H3K4me3 mediates the NF-κB p50 homodimer binding to the pdcd1 promoter to activate PD-1 transcription in T cells

Priscilla S. Redd\textsuperscript{a,b,c}, Chunwan Lu\textsuperscript{a,b,c}, John D. Klement\textsuperscript{a,b,c}, Mohammed L. Ibrahim\textsuperscript{a,b}, Gang Zhou\textsuperscript{b}, Takumi Kumai\textsuperscript{b}, Esteban Celis\textsuperscript{a}, and Kebin Liu \textsuperscript{a,b,c}

\textsuperscript{a}Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA; \textsuperscript{b}Georgia Cancer Center, Medical College of Georgia, Augusta, GA, USA; \textsuperscript{c}Charlie Norwood VA Medical Center, Augusta, GA, USA

\textbf{ABSTRACT}

PD-1 is a co-repressive receptor that curbs T cell activation and thereby serves as a protection mechanism against autoimmunity under physiological conditions. Under pathological conditions, tumor cells express PD-L1 as an adaptive resistant mechanism to suppress PD-1+ T cells to evade host immune-surveillance. PD-1 therefore is a key target in cancer immunotherapy. Despite the extensive studies of PD-1 expression regulation, the pdcd1 transcription machinery and regulatory mechanisms are still not fully understood. We report here that the NF-κB p50 homodimer is a transcription regulator of PD-1 in activated T cells. A putative κB sequence exists at the pdcd1 promoter. All five NF-κB Rel subunits are activated in activated T cells. However, only the p50 homodimer directly binds to the κB sequence at the pdcd1 promoter in CD4+ and CD8+ T cells. Deficiency in p50 results in reduced PD-1 expression in both CD4+ and CD8+ T cells. In an in vitro mixed bone marrow chimera mouse model, we show that p50 regulates PD-1 expression in a cell-intrinsic way and p50 deficiency leads to decreased PD-1 expression in both antigen-specific CD4+ and CD8+ T cells in vivo. The expression levels of H3K4me3-specific histone methyltransferase increased significantly, resulting in a significant increase in H3K4me3 deposition at the pdcd1 promoter in activated CD4+ and CD8+ T cells. Inhibition of H3K4me3 significantly decreased p50 binding to the pdcd1 promoter and PD-1 expression in a T cell line. Our findings determine that the p50-H3K4me3 axis regulates pdcd1 transcription activation in activated T cells.

\section*{Introduction}

T cell activation is an essential initial step of an immune response to infection or other diseases, such as cancer, and is controlled by sequential costimulatory and co-inhibitory signaling pathways to ensure effective effector function while preventing autoimmunity.\textsuperscript{1} PD-1 is a co-inhibitory receptor of the CD28 family and is expressed on T cells in response to antigenic stimulation such as infection and cancer.\textsuperscript{2} In contrast to CD28 which produces a positive stimulatory signal to enhance T cell receptor (TCR)-mediated activation of naive T cells, PD-1 produces a negative inhibitory signal that inhibits T cell activation and thereby suppresses the T cell effector function.\textsuperscript{1,3,4} PD-1 receptor engagement by its physiological ligand PD-L1 or PD-L2 leads to activation of phosphatases SHP1 and SHP2, particularly SHP2, that dephosphorylates TCR-associated zeta chain, and other signaling mediators such as ZAP70, of the TCR signaling pathway.\textsuperscript{1,5} PD-1-associated SHP2 also dephosphorylates the costimulatory receptor CD28 to reduce overall CD28 phosphorylation and its binding of the PI3K, thereby inactivating CD28 signaling to suppress T cell function.\textsuperscript{3,4,6} Targeting PD-1 expression has recently shown great promise in enhancing T cell activation and suppressing tumor growth.\textsuperscript{7,8} The nature of PD-1 function thus makes the underlying mechanism of PD-1 expression regulation the subject of extensive studies.\textsuperscript{7-15} PD-1 expression is apparently coupled with T cell activation through the TCR signaling pathways.\textsuperscript{2,16,17} In an acute response, PD-1 is transiently expressed and rapidly downregulated in T cells.\textsuperscript{18} In contrast, chronic exposure to antigen, including persistent viral infections and cancer leads to sustained up-regulation of PD-1 expression in T cells resulting in impaired T cell effector function.\textsuperscript{4,14,15,18-20} Furthermore, tumor-infiltrating cytotoxic T lymphocytes (CTL) express various levels of PD-1 in the same tumor microenvironment.\textsuperscript{20} Therefore, PD-1 expression is apparently regulated by multiple transcriptional mechanisms depending on the cellular context.\textsuperscript{7-15}

NF-κB is a master transcription factor.\textsuperscript{21} Constitutive but low levels of NF-κB activity are often detectable in many types of cancers and compelling experimental data from both human patients and mouse models have shown that NF-κB activation promotes tumor growth and progression.\textsuperscript{22} This provides a strong rationale for anticancer strategies that inhibit NF-κB signaling. In fact, several hundred NF-κB inhibitors have been developed.\textsuperscript{23} However, extensive studies have also shown that NF-κB may harbor dual functionality by acting to promote
apoptosis and senescence in multiple types of cells, including cancer cells and thereby acts as a tumor suppressor. Furthermore, in addition to its essential function in tumor cells, the NF-κB pathway also exerts an essential function in the regulation of the immune response, particularly in T cell differentiation and function. The mammalian NF-κB complex consists of five Rel family proteins: p65 (RelA), RelB, cRel, p50 (NF-κB1), and p52 (NF-κB2), which are capable of forming homodimers and heterodimers in any combinations to regulate distinct gene transcription. There is potential for up to fifteen different dimer combinations and ten NF-κB dimers have been identified. All five Rel family members contain a N-terminal Rel homology domain (RHD) that binds to the consensus DNA sequence motif (κB elements), that forms dimers with each other, and that interacts with the 1κB inhibitors before nuclear translocalization. In T cells, NF-κB is coupled with the TCR signaling pathway and T cell activation. The parallel activation of NF-κB and PD-1 during T cell activation led us to hypothesize that NF-κB may directly regulate PD-1 expression in T cells. To this end, we determined that the p50 NF-κB homodimer selectively binds to the κB element at the pdcd1 promoter and functions as a specific transcriptional activator of PD-1 in T cells. Furthermore, we observed that H3K4me3 is essential for p50 homodimer binding to the κB element and function in the activation of pdcd1 transcription. We therefore determined that p50 homodimer and H3K4me3 act in concert to activate pdcd1 transcription in activated T cells.

Material and methods

Mouse models and cell line

Female C57BL/6 (B6) mice aged 6–8 weeks were obtained through Jackson Laboratory. Male B6 CD45.1, Pep Boy (SJL) mice aged 6–8 weeks were also obtained through Jackson Laboratory. The murine T lymphoma cell line EL4 was obtained from and validated by ATCC (Manassas, VA). All mouse studies were approved by the Augusta University Institutional Animal Care and Use Committee.

Electrophoretic mobility shift assay (EMSA) of NF-κB activation

T cells were purified from spleen and lymph nodes using the MoJoSort CD3+, CD4+, and CD8+ T Cell Isolation Kits (BioLegend). T cells were activated in anti-CD3 and anti-CD28 mAbs-coated plates. EL4 cells were either unstimulated or stimulated with PMA and ionomycin. NF-κB activation was analyzed using NF-κB probe (Santa Cruz Biotech) and probes with NF-κB consensus sequences of the PD-1 promoter region (Table S1) as previously described.

Cell treatment

To determine H3K4me3 function in PD-1 expression, EL4 cells were plated at a concentration of 2 × 10^5 cells per well in 100 µl of medium. Cells were either untreated or treated with Chaetocin (LC laboratories). Cells were collected and stained with anti-PD-1, anti-PD-L1 fluorescent antibodies and Zombie Green (BioLegend). The samples were analyzed by flow cytometry on a FACS Calibur (BD Biosciences).

Chromatin immunoprecipitation (ChiP) assay

ChiP assays were carried out using the anti-p50 (Santa Cruz Biotech), anti-H3K4me3 (Millipore, MA), anti-H3K9me3 (Abcam, MA), anti-H3K36me3, and anti-H3K27me3 (Cell Technology, MA) antibodies, and protein A-agarose beads (Millipore). The mouse PD-1 promoter DNA was detected by quantitative PCR and semi-quantitative PCR using genespecific primers (Table S1).

In vitro T-cell activation and PD-1 kinetics

Purified T cells were cultured in anti-CD3 (0.8 µg/ml) and anti-CD28 (10 µg/ml) mAbs-coated plates at 2 × 10^6 cells/well. Cells were collected and stained using the following antibodies: CD25, CD8, CD4, and PD-1 (BioLegend). All flow cytometry was done on a LSR II (BD Biosciences) flow cytometer.

RT-PCR analysis

Purified CD3+ T cells were stimulated in anti-CD3 and anti-CD28-coated plates for 3 days. EL4 cells were treated with PMA and ionomycin for 3 days. The cells were collected and analyzed the expression levels of mll1, mll2, mll3, seld1a and seld1b by qPCR using primers as listed in Table S1.

Mixed bone marrow chimera mouse model and immunizations

Mice were irradiated at 850 Rad using a JL Shepherd Irradiator. Bone marrow mixture (1:1) of p50 KO and SJL mice was given intravenously to irradiated recipient mice. The blood samples were analyzed by flow cytometry using the following antibodies: Zombie Violet, CD45.2, CD45.1, CD4, and CD8 (BioLegend). The mice were given immunizations with one of two peptide vaccines to induce either CD4 or CD8 activation. The CD4 vaccine uses the 2W1S peptide (EAWGALANWAVDSA) and the CD8 vaccine uses the OVA peptide (SIINFEKL). Both peptide vaccines consist of a prime followed by a boost fourteen days later, and they are given through the vaccine strategy, TriVax. TriVax consists of a mixture of the peptide (2W1S: 150 µg; OVA: 100 µg), CD40 mAb (prime: 100 µg; boost: 25 µg), and poly-IC (50 µg). The CD4 vaccine also consisted of an intraperitoneal injection of OX40 mAb (200 µg). Seven days after each vaccination, blood samples were examined by flow cytometry using the following antibodies: MHCII, CD45.2, CD45.1, CD4/CD8, and PD-1 (BioLegend). The 2W1S/OVA tetramer antibodies were also included in each stain and were kindly provided by the NIH Tetramer Core Facility. An Fc block (2W1S: 1 µg; OVA: 0.5 µg) was used jointly with the tetramer antibodies. All flow cytometry was done on a LSR II (BD Biosciences) flow cytometer.
Flow and statistical analysis

All flow analysis was done using FlowJo, LLC (version 10). All statistical analyses were performed by two-sided Student t test using the GraphPad Prism program (GraphPad Software, Inc.). *p* < 0.05 is considered as statistically significant.

Results

All five Rel subunits of the NF-κB complexes are activated during T cell activation

It is known that NF-κB is an essential transcription factor not only for T cell lineage differentiation but also for T cell activation. Therefore, T cell activation is typically coupled with PD-1 transcriptional activation. We first sought to determine which Rel subunits are activated in activated T cells and used an electrophoretic mobility shift assay (EMSA) to determine protein-DNA interactions using a NF-κB-binding consensus sequence DNA probe. Consistent with the literature that NF-κB is engaged during T cell activation, we observed rapid activation of NF-κB in CD3+ T cells after stimulation with anti-CD3 and anti-CD28 antibodies in vitro (Figure 1A). Co-incubation of Rel subunit-specific antibodies resulted in super-shift of p65 and p50 and decreased binding of p52, RelB, and cRel to the κB element DNA (Figure 1B). These observations indicate that all five NF-κB Rel subunits are activated in activated T cells in vitro.

The p50 NF-κB homodimer specifically binds to the pdcd1 promoter region

The p65 NF-κB complex has been shown to bind to the promoter region of pdcd1, the gene that encodes PD-1, in macrophage. We then analyzed the promoter region of pdcd1 and identified a putative NF-κB-binding consensus κB element (Figure 2A). The existence of this putative κB element in the pdcd1 promoter region suggests that NF-κB is potentially a transcriptional regulator of PD-1 expression in T cells. To test this hypothesis, we sought to determine whether NF-κB directly binds to the pdcd1 promoter DNA and, if so, which subunits regulate pdcd1 transcription. We synthesized the putative NF-κB-binding consensus sequence κB element and used it as probe to examine protein-DNA interactions by EMSA. CD3+ T cells were activated in anti-CD3 and anti-CD28 antibody-coated plates and used to determine NF-κB binding to the pdcd1 DNA promoter. NF-κB binding activity to the pdcd1 DNA promoter was detected as early as 6 h after T cell activation and peaked at day 3 (Figure 2B). Interestingly, amongst the five NF-κB Rel subunits, only the p50 subunit (in its p50/p50 dimer form) was detected to bind to the pdcd1 DNA promoter sequence.

To determine whether p50 binds to the pdcd1 promoter region in both CD4+ and CD8+ T cells, we activated CD4+ and CD8+ T cells and analyzed NF-κB-pdcd1 κB DNA interactions by EMSA. In CD4+ T cells, the p50 homodimer was activated at about 22 h and is still activated 3 days after stimulation (Figure 2C). A similar p50 homodimer activation kinetics was observed in CD8+ T cells (Figure 2C).

The complimentary chromatin immunoprecipitation (ChIP) approach was then used to validate p50 binding to the pdcd1 promoter. In vitro activated CD4+ and CD8+ T cells were analyzed using p50-specific antibody by immunoprecipitation of the crosslinked and fragmented chromatin. Analysis of the p50-associated chromatin DNA fragments by qPCR using a pair of PCR primers flanking the NF-κB-binding consensus sequence κB element of the pdcd1 promoter revealed that indeed, p50 binds to the chromatin in the promoter region of pdcd1 in both CD4+ and CD8+ T cells (Figure 3A). We then repeated the ChIP analysis and analyzed the p50-associated chromatin by semi-quantitative PCR using PCR primers that cover the κB element of the pdcd1 promoter. It is clear that p50 is associated with the pdcd1 promoter (Figure 3B). Thus, our ChIP data validate the above EMSA analysis that the p50 NF-κB homodimer directly binds to the κB element chromatin of the pdcd1 promoter in both CD4+ and CD8+ T cells ex vivo.

p50 regulates PD-1 transcription activation during T cell activation

We next sought to determine whether the p50 homodimer binds to the putative κB element to activate or repress pdcd1 transcription in activated T cells. We examined the PD-1 transcriptional activation using EMSA with the NF-κB-binding consensus sequence DNA probe.
expression kinetics in WT and p50 knockout (p50 KO) T cells during T cell activation in vitro. The rationale is that PD-1 expression should be higher in p50 KO T cells than in WT T cells if p50 acts as a transcriptional repressor.\(^{37,38}\) Conversely, PD-1 expression level should be diminished in p50 KO T cells if p50 is a transcriptional activator.\(^{39-41}\) Purified CD3\(^+\) T cells from WT and p50 KO mice were stimulated with anti-CD3 and anti-CD28 antibodies and CD25 expression was used as a marker for T cell activation. Activated CD4\(^+\)CD25\(^+\) and CD8\(^+\)CD25\(^+\) T cells were then analyzed for PD-1 expression levels. Flow cytometry analysis validated that resting CD4\(^+\) T cells are essentially PD-1 negative (Figure S1). CD4\(^+\) T cell activation was observed as early as 6 hours after stimulation and these cells expressed PD-1 at low levels. CD4\(^+\) T cell activation (percentages of CD25\(^+\) cells) levels increased with time and plateaued at about 2 days after stimulation. PD-1 expression levels on the activated CD4\(^+\) T cells also increased with time and plateaued at about 2 days after stimulation. Significant lower levels of both percentages of PD-1\(^+\) cells and PD-1 protein levels (as mean fluorescent intensity or MFI) on PD-1\(^+\) cells were observed in p50 KO CD4\(^+\) T cells as compared to WT CD4\(^+\) T cells (Figure 4A). However, this difference disappeared 3 days after stimulation (Figure 4B, Figure S1), suggesting a temporal regulation mechanism of PD-1 expression by p50 in CD4\(^+\) T cells during activation. WT CD8\(^+\) T cells exhibit similar PD-1 expression kinetics as CD4\(^+\) T cells (Figure 4C & D). However, p50 deficiency resulted in decreased PD-1 activation in CD8\(^+\) T cells in the entire 4 day stimulation period (Figure 4D, Figure S1).

**p50 mediates maturation and survival of CD8\(^+\) but not CD4\(^+\) T cells in vivo**

To determine the function of p50 in PD-1 expression during antigen-specific T cell activation in vivo, we made use of two vaccination models that stimulate antigen-specific CD4\(^+\) and CD8\(^+\) T cell responses in vivo. In one model, vaccination of WT and p50 KO mice was done using the 2W1S peptide, which activates CD4\(^+\) T cells,\(^{42}\) whereas in the other model, mice were vaccinated with the OVA\(_{257-264}\) peptide, which activates CD8\(^+\) T cells.\(^{43}\) p50 KO mice exhibit certain levels of immunodeficiency that could affect T cells and non-T cells (e.g., dendritic cells).\(^{44}\) Thus, to determine p50 function in regulating PD-1 expression within the same cellular...
microenvironment, we created mixed bone marrow chimeras with WT and p50 KO mice (Figure S2A). The mixed chimeric mice had both WT and p50 KO CD4+ and CD8+ T cells (Figure S2B & C) and the levels of WT and p50 KO CD4+ T cells were not significantly different (Figure S2D). In contrast, the percentages of p50 KO CD8+ T cells were significantly lower than the WT CD8+ T cells (Figure S2D) even though a 1:1 ratio of BM were adoptively transferred to the recipient mice, validating the observations that p50 functions in CD8+ T cell thymic negative selection and post-selection maturation. Therefore, our data indicate that the p50 NF-κB homodimer may play a role for the maturation or survival of CD8+, but not CD4+ T cells during T cell lineage differentiation and homeostasis under physiological conditions.

**p50 regulates PD-1 expression in response to antigenic stimulation in vivo**

To determine the function of p50 in regulating PD-1 expression in antigen-stimulated CD4+ and CD8+ T cells in vivo, the mixed BM chimera mice were vaccinated with 2W1S and OVA257-264 peptides followed by a boost with the same peptides (Figure S2A). Antigen-specific WT and p50 KO CD4+ and CD8+ T cells were identified and quantitated by flow cytometry using tetramer staining. Interestingly, significantly lower numbers of antigen-specific p50 KO CD4+ T cells were generated after 2W1S vaccination as compared to WT CD4+ T cells (Figure 5A). Similar phenomenon was observed with the antigen-specific CD8+ T cells (Figure 5C). Because both CD4+ and CD8+ p50 KO T cells were as responsive to

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**Figure 3.** p50 binds to the pdcd1 promoter chromatin in activated T cells. A. CD4+ and CD8+ T cells were isolated from spleens and lymph node and stimulated on an anti-CD3 and anti-CD28 coated plate. ChIP analysis of p50 association with the pdcd1 gene promoter was done one day after CD4+ T cell stimulation and three days after CD8+ T cell stimulation. The immunoprecipitated DNA was then analyzed by qPCR using a pair of pdcd1 promoter-specific qPCR primers (PD-1-P-Chipa-F & PD-1-P-Chip-B, Table S1). The Input was arbitrarily set to 1. Column, Mean; Bar, SD. B. CD4+ and CD8+ T cells were prepared and stimulated as in A. ChIP analysis of p50 NF-κB association with the pdcd1 gene promoter was done one day after CD4+ T cell stimulation and three days after CD8+ T cell stimulation. The immunoprecipitated DNA was then analyzed by semi-quantitative PCR using a pair of pdcd1 promoter-specific PCR primers (PD-1-Chip3-F & PD-1-Chip3-B, Table S1). Input DNA was used as a normalization control.

**Figure 4.** p50 regulates PD-1 transcription activation during T cell activation in vitro. A. CD3+ T cells were isolated from spleens and lymph nodes of two WT and two p50 KO mice and stimulated on anti-CD3 and anti-CD28-coated plates. After one day of stimulation, the T cells were collected and analyzed by flow cytometry. CD4+ T cells were first gated out and analyzed for CD25+ activated cells. The CD4+CD25+ cells were gated out and analyzed for MFI of PD-1. B. CD3+ T cells were isolated and stimulated as in A and analyzed for percentage of CD4+CD25+PD-1+ (left panel) and PD-1 MFI (right panel) in activated CD4+CD25+ cells at the indicated time points. Each data point represents an average of the triplicates collected for each time point. WT1: Wild type C57BL/6 mouse 1, WT2: Wild type C57BL/6 mouse 2, p50 KO1: p50 KO mouse 1, and p50 KO2: p50 KO mouse 2. C. CD3+ T cells were isolated and stimulated as in A. After one day of stimulation, the T cells were collected and analyzed by flow cytometry. CD8+ T cells were first gated out and analyzed for CD25+ activated cells. The CD8+CD25+ cells were gated out and analyzed for MFI of PD-1. D. CD3+ T cells were isolated and stimulated as in A and analyzed for percent CD8+CD25+PD-1+ (left panel) and PD-1 MFI (right panel) in activated CD8+CD25+ T cells at the indicated time points. Each data point represents an average of the triplicates collected for each time point. WT1: Wild type C57BL/6 mouse 1, WT2: Wild type C57BL/6 mouse 2, WT3: Wild type C57BL/6 mouse 3, WT4: Wild type C57BL/6 mouse 4, p50 KO1: p50 KO mouse 1, and p50 KO2: p50 KO mouse 2.
stimulation to become activated as compared to the WT T cells (Figure 4 & S1), the decreased number of antigen-specific p50 KO T cells in the mixed BM chimera mice suggests that, in addition to a key role in CD8\(^+\) T cell maturation and survival, the p50 NF-\(\kappa\)B homodimer is essential for the survival of antigen-specific CD4\(^+\) and CD8\(^+\) T cells during T cell antigen-mediated activation in vivo.

Next, we analyzed PD-1 expression in the antigen-specific CD4\(^+\) and CD8\(^+\) T cells. All antigen-specific CD4\(^+\) and CD8\(^+\) T cells in the mixed bone marrow chimera mice were PD1\(^+\) (Figure 5B & 5D). However, the PD-1 protein levels, as measured by MFI, were significantly lower in antigen-specific p50 KO CD4\(^+\) T cells as compared to their WT counterparts (Figure 5B). Similar to what was observed in CD4\(^+\) T cells, the antigen-specific p50KO CD8\(^+\) T cells also exhibited significantly lower PD-1 expression levels as compared to the antigen-specific WT CD8\(^+\) T cells (Figure 5D). Our findings therefore demonstrate that p50 is a pdcd1 transcriptional activator in both CD4\(^+\) and CD8\(^+\) T cells during their activation in response to antigen in vivo.

**H3K4me3 is enriched in the pdcd1 promoter chromatin**

Posttranslational modification of histones at specific genomic locations is an important mechanism of gene transcriptional regulation in T cells.\(^{45,46}\) H3K4me3 deposition in the promoter regions of T cell effector genes has been observed during T cells activation.\(^{45}\) Chromatin modifications have been shown to be associated with pdcd1 gene expression regulation in T cells.\(^{10}\) To determine whether H3K4me3 contributes to pdcd1 transcription activation during T cell activation, we first analyzed the expression of H3K4me-specific histone methyltransferases mll1, mll2, mll3, setd1a, and setd1b. PCR analysis revealed that low levels of mll1, mll2, mll3, setd1a, and setd1b are expressed in resting CD3\(^+\) T cells and the expression levels of these five histone methyltransferase are all significantly increased in activated T cells (Figure S3A). All five histone methyltransferases are highly expressed in the T cell leukemia EL4 cell line and stimulation does not further increase the expression levels of these histone methyltransferases (Figure S3B). ChIP analysis of H3K4me3 indicates low levels of H3K4me3 deposition at the pdcd1 promoter region in resting CD4\(^+\) and CD8\(^+\) T cells (Figure 6B). Consistent with the up-regulation of histone methyltransferases, H3K4me3 deposition is significantly increased at the pdcd1 promoter region surrounding the transcription initiation site one day after stimulation and maintained at high level 3 days after stimulation in both CD4\(^+\) and CD8\(^+\) T cells (Figure 6B). Also consistent with the high expression levels of histone methyltransferases, H3K4me3 levels are high in the pdcd1 promoter region in both unstimulated and stimulated EL4 cells (Figure 6C).

We also analyzed H3K9me3, H3K36me3, and H3K27me3 depositions in T cells. ChIP assays indicated that levels of these three histone modification markers are lower at the pdcd1 promoter region near the transcription initiation site in resting CD4\(^+\) and CD8\(^+\) T cells (Figure S4A & B). Stimulation did not increase the levels of these markers in CD4\(^+\) and CD8\(^+\) T cells (Figure S4A & B).

**H3K4me3 is essential for p50 function in activation of PD-1 expression**

The above observations that H3K4me3 deposition is high in the pdcd1 promoter region near the \(\kappa B\) element in activated T cells suggest that H3K4me3 might mediate p50 function in the pdcd1 promoter region. We therefore sought to determine whether H3K4me3 mediates PD-1 expression in T cells using the EL4 T cell line. Our previous study has shown that H3K4me3 regulates PD-L1 expression in tumor cells,\(^{47}\) we thus analyzed PD-L1 expression in EL4 cells. EL4 cells express both PD-L1 and PD-1, and in vitro stimulation up-regulated both PD-L1 and PD-1 expression in EL4 cells (Figure S5A). Treatment of EL4 cells with H3K4me3-specific histone methyltransferase inhibitor chaetocin\(^{48,49}\) decreases PD-L1 expression level in activated EL4 cells (Figure S5B). Chaetocin treatment significantly decreases PD-1 expression level in EL4 cells as well.
EMSA analysis revealed that, as in activated primary T cells, the p50 homodimer binds to the κBelemata the pdcd1 promoter DNA in EL4 cells (Figure 7B). ChIP analysis determined that, as expected, inhibition of these H3K4me3-specific histone methyltransferases significantly decreased H3K4me3 level at the pdcd1 promoter region in EL4 cells (Figure 7C). ChIP analysis also revealed that decreased H3K4me3 deposition in the pdcd1 promoter region is accompanied by a significant decrease of p50 binding to the pdcd1 promoter region in vivo (Figure 7C). Taken together, our data indicate that T cell activation leads to: 1) up-regulation of H3K4me3-specific histone methyltransferase and resultant
H3K4me3 enrichment at the pdcd1 promoter region near the κB element; and 2) the NF-κB p50 homodimer and H3K4me3 cooperate to activate pdcd1 transcription in activated T cells (Figure S6).

**Discussion**

Multiple layers of transcriptional regulatory mechanisms have been identified in the regulation of PD-1 expression in CD8\(^+\) T cells.\(^9\)\textsuperscript{-13} TCR signaling pathway-activated NFATc1 directly binds to the pdcd1 promoter to couple T cell activation with PD-1 transcriptional activation.\(^10\)\textsuperscript{-13} This NFATc1-mediated PD-1 expression can be further amplified by IL6 and IL12-activated STAT3 and STAT4.\(^10\) On the other hand, Blimp-1 and T-bet directly repress pdcd1 transcription.\(^9\) More recently, it was observed that the chromatin organizer SATB1 acts as an epigenetic repressor of pdcd1 transcription.\(^12\) These findings indicate that PD-1 expression is regulated by complex mechanisms. In this study, we observed a coordinate genetic (p50 homodimer) and epigenetic (H3K4me3) regulatory mechanism of pdcd1 transcription in activated T cells. We determined that although all five NF-κB subunits are activated in activated T cells, only the p50 NF-κB homodimer selectively binds to the κB element of the pdcd1 promoter to activate pdcd1 transcription in CD4\(^+\) and CD8\(^+\) T cells. Therefore, the p50 homodimer is a new transcription activator in activated CD4\(^+\) and CD8\(^+\) T cells.

Although p50 exhibited similar binding kinetics to the pdcd1 promoter κB DNA element in both CD4\(^+\) and CD8\(^+\) T cells in vitro, p50 deficiency resulted in a greater decrease in PD-1 expression activation in CD8\(^+\) T cells than in CD4\(^+\) T cells. Our WT and p50 KO competitive BM reconstitution studies indicate that p50 plays an important role in CD8\(^+\) T cell survival in vivo. It is therefore possible that, in addition to binding to the κB element of the pdcd1 promoter to directly regulate PD-1 expression, p50 may also mediate the CD8\(^+\) T cell survival signaling pathways during CD8\(^+\) T cell activation to indirectly regulate PD-1 expression in CD8\(^+\) T cells.

Previous studies have shown that H3K4me3 enrichment within the gene promoter is correlated with increased mRNA levels in effector and memory CD8\(^+\) T cells.\(^45\) Chromatin architecture of the pdcd1 locus is enhanced when pdcd1 is activated in primary CD8\(^+\) T cells.\(^10\) In macrophages, it was observed that H3K27ac, but not H3K4me3, is enriched at the pdcd1 promoter region.\(^36\) In this study, we observed that H3K4me3 is enriched in activated CD4\(^+\) and CD8\(^+\) T cells. Our data thus suggest that histone modifications may regulate the chromatin architecture at the pdcd1 promoter region and the specific types of histone modifications at the pdcd1 promoter region are cell type-dependent. H3K4me3 is enriched at the pdcd1 promoter region near the κB consensus-binding element in activated CD4\(^+\) and CD8\(^+\) T cells. H3K4 methylation is catalyzed by multiple histone methyltransferases.\(^50\)\textsuperscript{-53} Chaetocin inhibits MLL1, one of the histone methyltransferase for H3K4me3.\(^48,54\) Therefore, it is interesting that...
chaetocin is effective in decreasing H3K4me3 level at the pdcd1 promoter region to repress PD-1 expression in T cells. Furthermore, decreased H3K4me3 level in the pdcd1 promoter in chaetocin-treated cells leads to a decrease in both p50 binding to pdcd1 promoter and PD-1 expression in T cells. These observations thus indicate that H3K4me3-mediated chromatin conformation enhances p50 binding to the kB element at the pdcd1 promoter in T cells. Multiple histone methyltransferases catalyze H3K4me3 formation.  

The next challenge will be to determine whether other histone methyltransferases mediate H3K4me3 at the pdcd1 promoter in T cells and whether histone methyltransferases directly interact with p50 at the pdcd1 promoter to regulate PD-1 expression in T cells.

In macrophages, it is the p65 NF-κB complex that binds to the kB element at the pdcd1 promoter region.  

We determined here that the p50 homodimer binds to the pdcd1 promoter region to activate pdcd1 transcription in activated T cells. Unlike p65, RelB, and cRel subunits which possess a C-terminal transcription transactivation domain (TAD) and typically function as transcriptional activators, the p50 subunit lacks a TAD and is therefore considered a transcriptional repressor.  

The function of the p50 homodimer as a transcription factor is therefore cellular context-dependent.\textsuperscript{21,36} The p50 homodimer can act as a transcriptional activator by associating with transcriptional coactivators.\textsuperscript{39,41} In addition, the p50 homodimer can also function to repress transcription of kB element-dependent target genes through excluding transcriptionally active NF-κB heterodimers binding to the kB sites or by recruiting HDACs to kB element-containing promoters to decrease chromatin histone acetylation.\textsuperscript{37} Therefore, it is possible that different co-factors are associated with the p50 NF-κB and p65 NF-κB complexes at the pdcd1 promoter region in activated T cells and macrophages, respectively. It is also possible that H3K4me3 and H3K27ac mediate binding of these unique p50 and p65 NF-κB complexes to the pdcd1 promoter DNA to regulate PD-1 expression in activated T cells and macrophages.\textsuperscript{36}

PD-1 is a key target in cancer immunotherapy, current strategy has been focused on blocking PD-1 interaction with PD-L1, which has proven to be effective in reversing PD-1-mediated T cell immune suppression in the tumor microenvironment.\textsuperscript{1,2} Our findings that the p50 homodimer NF-κB regulates PD-1 expression in activated T cells suggest that targeting p50 may be a complimentary approach to PD-1 blocking antibody-based cancer immunotherapy. It has been shown that targeting PD-1 transcriptional activation to reduce PD-1 expression is as effective as anti-PD-1 and anti-PD-L1 blocking antibodies in the control of tumor growth.\textsuperscript{7,8} NF-κB is currently considered a key target in cancer and more than 785 NF-κB small molecule inhibitors have been developed for cancer therapy.\textsuperscript{23} Our data suggest that targeting the specific p50 NF-κB subunit, not the pan NF-κB complex, may represent a more attractive approach in reducing PD-1 expression level in T cells. Particularly, the p50-H3K4me3 axis at the pdcd1 promoter might be another molecular target for reducing PD-1 expression in activated T cells as an alternative strategy for anti-PD-1 and anti-PD-L1 blocking antibody cancer immunotherapy. In addition, NF-κB is known to regulate chronic inflammation to promote cancer and play a critical role in T cell lineage differentiation, activation, and survival.\textsuperscript{21,34} The function of the p50 homodimer in regulating PD-1 expression in CD8\textsuperscript{+} T cells may have a key impact on CD8\textsuperscript{+} T cell exhaustion under chronic inflammation conditions, such as in the tumor microenvironment.

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Author biographies

P.S.R., C.L., and J.D.K.: performed experiments. P.S.R., C.L., J.D.K. M.L. I., and T.K: developed methods. E.C.: provide key reagents and developed concept. G.Z., and K.L.: Concept development. P.S.R., and K.L.: designed studies and wrote manuscript.

ORCID

Kebin Liu http://orcid.org/0000-0003-1965-7240

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