intensity (MFI) of each sample was normalized to average MFI of control populations and graphed with standard deviation.
Supplemental Figure 2. BMS 345541 and helenalin are non-toxic to macrophages following short treatments.
Three independent populations of RAW264.7 cells were treated with NF-κB inhibitors BMS 345541 and helenalin for 0, 4, 8, and 24 hours. Immediately following treatment, cells were stained with Annexin V (identifying apoptotic cells) for 10 minutes, followed by 7-AAD (identifying dead cells with disrupted membranes) for 10 minutes, and immediately analyzed by flow cytometry. Representative flow plots at each time point as well as from unstained cells are shown, and frequency of viable cells (Annexin V- 7-AAD-) from each time point is graphed (bottom right).
### RT-PCR Primers

| Mouse 18s Forward          | 5'-GTAACCGGGTGAACCCCATT-3' |
| Mouse 18s Reverse          | 5'-CCATCCATCGGGTAGGCGCAG-3' |
| Mouse PD-1 Forward         | 5'-GCTGAGAGCTCTCTTCTGTACAT-3' |
| Mouse PD-1 Reverse         | 5'-AGATATCCACAGGCTCTGCCC-3' |
| Mouse IL-2 Forward         | 5'-ACCCACTTCAAGCTCCACTTTA-3' |
| Mouse IL-2 Reverse         | 5'-TGCCCTGCTGGGCAAGTAAA-3' |

### Bisulfite Cloning

| Mouse CR-B Forward         | 5'-GGGGGTTTTTATTTTTAGGGATTGAGG-3' |
| Mouse CR-B Reverse         | 5'-CTAAAAACTAAAACCACACTTTATCCC-3' |
| Mouse CR-C Forward         | 5'-ATTTTTTTAGTTTTTGTTTATAGGTTTTAT-3' |
| Mouse CR-C Reverse         | 5'-TTTCTTCCCCCTAAAAAAAAACCTAACACCA-3' |

### Mutant vector cloning

| CR-B/C bounding Forward   | 5'-GTTCCCTCGAGGGAGACCTTCTGAGGACTTC-3' |
| CR-B/C bounding Reverse   | 5'-ACGACTCGAGGGCAATGTCGCCTTCAGT-3' |
| NK-1 mutant Forward       | 5'-GCTAGAATTGCTACATCCCCACGCTGGCTGTTA-3' |
| NK-1 mutant Reverse       | 5'-AATCCTCTCTAACCTCTTACTAGCCAGCCAG-3' |
| NK-2 mutant Forward       | 5'-ATTTCTAGAGGTTAGATGATTTAATCATCTGTCTCTG-3' |
| NK-2 mutant Reverse       | 5'-TTTCTTCCCCCTAAAAAAAAACCTAACACCA-3' |

### ChIP Primers

| CR-C Forward              | 5'-CCTCACCTCCTGGCTTCTTC-3' |
| CR-C Reverse              | 5'-GTGAGACCCACACATCTCSGCTC-3' |
| CR-B Forward              | 5'-CTCTGACTAGCTGTCCCTTGGCC-3' |
| CR-B Reverse              | 5'-CTCTGAGAACCCCCCTCCTGCAAG-3' |
| 3.7 Forward               | 5'-TCTCTGCCCTGACTCTGCCCTTGA-3' |
| 3.7 Reverse               | 5'-AAAGCTCTGACTGAGCTGTCAGTA-3' |
| 17.1 Forward              | 5'-TGAGAGGAGAGAGGAAAG-3' |
| 17.1 Reverse              | 5'-CAAACGCGTGCTCATGTCCTC-3' |
| PD-1 Cont Forward         | 5'-GGCTCTGGGAATAGACTGAGG-3' |
| PD-1 Cont Reverse         | 5'-AGTGCAAGTGGTTGCTTGC-3' |
| SOD2 Forward              | 5'-TAACTGCGCAATCCAGAAGGAGA-3' |
| SOD2 Reverse              | 5'-GAATGCTTTCCCCAAGTGCTGCT-3' |

*Blue colors indicate XhoI restriction enzyme sites

**Red colors indicate mutated sequences