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_________________________________ _______________
James Wesley Austin Date

Regulation of Programmed Cell Death-1 In CD8 T Cells

By

James Wesley Austin

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Genetics and Microbiology

____
Jeremy M Boss, Ph.D.
Advisor

____
John Lucchesi, Ph.D.
Committee Member

____
Daniel Reines, Ph.D.
Committee Member

____
Samuel Speck, Ph.D.
Committee Member

____
Paula Vertino, Ph.D.
Committee Member

Accepted:

____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

____
Date

Regulation of Programmed Cell Death-1 In CD8 T Cells

By

James Wesley Austin

B.S. University of Miami, 2006

Advisor: Jeremy M. Boss, Ph.D.

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Abstract

Regulation of Programmed Cell Death-1 In CD8 T Cells

By: James Austin

Programmed Death-1 (PD-1) is a transmembrane signaling molecule that negatively regulates effector functions of lymphocytes. Expression and subsequent signaling through PD-1 has been shown to mediate T cell exhaustion, a process that results in the loss of T cell effector function. This dissertation explores the mechanism by which PD-1 is regulated. Using an unbiased, PCR based approach, multiple DNase I hypersensitive sites (DHS) were discovered across the PD-1 locus. DHS, which are indicative of potential regulatory regions, were tested for regulatory activity with two regions showing enhancer function. IL-6 and IL-12, cytokines that activate STAT3 and STAT4 respectively, prolonged and increased PD-1 expression of activated T cells, but cytokine treatment alone was not sufficient to activate PD-1 expression. The transcription factors NFATc1 (T-cell receptor), STAT3 (IL-6), and STAT4 (IL-12) were shown to bind to the enhancer regions in response to their stimuli and were associated with a change in histone modifications at the regions. Both of the regulatory regions interact with the PD-1 promoter, providing a mechanism for their action. NFATc1 and STAT binding is not dependent on each other, although the regulatory function of the region is dependent on both factors being bound. The CCCTC-binding factor (CTCF) bound two locations in the PD-1 locus, flanking the regulatory regions and formed a constitutive regulatory loop, isolating the PD-1 locus from the surrounding chromatin. DNA methylation of the known regulatory regions of PD-1 showed a dynamic pattern that inversely correlated with PD-1 expression during acute infection. In chronic infections with low antigen levels, PD-1 expression is decreased, but without an accompanying increase in DNA methylation. Thus, the inverse correlation of PD-1 expression and DNA methylation was lost in chronic infections. Through this work multiple regulatory mechanisms of PD-1 have been established and provide additional targets for molecular therapies designed to reinvigorate exhausted T cells.

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Chapter 1. Introduction

This introduction will cover a number of topics. It will start with an examination of the immune system and programmed cell death-1's (PD-1) function. Following that, the cellular expression and signaling of PD-1 and its ligands will be discussed. As will be shown, T cell development and differentiation are regulated by PD-1, thus, those processes and PD-1's role in regulating them will be examined. PD-1's role in autoimmune diseases, cancers, and chronic viral infections will also be explored. The introduction will conclude with an in depth review of the cytokine signals and transcription factors that modulate PD-1 expression.

PD-1 Function and its Role in The Immune System

The immune system uses a complex network of molecular and cellular signals to protect higher eukaryotes from disease causing agents. It does this by discriminating self from non-self molecules and neutralizing or clearing potentially harmful substances. Parasitic worms, pathogenic bacteria, viruses, and more must be recognized as all can cause harmful infections. Foreign molecules, termed antigens, are detected through interactions between the particles and cell surface proteins from host cells. Following recognition, signals are transmitted through the outer membrane leading to changes in transcription that activate the defensive functions of the immune cells.

The immune system is comprised of two arms, innate and adaptive. The innate arm rapidly responds to pathogens in a non-specific manner. Innate responses are non-discriminate and have no long lasting memory. The adaptive immune system responds in an antigen-specific manner. The delayed response of the adaptive arm recognizes molecular

characteristics of an antigen through cell surface receptors unique to each type of immune cell. The antigenic recognition receptor of the T cell, the T-cell receptor (TCR), is created through genetic recombination of gene segments, allowing for extraordinary diversity (Chien et al., 1984). T cells expressing the antigen-specific receptor become activated upon recognition of the proper antigen when presented by major histocompatibility complex (MHC) protein on antigen-presenting cells (APCs) (Waldron et al., 1973). Signaling molecules, such as cluster of differentiation (CD) 3 interact with the TCR to relay signals that lead to proliferation and differentiation (Chang Tw Fau - Testa et al.; Tax Wj Fau - Willems et al.). In many cases this proliferation is extensive, with 12-15 doublings occurring over 5-8 days. Following antigen clearance, a small subset of T cells differentiate into memory cells, a population of long-lived cells that can rapidly respond in a greater magnitude upon reencountering the same antigen (Sallusto et al., 2010).

A critical negative regulator of immune cell function is PD-1. PD-1 was discovered in 1992 as a molecule that was overexpressed in two cell lines, the hematopoietic cell line LyD9 and the T cell hybridoma line 2B4.11 that were stimulated to undergo apoptosis (Ishida et al., 1992). Apoptosis was stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) treatment or interleukin 3 (IL-3) deprivation in 2B4.11 and LyD9 cells, respectively. Despite this evidence it was later shown that overexpression of PD-1 in these cell lines does not induce apoptosis (Agata et al., 1996). PD-1 was finally ascribed its function of negatively regulating the immune response in 1999 when it was discovered that PD-1 knockout mice developed lupus-like arthritis and glomerulonephritis (Nishimura et al., 1999). Finally, in 2006, PD-1 was discovered to be overexpressed in exhausted CD8 T cells (Barber et al., 2006). More importantly, PD-1 was shown to be functionally important in T cell exhaustion, as blocking PD-1 interactions with its ligand PD-ligand 1 (PD-L1)

reinvigorated the functionality of exhausted CD8 T cells. As will be discussed below, PD-1 has been shown to be important in T cell development and differentiation, autoimmune disease, anti-tumor immunity, and in viral infection. In addition to exhausted cells, PD-1 is expressed on activated T cells, B cells, monocytes, dendritic cells (DC), natural killer (NK) cells, and natural killer T (NKT) cells (Agata et al., 1996; Keir et al., 2008; Moll et al., 2009; Yamazaki et al., 2002).

PD-1 expression is controlled through a complex, hierarchical set of factors. Transcription factors such as B lymphocyte-induced maturation protein-1 (Blimp-1), nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1 (NFATc1), and T-box expressed in T cells (T-bet) have been shown to directly regulate PD-1 through a set of conserved sequences located upstream of the transcriptional start site (Kao et al., 2011; Lu et al., In press; Oestreich et al., 2008). Dynamic histone modifications and DNA methylation are associated with the regulatory regions of PD-1 (Lu et al., In press; Oestreich et al., 2008; Youngblood et al., 2013; Youngblood et al., 2011). Additionally, cytokines such as IL-2, IL-7, IL-12, and IFN-α have been shown to play a role in PD-1 expression and programming of cells to secondary responses (Gerner et al., 2013; Kinter et al., 2008; Terawaki et al., 2011). How all of the regulatory components interact to control PD-1 expression is still not understood. Complicating the matter is the disjoint between the roles of the above factors during acute versus chronic infections. PD-1 regulation will be described in detail below.

Cellular Expression of PD-1 and its Ligands

PD-1 was first described as being expressed in T cells stimulated with concanavalin A or PMA/Io and in B cells upon anti-IgM stimulation (Agata et al., 1996; Vibhakar et al., 1997). It is important to note that PD-1 is expressed at very low levels on naïve B or T cells, but is

inducible within 24 hours (Okazaki et al., 2001). In humans, the peak of PD-1 mRNA expression in T cells, stimulated *ex vivo* with anti-CD3/CD28 antibodies, was 48 hours post stimulation (Chemnitz et al., 2004). In contrast, using the same system in mice, PD-1 expression was maximal after 24 hours of stimulation (Lu et al., 2014). Murine DCs and macrophages express PD-1 following infection with *Listeria monocytogenes* or through stimulation of toll-like receptor 2 (TLR2), TLR3, TLR4 or the nucleotide binding oligomerization domain (NOD) protein (Cho et al., 2008; Yao et al., 2009). PD-1 is inducibly expressed in human monocytes by lipopolysaccharide and on myloid $CD11c^{+}$ DCs through TLR7 and TLR8 (Petrovas et al., 2006). PD-1 is also induced on NKT cells stimulated with α-galactosylceramide (Chang Ws Fau - Kim et al.) and on CD1d-restricted invariant NKT cells during human immunodeficiency virus (HIV) infection (Moll et al., 2009). In summation, multiple cells of the immune system inducibly express PD-1 following cell-specific activation signals.

There are two known ligands of PD-1, PD-L1 (Freeman et al., 2000) and PD-L2 (Latchman et al., 2001). PD-L1 is expressed on a multitude of immune cells, including T cells, B cells, dendritic cells (DCs), macrophages, and mast cells. (Keir et al., 2008; Yamazaki et al., 2002). PD-L1, which has a higher basal expression in mice than in humans, is also expressed on non-hematopoietic cell types, such as pancreatic islet cells, neurons, fibroblastic reticular cells, epithelia, vascular endothelial cells, mesenchymal stem cells, and keratinocytes (Eppihimer et al., 2002; Keir et al., 2008; Schreiner et al., 2004). PD-L2 expression is limited to DCs, macrophages, bone-marrow derived mast cells, and 60% of B1 B cells (Keir et al., 2008; Zhong et al., 2007). Both molecules have been found to interact with PD-1 and downregulate T cell responses. Interestingly, at the same time that PD-L1 and PD-L2 were discovered to negatively regulated T cells, other groups reported that B7-

H1 (Dong et al., 1999) and B7-DC (Tseng et al., 2001), molecules identical to PD-L1 and PD-L2, respectively, costimulated T cells. The mechanism for costimulation is still unknown. Comparing PD-L1 and PD-L2 knockout mice or studies using blocking antibodies revealed several overlapping functions between the ligands (Habicht et al., 2007; Kanai et al., 2003; Keir et al., 2006; Matsumoto et al., 2004). Despite this, there are still unique functions of each molecule in a tissue-specific manner. Ligation of PD-1 on a T cell with one of its ligands transmits a signal across the membrane of the T cell, leading to down-modulation of TCR signaling (Chemnitz et al., 2004; Parry et al., 2005; Sheppard et al., 2004). Down-modulation of TCR signaling by PD-1 is accomplished through the recruitment of the phosphatase SHP2, which inhibits the activity of both Zap70 and phosphoinositide 3-kinase (PI3K) (Figure1-1) (Parry et al., 2005; Sheppard et al., 2004). Inhibition of this positive signaling pathway to the T cell is important for proper T cell formation and function.

PD-1 Structure And Signaling

PD-1 is a member of the B7/CD28 superfamily of type 1 transmembrane glycoprotein receptors. Structurally, PD-1 shares between 21-33% sequence identity in its immunoglobulin variable (IgV) domain with other CD28 family members cytotoxic T-lymphocyte Antigen 4 (CTLA-4), CD28, and inducible T-cell costimulator (ICOS) (Greenwald et al., 2005). Unlike other CD28 family members, PD-1 lacks a membrane proximal cysteine residue; thus, PD-1 does not form homodimers like the other family members (Zhang et al., 2004). The cytoplasmic domain of PD-1 contains both an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM), which is necessary for PD-1's inhibitory function (Long, 1999;

Sidorenko and Clark, 2003). PD-L1 and PD-L2 are also type 1 transmembrane glycoproteins in the B7/CD28 superfamily. Both are composed of an immunoglobulin constant (IgC) and an IgV domain, which share approximately 40% amino acid identity. PD-L1 contains a well-conserved cytoplasmic domain comprising approximately 30 amino acids (Dong et al., 1999; Freeman et al., 2000). PD-L2's cytoplasmic domain is also approximately 30 amino acids in most mammals, with rodents having a shortened domain of 4 amino acids (Latchman et al., 2001; Tseng et al., 2001). Despite the ligands similarity, the affinity of PD-1 for each ligand is different with PD-1 having a three fold higher affinity for PD-L1 compared to PD-L2. Another member of the B7/CD28 family, CD80 (B7-1), has been shown to interact with PD-L1, but not PD-L2, to transduce an inhibitory signal to regulate T cells, although the mechanism of inhibition is unknown (Butte et al., 2007).

The mechanism by which PD-1 inhibits immune function was first characterized in the B cell line IIA1.6 (Okazaki et al., 2001). B-cell receptor (BCR) crosslinking induced Ca^{2+} signaling, which led to tyrosine residues in both the ITIM and ITSM of PD-1 being phosphorylated by the kinase Lyn. ITSM but not ITIM phosphorylation led to recruitment and phosphorylation of the SH2-domain containing tyrosine phosphatase 2 (SHP-2). Phospho-SHP-2 dephosphorylated the BCR signaling molecules Syk, Igα, and Igβ. Dephosphorylation of these molecules led to a decrease in signaling of downstream activation molecules, such as extracellular-signal-regulated kinase (ERK), Phosphoinositide 3-kinase (PI3K), and Phospholipase C (PLC). PD-1 inhibition of TCR signaling was later shown to be through similar mechanisms (Parry et al., 2005; Sheppard et al., 2004) (Figure 1-1). One difference in the T cell system is the recruitment of SHP-1 in addition to SHP-2 to the phosphorylated ITSM domain of PD-1 (Chemnitz et al., 2004). The role of SHP-1 in PD-1 signaling is still not fully understood. A hallmark of an ITSM is its ability to switch

between positive and negative signals (Sidorenko and Clark, 2003). In this regard, the ITSM of PD-1 may not be a true ITSM as its ability to switch to transduce a positive signal is potentially inhibited due to its inability to bind SLAM-associated protein (SAP) (Chemnitz et al., 2004), an adapter protein previously described to be critical for ITSM switching (Shlapatska et al., 2001).

T Cell Development

Development of T cells starts upon population of the thymus by T cell progenitors, a group of cells derived from hematopoietic stem cells (Fowlkes et al., 1985). The hematopoietic progenitors divide to give rise to immature thymocytes, also known as T cell precursors. T cell precursors express neither CD4 nor CD8, two lineage defining co-receptors, and thus are known as double negative (DN). There are four stages of DN cells, based upon expression of various cell surface molecules (Godfrey et al., 1993). During the transition from DN2 to DN3 the β chain of the TCR begins to undergo rearrangement (Godfrey et al., 1994). The TCRβ locus contains a number of variable (V) segments preceded by a leader (L) sequence, then two distinct clusters, which contain a single diverse (D) segment, several joining (J) segments, and an individual constant (C) segment (Kurosawa et al., 1981). Through a series of well-characterized rearrangements, an individual D segment is joined to a J segment. The newly rearranged (D)J segment then undergoes recombination with a V segment using the same procedure, creating a recombined V(D)J β chain.

Cells that fail to properly rearrange the β chain stay in the DN3 stage and die unless the cell is able to rearrange both the γ and δ loci, whereas cells that have properly rearranged the β chain move into the DN4 stage (Dudley et al., 1994). γ:δ T cells are a distinct population of T cells; however, as they were not considered in this study they will not be

discussed further. Rearranged TCR β chains from DN3 thymocytes interact with the pre-T-cell α (pTα), a surrogate α chain that leads to the production of the pre-T-cell receptor (Groettrup et al., 1993). A faulty rearrangement of the β chain leads to improper signaling through the pre-TCR. PD-1 negatively regulates TCR β selection by inhibiting pre-TCR signals, thereby ensuring a TCR β chain was produced that is able to transduce strong activation signals (Nishimura et al., 2000). Proper pre-TCR signals induce cell proliferation and expression of CD4 and CD8 (Levelt et al., 1993). Cells expressing both CD4 and CD8 are known as double-positive cells. Following proliferation the double-positive thymocytes undergo rearrangement of their TCR α chain.

The TCR α chain locus is organized in a different fashion than the β chain locus. Noticeably, there is a lack of a D segment, thus the TCR α chain only contains V and J segments (Yoshikai et al., 1985). Recombination of the V and J chains produces an α chain that pairs with the previously made β chain, replacing the pTα by outcompeting it during TCR production in the endoplasmic reticulum (Trop et al., 2000). As briefly mentioned above, functional TCRs recognize a foreign antigen in the context of self-presentation by an MHC molecule. A critical step in T cell development is the recognition of MHC, a process known as positive selection. (Bevan, 1977)

If the TCR formed from the previously made β chain and the newly made α chain recognizes self-MHC when presenting antigen, a signal is transmitted into the cell leading to cessation of further α chain rearrangement. If the newly formed TCR does not recognize the MHC molecule the α locus undergoes another round of recombination to produce a different α chain (Petrie et al., 1993). TCR α chain rearrangement continues until a TCR is produced that recognizes MHC or the cell undergoes cell death due to a lack of positive signals from the TCR. During the selection process, thymocytes express both CD4 and

CD8, but mature cells express only one of these costimulatory molecules. Which molecule is expressed depends on the interaction of the molecule with MHC. There are two classes of MHC, known as class I, which interacts with CD8 and class II, which interacts with CD4. Interaction of MHC with the costimulatory molecule sends signals that inhibit expression of the other co-stimulatory molecule, leading to mature T cells that express only one co-stimulatory molecule (von Boehmer et al., 1989).

Positive selection of thymocytes is critical for formation of T cells that recognize antigens presented by MHC. However, recognition of self-antigens leads to autoimmune disorders. Negative selection of thymocytes occurs to delete or anergize cells that express TCRs that recognize self-antigens (Kappler et al., 1987). Self-antigens are presented through MHC to developing T cells, with a strong recognition of the self-antigen leading to apoptosis of the T cell. Positive and negative selection occurs simultaneously. This is thought to be due to either the strength or quality of the TCR signal received by the developing T cell (Ashton-Rickardt et al., 1994). ERK signaling has been implicated in the selection process as high levels of ERK, c-Jun NH(2)-terminal kinase (JNK), and p38 activation led to negative selection, while lower levels of ERK activation led to positive selection (Alberola-Ila et al., 1996).

PD-1 knockout mice spontaneously develop autoimmune disorders, thus it came as no surprise when it was discovered that PD-1 regulates both positive and negative selection of thymocytes (Nishimura and Honjo, 2001; Nishimura et al., 1999). It was shown that PD-1:PD-L1 but not PD-1:PD-L2 interactions are critical during positive selection (Keir et al., 2005). PD-1 inhibitory signals help control TCR thresholds for selection of thymocytes through suppression of Bcl-2 and ERK phosphorylation. PD-1 has also been demonstrated to act in negative selection as there was an increase in DN thymocytes that underwent

positive selection in PD-1 knockout mice (Nishimura et al., 1999), but there was also an increase in death rates among DP thymocytes, suggesting an increase in negative selection (Blank et al., 2003). A possible explanation for these results is that absence of PD-1 lowers the TCR threshold, allowing positive selection of weakly interacting DN thymocytes. Lack of PD-1 also increases negative selection as moderate TCR signals under normal conditions are now much stronger, therefore the developing thymocytes are stimulated to undergo apoptosis.

T Cell Differentiation

Fully developed, mature T cells that have undergone the selection process, leave the thymus as naïve T cells. Naïve T cells circulate through the circulatory and lymphatic systems, interacting with APCs in secondary immune tissues, such as lymph nodes, that present both self and foreign antigens. Distinguishing between harmful and non-harmful antigens is necessary to prevent an immune response against innocuous substances. To keep aberrant activation from happening, T cells require three signals to become activated: signal 1, signal 2, and signal 3. Signal 1, the interaction of the TCR with MHC:peptide complexes, is needed to ensure specificity of the immune response. Signal 2 promotes survival through the interaction of the B7 family of co-receptors on the APC with CD28 on the T cell (Gonzalo et al., 2001). The final signal received by T cells, signal 3, which is provided by cytokines, controls the differentiation of CD4 T cells. CD8 T cells have a relatively uniform cytotoxic phenotype, whereas CD4 T cells can differentiate into multiple subtypes, including T helper 1, 2, and 17 (Th1, Th2, and Th17, respectively) T regulatory (Treg), and T follicular helper (Tfh) cells. Differentiation of naïve CD4 T cells into the appropriate effector CD4 subtype is accomplished via the strength of TCR signal and programming through cytokine

stimulation (Yamane and Paul, 2013). Once differentiated, distinguishing between the various CD4 subsets is accomplished by detection of the unique cytokine profiles produced by each cell subtype.

There is approximately a 10,000 fold expansion of T cells upon naïve T cell activation (Figure 1-2). Antigen clearance necessitates both a contraction and a shut down of effector function of the antigen-specific T cells. A critical step in controlling the immune response is the expression of inhibitory receptors. Receptors such as PD-1 (Nishimura et al., 1999), CTLA4 (Linsley et al., 1992), T-cell immunoglobulin domain and mucin domain 3 (TIM3) (Zhu et al., 2005), and killer cell lectin-like receptor subfamily G member 1 (KLRG1) (Robbins et al., 2002) downregulate T cell effector functions to inhibit immunopathology. A complete loss of antigen-specific cells would lead to susceptibility to reinfection with the same pathogen, therefore a population of cells needs to be maintained to protect against reinfection. These long-lived cells that protect upon reinfection are known as memory T cells (Figure 1-2). Although there is a contraction of antigen-specific cells, the frequency of memory antigen-specific cells is approximately 100-1,000 fold higher than that of the naïve-precursor population.

Memory T cells can be divided into two distinct populations, known as effector memory and central memory T cells (Sallusto et al., 1999). Effector memory cells rapidly differentiate into effector cells upon restimulation, secrete effector cytokines, such as interferon-γ (IFN-γ), IL-4, and IL-2, and are targeted to inflamed tissues. Central memory cells take longer to differentiate into effector T cells than effector memory and reexpress effector cytokines slower. Additionally, central memory cells express receptor molecules that target them to lymph nodes. Thus, two populations of memory cells allow for both a

rapid response at sites of infection, as well as a population that can survey draining lymph nodes.

Anergic T cells are cells that have encountered antigen but remain functionally inactive (Chappert and Schwartz, 2010). Anergic cells survive for an extended period of time, but remain hyporesponsive. Chikuma et al. showed that PD-1 is necessary for induction of anergy of CD8 T cell *in vivo* (Chikuma et al., 2009). Using the alloreactive 2C T cell model, it was shown that PD-1-deficient CD8 T cells dysregulated IL-2 upon anergy induction. Blockade of IL-2 resulted in the induction of anergic PD-1 null cells, whereas introduction of IL-2 to PD-1-sufficient cells resulted in a decrease in anergic T cells. Together this suggests that PD-1 signaling acts to inhibit autocrine signaling from IL-2. Indeed, using an *in vitro* CD4 T cell model, it was shown that PD-1 signaling decreased IL-2 production (Bishop et al., 2009). The exact mechanism for how PD-1 regulates IL-2 is unclear.

Regulatory T cells (Tregs) constitute a CD4 subset that suppresses T cell effector functions in the periphery (Asano et al., 1996; Mason and Powrie, 1998; Sakaguchi et al., 1995; Sakaguchi et al., 1996). A hallmark of Tregs is the expression of the transcription factor forkhead box protein 3 (Foxp3), the critical mediator of Treg differentiation (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). Tregs can be divided into two categories, natural (nTreg) and induced (iTreg). nTregs are generated during T cell development in the thymus when Foxp3 is inducibly expressed by double positive cells upon recognition of self-antigens (Fontenot et al., 2003). iTregs, which develop in the periphery from Foxp3^{-} naïve cells, are dependent on TGF-β and IL-2 for *in vitro* differentiation (Chen et al., 2003b; Davidson et al., 2007; Rubtsov and Rudensky, 2007). iTreg production is dramatically increased when PD-L1 is used in combination with TGF-β and TCR

stimulation to induce Treg differentiation (Francisco et al., 2009), and studies have shown that PD-1 signaling helps maintain Foxp3 expression and enhances the effectiveness of iTregs (Francisco et al., 2009). PD-1 signaling attenuates Akt-mTOR signaling while upregulating PTEN, which has been shown to be critical for iTreg formation (Haxhinasto et al., 2008; Sauer et al., 2008), providing a possible mechanism for PD-1 signaling mediated Treg development.

PD-1 in Chronic Viral Infection

Functional memory formation is accomplished upon antigen clearance. Chronic infections can lead to a phenotype known as functional exhaustion, wherein effector T cell function is lost without the formation of functional memory cells (Zajac et al., 1998) (Figure 1-2). Exhaustion, which has been characterized in a multitude of infections, such as lymphocytic choriomeningitis virus (LCMV) (Barber et al., 2006; Wherry et al., 2007), simian immunodeficiency virus (SIV) (Velu et al., 2007), HIV (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006), hepatitis C virus (HCV) (Kasprowicz et al., 2008; Urbani et al., 2006b), and hepatitis B virus (HBV) (Boettler et al., 2006; Boni et al., 2007; Peng et al., 2008), leads to a hierarchical loss of cytokine and effector functions in CD8 T cells. In each of these systems, exhausted T cells display high surface expression of inhibitory receptors (e.g. PD-1, CTLA4, and TIM3), with higher inhibitory receptor expression correlating with more severe exhaustion (Blackburn et al., 2008; Blackburn et al., 2009). As they become exhausted, T cells sequentially lose IL-2 production, cell proliferation, cytotoxic effects and tumor necrosis factor (TNF) production (Fuller and Zajac, 2003; Wherry and Ahmed, 2004; Wherry et al., 2003; Zajac et al., 1998). Severe exhaustion leads to a loss of IFN-γ and the inability to degranulate, culminating in the deletion of virus-specific T cells. CD4 T cells also

lose effector function during chronic infections; however, less is known about exhaustion of CD4 T cells compared to CD8 T cells (Brooks et al., 2005; Kaufmann et al., 2007; Oxenius et al., 1998; Urbani et al., 2006a).

In HIV infection, viral load, CD4 T cell counts, and CD8 T cell proliferation all correlate with the percentage of HIV-specific CD8 T cells that express PD-1 (D'Souza et al., 2007; Day et al., 2006). Streeck et al. showed that PD-1 expression on HIV-specific CD8 T cells decreased for epitopes that had undergone escape; however, T cells specific for conserved epitopes showed an increase in PD-1 expression over time (Streeck et al., 2008). This data suggests that continued antigen stimulation is important for overexpression of PD-1 in chronic infections. Upregulation of PD-1 is beneficial to HIV as a way to escape targeting by CD8 T cells. Hence, it is not surprising that HIV targets PD-1 for upregulation through negative factor (Nef) using a p38 mitogen-activated protein kinase-dependent mechanism (Muthumani et al., 2008). Another virus that takes advantage of the PD-1 pathway to escape the immune system is HCV. HCV-specific T cells showed a correlation between PD-1 expression and effector function (Nakamoto et al., 2009), and it was demonstrated that HCV-core protein can induce PD-1 expression upon binding to the gC1q complement receptor (Yao et al., 2007b).

The discovery that PD-1 marks exhausted T cells and acts as a negative regulator of T cell function led to studies looking at ways to abrogate the PD-1 pathway to reinvigorate T cell function. The first report of reinvigoration of exhausted T cells was accomplished through the blockade of the PD-1:PD-L1 interaction in the LCMV model (Barber et al., 2006). Blocking the PD-1:PD-L1 interaction via antibodies to either PD-1 or PD-L1 restored cytokine production, increased LCMV-specific T cell numbers, and reduced viral loads (Figure 1-2). Subsequently, blockade studies using an anti-PD-1 or anti-PD-L1

antibody in the SIV and HIV models have demonstrated reduced viral loads, increased proliferation, and an increase in effector function of virus-specific cells (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Velu et al., 2009). Despite this success, anti-PD-1/L1 treatments do not cure disease. A possible explanation is that not all exhausted T cells can be invigorated by anti-PD-1 or anti-PD-L1 treatment. In both LCMV and HCV, two populations of virus-specific cells exist based on PD-1 levels. Cells that cannot be reinvigorated are PD-1^{hi} and show more severe exhaustion than PD-1^{int} cells, which can be reinvigorated by PD-1 blockade. In addition, as discussed below, PD-1 is a mediator of autoimmune disorders. Treatments need to be modulated so that T cell effector function is restored but immunopathology is limited.

PD-1 in Autoimmunity

PD-1 knockout mice develop strain-specific autoimmune disorders. C57Bl/6 mice develop lupus-like glomerulonephritis and arthritis (Nishimura et al., 1996; Nishimura et al., 1999), while BALB/c, nonobese diabetic (NOD), and Murphy Roth's Large (MRL) display dilated cardiomyopathy and gastritis (Nishimura et al., 2001), subacute type I diabetes (Wang et al., 2005), and lethal myocarditis (Wang et al., 2010), respectively. Pancreatic islet cells from NOD mice upregulate PD-L1 to inhibit T cell mediated killing of islet cells, thereby slowing the progression of diabetes (Liang et al., 2003). Blockade of PD-1:PD-L1 interactions in these mice led to an accelerated onset of disease (Ansari et al., 2003). Like the diabetes model, in the experimental autoimmune encephalomyelitis (EAE) model the blockade of PD-1 signaling led to a more rapid onset and more severe disease (Salama et al., 2003). In this model, PD-L1 on T cells, APCs, and host tissue was critical to restrict myelin-specific T cell effector function (Latchman et al., 2004). The EAE model displays a strain-specific

phenotype where blockade of PD-L1 but not PD-L2 exacerbates disease in BALB/c and SJL/J mice whereas in C57Bl/6 mice, PD-L2 but not PD-L1 blockade worsens the disease (Salama et al., 2003; Zhu et al., 2006). One possible explanation for the strain-specific effect was differential expression of PD-L1 and PD-L2, but it was shown that expression of the ligands was comparable between mouse strains (Zhu et al., 2006). The strain-specific effects of PD-L1 and PD-L2 blockade are still not understood.

In humans, there have been several reports of SNPs in the PD-1 gene linked to various autoimmune diseases, including multiple sclerosis (Kroner et al., 2005), rheumatoid arthritis, type I diabetes (Nielsen et al., 2003), Graves disease (Hayashi et al., 2008), systemic lupus erythematosus (Prokunina et al., 2002), and ankylosing spondylitis (Lee et al., 2006a). A SNP in intron 4 of PD-1, named PD1.3, occurs in a putative binding site for runt-related transcription factor 1 (RUNX1) (Prokunina et al., 2002). The A (risk) allele shows a decrease in PD-1 induction compared to the G (non-risk) allele in *in vitro* settings (Bertsias et al., 2009). This same SNP was also associated with increased IFN-γ production in multiple sclerosis (MS) patients (Kroner et al., 2005). Despite this evidence, to date no divergent regulatory activity in RUNX1 binding activity *in vivo* has been found associated with this SNP (Oestreich and Boss, unpublished data). Unsurprisingly, blockade of the PD-1:PD-L1 pathway contributes to some disease progression in humans. In patients with rheumatoid arthritis, autoantibodies to PD-L1 have been discovered and correlate with disease progression (Dong et al., 2003). However, it is not clear that all of their autoantibodies are functioning to block PD-1 signaling.

As discussed above, PD-1 is critical in selection of developing thymocytes and in formation of Tregs. This implicates PD-1 in both central and peripheral tolerance. However, PD-1 also functions in preserving immune privileged sites (Hori et al., 2006).

Immune privileged sites, such as the eye, testes, and placenta, are protected against inflammation and immune responses that could potentially damage critical tissues. PD-L1 is constitutively expressed in the cornea, and its interaction with PD-1 on T cells protects the eye (Meng et al., 2006; Sugita et al., 2009; Watson et al., 2006). PD-L1 was also shown to play a role in fetal-maternal tolerance as PD-L1 was expressed on placental and vascular endothelial cells during gestation (Guleria et al., 2005; Holets et al., 2006).

PD-1 in Cancer

In much the same way that viruses upregulate PD-1 to escape the immune system, cancer cells take advantage of PD-1's inhibitory effects to modulate the immune response. PD-L1 and PD-L2 expression in multiple types of cancers have been shown to correlate with an unfavorable outcome. Studies on renal cell carcinoma have shown that patients with high PD-L1 expression on tumors and/or lymphocytes succumbed to their cancer more rapidly than patients with low PD-L1 expression (Thompson et al., 2004). In ovarian cancer a similar pattern emerged where patients that had tumors with high levels of PD-L1 had a shorter survival time than patients with low PD-L1 (Hamanishi et al., 2007). Moreover, patients with tumors that expressed PD-L1 and PD-L2 had a mortality rate almost double that of patients that lacked expression of the ligands. In addition to ovarian and renal cancers, PD-L1 has been shown to be an indicator for prognosis in esophageal (Ohigashi et al., 2005), gastric (Wu et al., 2006), malignant melanoma (Hino et al., 2010), bladder (Nakanishi et al., 2007), and pancreatic cancers (Nomi et al., 2007).

PD-L1 and PD-L2 expression on tumors can suppress cancer-specific T cell effector function as an evasion mechanism (Curiel et al., 2003; Hirano et al., 2005; Iwai et al., 2002). Blockade of PD-1 signaling of cancer-specific cells has been shown to be an efficacious

treatment. In addition to the use of anti-PD-1 or anti-PD-L antibodies (Blank et al., 2004; Iwai et al., 2002; Iwai et al., 2005), other systems such as RNA interference (Borkner et al., 2010), genetic ablation of PD-1 (Ding et al., 2010), DNA vaccination with the extracellular region of PD-1 (He et al., 2004), and using the extracellular domain of PD-1 or PD-L1 (Terawaki et al., 2007) have been tried as blocking mechanisms. Recently, phase 1 clinical trials have been conducted using anti-PD1 or anti-PD-L1 as a treatment for a variety of cancers (Brahmer et al., 2012; Hamid et al., 2013; Topalian et al., 2012b). All standard therapies used to treat these cancer patients have failed; however, the PD-1 blockade studies have shown remarkable efficiency, leading to remission in a multitude of cancer types including lung cancer, renal carcinoma, and melanoma.

Modulation of PD-1 and its Ligands by Cytokines

IFN-α positively regulates PD-1 expression in both macrophages and T cells by inducing multiple transcription factors to bind to a conserved regulatory region of PD-1 (Cho et al., 2008; Terawaki et al., 2011). A detailed discussion of these factors and the regulatory region is presented in the following section. In addition to IFN-α positively regulating PD-1 on macrophages, the other type I interferon, IFN-β, also stimulates PD-1 expression on macrophages and DCs (Jin et al., 2013). Recently it was discovered that programming of CD8 T cells by IFN-α led to an increase in PD-1 expression and decrease in effector function upon antigen re-encounter (Gerner et al., 2013). Blockade of PD-1 signaling led to an increase in functionality, suggesting that the increased PD-1 was the cause of the functional impairment in the IFN-α programmed cells. In contrast, programming by IL-12 showed lower levels of PD-1 than IFN-α programmed cells upon restimulation, and there was no functional impairment of IL-12 programmed cells as blockade of PD-1 had no

effect. Cells programmed in both IFN-α and IL-12 adopted the IL-12 phenotype, exhibiting that there is no need to eliminate type I interferon signaling during T cell priming to obtain more functional T cells. In agreement with these results, Schurich et al. showed that HBV-specific CD8 T cells from chronically-infected patients produced more IFN-γ and less PD-1 upon restimulation with antigen and IL-12 as compared to antigen only stimulated cells (Schurich et al., 2013). Restimulation of the HBV-specific cells with IFN-α and antigen showed no difference from cells stimulated with antigen alone. Interestingly, the effect of IL-12 appears to be specific for exhausted cells, as cytomegalovirus (CMV)-specific cells, which are not considered to be exhausted, showed no effect of IL-12 on PD-1 expression upon restimulation. A negative regulator of PD-1 expression, T-bet (Kao et al., 2011), was more highly expressed in IL-12 stimulated cells, providing a mechanism for the decrease in PD-1 expression (Schurich et al., 2013). T-bet's role as a direct repressor of PD-1 expression is discussed in detail below.

The cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 share a common γ-chain receptor subunit (Rochman et al., 2009). Together, these cytokines play key roles in T cell survival, function, and differentiation. *Ex vivo* culture of CD4 and CD8 central memory, effector memory, or effector T cells with the common γ-chain cytokines IL-2, IL-7, IL-15, or IL-21 led to an increase of PD-1 expression (Kinter et al., 2008). In contrast, cytokine treatment alone elicited no PD-1 expression on naïve T cells, implying T cell activation is necessary for cytokines to have and effect. IL-2 treatment has been shown to increase effector function of exhausted CD8 T cells (Blattman et al., 2003), implying that PD-1 expression would be lower in these reinvigorated cells. Indeed, a report by West et al. using the chronic LCMV clone 13 system showed that IL-2 treatment of infected mice led to a decrease in PD-1 on LCMV-specific CD8 T cells (West et al., 2013). As with IL-12

treatment of HBV-specific cells discussed above, IL-2 treatment increased T-bet expression, suggesting a possible mechanism for the decreased PD-1 expression. The use of IL-2 as a therapy to down-regulate PD-1 expression is not universally successful. Peripheral blood mononucleated cells (PBMCs) from HIV infected patients undergoing IL-2 immunotherapy had higher PD-1 expression following IL-2 therapy than prior to treatment (Kinter et al., 2008). It is possible that IL-2 treatment is effective in a species or virus-specific manner. Alternatively, IL-2 immunotherapy may only be effective on virus-specific CD8 T cells and not total PBMCs.

IL-7 treatment of infected mice led to clone 13 viral clearance and an increase in T cell effector function by antagonizing inhibitory pathways (Nanjappa et al., 2011; Pellegrini et al., 2009; Pellegrini et al., 2011). Analysis of splenic CD4 and CD8 T cells showed that following three weeks of IL-7 treatment, PD-1 expression was decreased compared to control treated animals. Due to the drop in viral load, the effect of IL-7 on PD-1 expression could be due to the loss of antigen stimulation and not a direct effect of IL-7. In agreement with this hypothesis, a phase I dose escalation study using recombinant human IL-7 in HIV infected individuals on highly active antiretroviral therapy (HAART) therapy showed no change in PD-1 expression on CMV, HIV, Epstein-Barr virus (EBV), or flu-specific CD8 T cells (Sereti et al., 2009). In addition to no change in PD-1 levels, there was no appreciable change in viral load in this study, providing more evidence that a decrease in viral antigen and not IL-7 effects PD-1 expression *in vivo*. One caveat to this explanation is that the human study used a single dose strategy instead of a multiple injection strategy as in the mouse experiment; therefore it's possible multiple IL-7 treatments are necessary to observe a change in PD-1 expression.

IL-21 production has been linked to control and functionality of CD8 T cells in HIV patients (Chevalier et al., 2011; Williams et al., 2011; Yue et al., 2010). In agreement with this observation, IL-21 receptor knockout mice developed more severe exhaustion following LCMV challenge (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009). Interestingly, although IL-21 led to an increase in PD-1 expression *ex vivo* (Kinter et al., 2008), loss of IL-21 signaling *in vivo* had no effect on PD-1 expression in CD8 T cells during chronic LCMV infection (Frohlich et al., 2009; Yi et al., 2009). Thus, while critical for proper CD8 functionality, the role of IL-21 in directly regulating PD-1 expression is still unclear.

Transforming growth factor-β (TGF-β), through phosphorylation of SMAD2 has been linked to T cell exhaustion and immune dysfunction in HCV and HIV (Alatrakchi et al., 2007; Garba et al., 2002; Tinoco et al., 2009). SMAD2 phosphorylation was increased in chronic versus acute LCMV infection, while blockade of TGF-β using a dominant negative TFG-β receptor (dnTGFβR) restored functionality of exhausted T cells. PD-1 expression was decreased in dnTGFβR CD8 T cells at 9 days post infection compared to WT CD8 T cells, however this was likely a consequence of viral clearance as PD-1 levels at day 5 post infection are similar (Tinoco et al., 2009). Thus, the decrease of PD-1 in dnTGFβR mice was likely a secondary effect. In agreement with this, WT and dnTGFβR CD8 T cells from mixed bone marrow chimeras showed comparable PD-1 levels.

Increased IL-10 production is associated with multiple chronic infections such as HCV (Hofer et al., 2005; Woitas et al., 2002), HIV (Graziosi et al., 1994; Zanussi et al., 1996), and LCMV (Ejrnaes et al., 2006). In HIV, the source of IL-10 production that most strongly correlated with viral replication and disease progression was CD4 T cells (Clerici et al., 1997; Orandle et al., 2001; Ostrowski et al., 2001). IL-10 signaling blockade, accomplished with an anti-IL-10 receptor (IL-10R) antibody, led to decreased PD-1

expression and viral clearance of clone 13 (Ejrnaes et al., 2006). Similar to clone 13, IL-10 blockade has been shown to be an effective treatment *ex vivo* in HIV (Clerici et al., 1994; Landay et al., 1996) and HCV (Rigopoulou et al., 2005). Not all chronic infections can be cleared through IL-10 signaling blockade. The chronic strain LCMV Docile causes more severe exhaustion than clone 13 (Moskophidis et al., 1995), and could not be cleared by IL-10 deficiency in mice (Richter et al., 2013).

IL-10 production and decreased IL-12 production in APCs is a consequence of TLR2 signaling (Hirschfeld et al., 2001; Re and Strominger, 2001). DCs that matured in the presence of IL-10 were poor at providing activation signals to cells and led to CD4 T cells that produced IL-10 (Akbari et al., 2001). IL-10 can positively regulate itself through a feedback mechanism implying that DCs and CD4 T cells can regulate activation of each other through a cytokine circuit. (Saraiva and O'Garra, 2010). In contrast, TLR4 (Hirschfeld et al., 2001; Re and Strominger, 2001) and IFN-γ (Hayes et al., 1998) signaling led to IL-12 production (Hirschfeld et al., 2001; Re and Strominger, 2001), which as described above, led to functionally superior programmed T cells. IL-12 stimulation increased IFN-γ production from T cells, thereby forming its own positive feedback circuit (Kusaba et al., 2005). IL-10R blockade led to a decrease of PD-1 expression, an increase in IFN-γ production by T cells and a decrease in IL-10 producing DCs. Therefore, the question of how IL-10 signaling blockade regulates PD-1 is still not resolved. It is unclear if the change in PD-1 expression following IL-10 blockade is a function of decreased IL-10, increased IL-12, or both.

Unstimulated Tregs express low levels of PD-1 on their surface, however PD-1 accumulates intracellularly (Raimondi et al., 2006). Accumulation of internal PD-1 can be augmented through estrogen receptor (ER) signaling (Polanczyk et al., 2007). ERα but not

ERβ knockout mice showed a decrease of intracellular PD-1 in Tregs, implying that ERα regulates PD-1 expression. Estrogen signaling can regulate gene expression through multiple mechanisms (Sanchez et al., 2002). Classical ER signaling occurs when DNA bound ER recruits coactivators, such as p160 or cAMP response element-binding proteins (CREB) (Shang et al., 2000; Xu et al., 1999). In addition, ER can interact with other DNA proteins, such as activator protein 1 (AP-1), to regulate gene expression without direct ER-DNA binding (Gaub et al., 1990; Kushner et al., 2000). A third pathway, known as the nongenomic pathway, involves the rapid activation of the mitogen-activated protein kinases (MAPK) or protein kinase C pathways through estrogen signaling (Kelly et al., 1999; Migliaccio et al., 1996). It is not known which of these pathways is involved in PD-1 expression through the estrogen receptor.

Many of the cytokines that regulate PD-1 expression also regulate expression of PD-L1 and PD-L2. The common γ-chain cytokines IL-2, IL-7, IL-15, and IL-21 regulate PD-L1 and PD-L2 on many cell types, including macrophages, monocytes, T cells and B cells. (Kinter et al., 2008). *In vitro* studies have shown that IFN-α, -β, and -γ increase PD-L1 expression on endothelial cells (Eppihimer et al., 2002), while IFN-β stimulates PD-L1 and PD-L2 expression on monocytes, DCs, and CD4 T cells (Schreiner et al., 2004; Wiesemann et al., 2008). Using IL-12 to stimulate the production of IFN-γ *in vivo*, it was shown that PD-L1 expression was increased in various tissues in the mouse (Gately et al., 1994). Importantly, IFN-γ knockout mice showed no increase in PD-L1 when given IL-12, indicating that the increase in PD-L1 expression is an indirect effect of IL-12. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has also been shown to activate PD-L1 and PD-L2 on macrophages and DCs through an unknown mechanism (Yamazaki et al., 2002).

Macrophages upregulate PD-L1 expression upon interaction with Th1 or Th2 cells, even in the absence of peptide, although addition of peptide leads to a stronger induction (Loke and Allison, 2003). PD-L2 is upregulated on macrophages by both Th1 and Th2 cells, but in a peptide-dependent manner. The upregulation by Th1 cells is at least partially dependent on signal transducer and activator of transcription 1 (STAT1) as STAT1-deficient macrophages have less PD-L1 expression following Th1 induction. IL-4 and STAT6 are the critical mediators of Th2-dependent upregulation as IL-4R and STAT6-deficient mice show an almost complete loss of PD-L2 induction on Th2 stimulated macrophages. In agreement with these studies, STAT6-dependent upregulation of PD-L2 was observed on macrophages following helminth infection (Huber et al., 2010). Further investigation showed that STAT6 was activated through production of IL-4 and IL-13 by CD4 T cells. There is no STAT6 binding site at the PD-L2 promoter, thus how STAT6 regulates PD-L2 is still unknown (Loke and Allison, 2003). IL-4 also positively regulates PD-L1 expression on macrophages in a STAT6-dependent manner (Huber et al., 2010; Loke and Allison, 2003).

IL-10 and IL-27 induce PD-L1 expression in a STAT3-dependent manner on macrophages and DCs (Karakhanova et al., 2011; Matta et al., 2012; Selenko-Gebauer et al., 2003; Wolfle et al., 2011). IL-6, another activator of STAT3, was also shown to be important in the upregulation of PD-L1 on microglia, macrophages, and DCs during Theiler's murine encephalomyelitis virus (Jin et al., 2013). In this system, overexpression of IL-6 led to an increase in PD-L1, while an IL-6 deficiency led to a decrease in PD-L1. As demonstrated, the expression of PD-1, PD-L1 and PD-L2 is effected by multiple cytokines. Several of the cytokines discussed enhance the expression of PD-1 and its ligands. Since PD-1 requires interaction with one of its ligands to downmodulate T cell effector function, the use of a

single cytokine to effectively upregulate PD-1, PD-L1 and/or PD-L2 allows for a rapid turning off of the local immune response.

Transcription Factors of PD-1 and its Ligands

TCR, BCR, and Fc receptor signaling leads to a rise in the concentration of intracellular Ca^{2+} (Loh et al., 1996; Rao et al., 1997). The rise in calcium activates the phosphatase calcineurin and subsequent dephosphorylation of NFAT proteins. NFAT proteins regulate multiple T cell effector genes, such as IL-2, IL-4, and IFN-γ (Campbell et al., 1996; Rooney et al., 1995; Weiss et al., 1996). Blocking calcineurin-dependent dephosphorylation by cyclosporine A (CsA) inhibits activation of NFAT target genes (Campbell et al., 1996; Flanagan et al., 1991; Fruman et al., 1992; Loh et al., 1996). Discovered in 1976 as a fungus metabolite (Ruegger et al., 1976), the small, cyclic peptide CsA was shown to have multiple anti-lymphocytic functions within two years of its discovery (Borel et al., 1976; Borel et al., 1977). In 2008, it was discovered that CsA inhibits induction of PD-1 during CD8 T cell activation (Oestreich et al., 2008). NFATc1 (or NFAT2) bound to a DNase I hypersensitive region upstream of the PD-1 transcription start site (TSS) termed conserved region C (CR-C) for its mammalian sequence homology (Figure 1-3A). Another site proximal to the TSS, termed CR-B was also sensitive to DNase I digestion but its specific function was not determined. Active histone modifications, such as histone H3 lysine 9 acetylation (H3K9ac), H4K16ac, and H3K4 trimethylation (me3) were also present in cells actively transcribing PD-1, but not in naïve, non-transcribing cells. NFATc1's role in activating PD-1 transcription was originally determined in CD8 T cells, but it has subsequently been shown that CD4 T cells and B cells also require NFATc1 to initiate PD-1 expression (Bally and Boss, personal communication). During chronic viral infection, NFAT translocation is impaired (Agnellini et al., 2007). This

suggests that while critical for activation, NFATc1 is not needed to maintain PD-1 expression in a chronic viral setting. Recently, NFκB was discovered to bind to CR-C in activated macrophages to promote PD-1 expression (Bally and Boss, personal communication). Using a combination of PMA and ionomycin to induce NFATc1 signaling was unsuccessful in activating PD-1, making macrophages the first cell type discovered to regulate PD-1 in an NFATc1-independent manner.

A frequent partner of NFAT in gene regulation is AP-1, a heterodimeric protein composed of Jun, Fos, Jun dimerization protein (JDP), or activating transcription factor (ATF) family members (Macian et al., 2001). AP-1 is activated through the mitogen-activated protein kinase (MAPK) pathway, which includes members ERK, JNK, Ras, and Raf (Karin, 1995). In the Lewis lung carcinoma model, an AP-1 component, c-Fos was found to be overexpressed in tumor infiltrating T cells and correlated with more robust tumor growth (Xiao et al., 2012). Overexpression of c-Fos led to an increase of PD-1 mRNA through an AP-1 binding site located in CR-B (Figure 1-3A). Deletion of the AP-1 binding site enhanced anti-tumor T cell function, presumably by downmodulating PD-1 expression and ultimately its negative affects on T cell function.

As discussed above, PD-1 expression is augmented in activated macrophages and T cells when stimulated with IFN-α (Cho et al., 2008; Terawaki et al., 2011). Analysis of the upstream regions of the PD-1 promoter identified a perfect consensus Interferon-sensitive response element (ISRE) located within CR-C. Type I interferons (IFN-α and IFN-β) can induce the formation of the interferon-stimulated gene factor 3 complex (ISGF3) (Horvath et al., 1996). This trimer is composed of STAT1, STAT2 and interferon regulatory factor 9 (IRF9). Although individually each factor can bind DNA, when in the ISGF3 complex, IRF9 acts as the DNA binding component (Veals et al., 1993). In IFN-α stimulated

macrophages, STAT1 and STAT2 were found to bind the region through electrophoretic mobility shift assays (EMSA) (Cho et al., 2008). The binding of IRF9 was not determined in macrophages, therefore it is unknown if STAT1 and STAT2 are binding the locus as a heterodimer or as part of the ISGF3 complex. The final component of ISGF3, IRF9, bound the ISRE in activated T cells stimulated with IFN-α as determined by chromatin immunoprecipitation (ChIP). Together the data indicates that ISGF3 regulates PD-1 in both macrophages and activated T cells exposed to type I interferons (Figure 1-3A).

The Notch signaling cascade has been implicated in regulating T cell effector functions (Cho et al., 2009; Maekawa et al., 2008). This observation led Mathieu et al. to examine the effects of Notch on PD-1 expression. PD-1 expression was decreased in activated CD8 T cells treated with Notch inhibitors DAPT or SAHM1 compared to controls, suggesting that Notch positively regulates PD-1(Mathieu et al., 2013). Notch target genes are activated when the Notch intracellular domain (NICD) translocates into the nucleus and interacts with recombination signal binding protein for immunoglobulin kappa J (RBP-J) (Borggrefe and Oswald, 2009). In the absence of NICD, RBP-J can recruit repressive complexes to repress gene expression. In agreement with Notch being an activator of PD-1, both RPB-J and NICD bound to two sites, located upstream of CR-C and in the first intron (Figure 1-3A). Interestingly, maximal binding of the factors was at 6 hours and was lost by 24 hours post stimulation. This suggests that Notch is involved in early expression of PD-1; however, the effects of Notch signaling blockade are seen 48 hours post activation. The reason why Notch binding is lost but its effects remain is still not understood. It is also important to note that RBP-J and NICD did not bind in naïve cells, suggesting a cooperative effect of NFATc1 and Notch as seen in keratinocyte growth and differentiation (Mammucari et al., 2005).

T-box expressed in T cells (T-bet) is expressed at higher levels in LCMV-specific CD8 T cells during acute Armstrong compared to chronic clone 13 infection (Kao et al., 2011). There was also an inverse correlation between PD-1 and T-bet in exhausted cells during chronic LCMV infection (i.e. PD-1^{hi} cells were T-bet^{lo}, while PD-1^{int} cells were T-bet^{hi}). Further, T-bet conditional knockout mice showed an increase in PD-1 surface expression compared to WT mice following clone 13 infection. T-bet bound to a region between CR-B and CR-C (Figure 1-3A) to directly repress PD-1 expression, with overexpression of T-bet leading to a skewing of LCMV-specific cells to the PD-1^{int} phenotype during clone 13 infection. T-bet can repress NFAT-mediated activation of IL-21 (Mehta et al., 2005), but what if any effect T-bet has on NFATc1 at PD-1 is unknown.

B lymphocyte-induced maturation protein-1 (Blimp-1) is induced following T cell activation, reaching a maximal expression approximately 8 days post infection (Rutishauser et al., 2009), a time at which PD-1 levels have declined (Youngblood et al., 2011). As with T-bet (Intlekofer et al., 2007; Joshi et al., 2007), Blimp-1 is also required for memory cell formation (Rutishauser et al., 2009). During chronic viral infection higher Blimp-1 expression correlated with higher PD-1 expression and deletion of Blimp-1 in antigen-specific cells during chronic infection led to a decrease in PD-1 surface expression (Shin et al., 2009). Blimp-1 is required for proper T cell function and memory formation (Kallies et al., 2009), leading to the hypothesis that Blimp-1 functions as a transcriptional rheostat where low Blimp-1 is necessary for proper T cell function and high Blimp-1 leads to exhaustion. Recently, during acute infection, Blimp-1 was found to be a direct repressor of PD-1 by using a feed-forward repressive transcriptional circuit (Lu et al., In press). Blimp-1 bound to a site adjacent to CR-C and directly repressed PD-1, while also repressing the activator NFATc1 (Figure 1-3A). Blimp-1 has been shown to recruit histone deacetylase

(HDAC) 1 and 2 (Yu et al., 2000), the histone methyltransferase G9a (Gyory et al., 2004), and the histone demethylase LSD1 (Su et al., 2009). Together, these enzymes change the chromatin structure from an active to a repressed state. Indeed, exogenous expression of Blimp-1 led to a repressed chromatin state of PD-1, while loss of Blimp-1 led to an active gene state and prolonged PD-1 expression during acute LCMV infection (Lu et al., In press). While Blimp-1 acts as a repressor in acute settings, its role in chronic settings is still not understood. It is possible that Blimp-1 is modified in chronic infection such that it no longer functions as a repressor. Alternatively, Blimp-1 function could be overridden by another transcription factor.

In addition to histone modifications, DNA methylation at CR-B and CR-C is dynamic during viral infection (Youngblood et al., 2011) (Figure 1-3A). The CR-B region in naïve CD8 T cells is almost completely methylated, while the CR-C region is only partially methylated. During acute LCMV infection at day 4, a time point where PD-1 expression is significantly induced, both CR-B and CR-C have a significant reduction in DNA methylation. As antigen is cleared, there is a remethylation of late effector cells (day 8), which correlates with reduced PD-1 levels. Memory cells, which no longer express PD-1, have remethylated CR-B to levels comparable to naïve cells. At CR-C, memory cells gain methylation compared to effector cells, however the levels and pattern of methylated CpG sites are distinct from naïve cells. Chronic infection led to a demethylated state at both CR-B and CR-C that was maintained throughout infection, correlating with the continued high PD-1 expression. Histone modifications during chronic viral infection are also maintained in the active state. DNA methyltransferase 3a (DNMT3a) isoform 2 was downregulated in exhausted CD8 T cells, suggesting a possible mechanism for the inability of cells to remethylate. Studies from HIV-infected patients show a similar trend with HIV-specific

CD8 T cells maintained in an unmethylated state (Youngblood et al., 2013). This unmethylated state persists even with successful HAART treatment, which reduces viral load. Additionally, CD8 T cells remain unmethylated in elite controllers, a small population of HIV-infected individuals who can control HIV viral loads in the absence of anti-retroviral therapy (Walker, 2007). Together, this shows that the epigenetic programming of CD8 T cells is set early in infection, and therapies directed at changing the epigenetic program could be successful.

Much less is known about the regulation of PD-L1 and PD-L2 compared to PD-1. NFκB was found to bind near the PD-L1 promoter to activate transcription, providing a mechanism for TLR induction of PD-L1 (Figure 1-3B) (Lee et al., 2005). In the lung carcinoma model, IRF1 bound to two sites located between 200-300 bp upstream of the TSS in a STAT-dependent manner to augment PD-L1 expression (Figure 1-1B) (Lee et al., 2006b). T cell lymphomas that contain the chimeric nucleophosmin (NPM)/anaplastic lymphoma kinase (ALK) overexpress PD-L1 (Marzec et al., 2008; Yamamoto et al., 2009). NPM/ALK leads to STAT3 phosphorylation and its subsequent binding to two sites near the PD-L1 promoter to activate gene expression (Figure 1-3B). Even less is known about the transcription factors regulating PD-L2. B1 B cells constitutively express PD-L2, as opposed to macrophages, which only express PD-L2 upon stimulation. In addition, the isoforms of PD-L2 differ between the cell types (Kaku and Rothstein, 2010). Cell type-specific isoform expression is driven through the use of two different promoters. B1 B cells use a promoter located between exons 1 and 2 (Figure 1-3C). Two octamer binding proteins, Oct1 and Oct2, bind to this promoter *in vitro*, but only Oct2 binding was detected *in vivo*. While many cytokines regulate both PD-1 and its ligands, there is not a great deal of overlap in the transcription factors that regulate the genes. Along with NFκB, the only other known factor

that regulates expression of both PD-1 and one of its ligands is STAT3. The role of STAT3 in regulating PD-1 will be discussed in detail in Chapter 2.

At the start of this project, the only known regulatory regions of PD-1 were CR-B and CR-C. NFATc1 was known to bind to CR-C, but the other regulatory factors had yet to be discovered. The first goal of this project was to determine if additional regulatory regions of PD-1 existed and to determine which factors bound to these regions. The second goal of this project was to determine the epigenetic modifications associated with the regulatory regions. As will be discussed in detail in Chapter 2, two additional regulatory regions, located -3.7 and +17.1 kb from the TSS, regulate PD-1 in an NFATc1 and STAT-dependent manner (Figure 1-3A). The STAT family member that binds to the -3.7 and +17.1 regions is dependent on the cytokine signals that the CD8 T cells receive. In addition, PD-1 expression is differentially augmented when various cytokine stimulations are used. The CCCTC binding factor (CTCF) also binds to two sites in the PD-1 locus at -26.7 and +17.5 kb. These sites form a structural loop that potentially segregates the PD-1 locus from the surrounding chromatin. Chapter 3 will discuss the epigenetic modification of DNA methylation, its role in silencing PD-1 expression during acute infections, and how DNA methylation is irreversibly lost during chronic infection. Chapter 4 details the role of multiple STATs in prolonging PD-1 expression. Finally, a discussion of all the results presented here, other potential regulatory factors, and the role of the newly discovered factors in overexpression of PD-1 during chronic infection is presented in Chapter 5.

Figure Legends

Figure 1-1. Diagram of the signaling pathways leading to PD-1 and PD-L1 expression. The TCR and JAK-STAT pathways stimulate PD-1 expression. TCR signaling

leads to an increase in intracellular calcium (Ca2+) and activation of the calmodulin (CaM)/calcineurin (CN) pathway. Dephosphorylation of NFAT by CN allows for its translocation into the nucleus where it binds to multiple regulatory regions of Pdcd1. Cytokine signals are recognized by their cognate receptors, leading to phosphorylation of the STAT proteins by the JAKs. Phosphorylated STATs dimerize and translocate into the nucleus where they bind to target sequences. Suppressor of cytokine signaling (SOCS) inhibits JAK phosphorylation of the STATs. PD-1 inhibits TCR signaling through the action of the phosphatase SHP2 by inhibiting both Zap70 and PI3K. In addition to the JAK-STAT pathway, the Toll-like receptor (TLR) pathway activates PD-L1 expression in antigen presenting cells (APC). Activation the TLR pathway signals through the adaptor protein MyD88, leading to phosphorylation of inhibitor of kappa B (IkB) by the IkB kinase. Phosphorylated IkB releases NFkB, which dimerizes then translocates into the nucleus and binds to the PD-L1 encoding gene *Cd274*.

Figure 1-2. Formation of memory or exhausted T cells is dependent on disease progression. Naïve T cells expressing an antigen-specific TCR (green cup) differentiate into effector T cells upon recognition of their specific antigen. T effector cells produce granzyme B (GzmB), IL-2, TNF-α and IFN-γ. PD-1 (blue arrow) expression is increase on effector compared to naïve T cells. Resolved, acute infections lead to formation of memory T cells, which express PD-1 at comparable levels to naïve cells. Memory cells are characterized by expression of multiple surface marks such as CD127 and CD122. Chronic infection leads to an overexpression of PD-1. PD-1-PD-L1/L2 signals cause functional exhaustion, characterized by a decrease in proliferation, cytokine production, and cytotoxicity. Blockade of PD-1-PD-L1/L2 interaction by anti-PD-1, anti-PD-L1, or anti-PD-L2 antibodies (red Y)

reinvigorates exhausted T cells. Reinvigorated cells partially recover their effector functions, including an increase in proliferation, cytokine production, and cytotoxicity.

Figure 1-3. Multiple regulatory regions and factors combine to control expression of the PD-1-PD-L1/L2 axis. **(A)** Schematic of transcription factors known to bind to the *Pdcd1* locus, which encodes PD-1. Location relative to the transcription start site (TSS) in kb is listed next to the binding sites. Conserved region (CR)-B and CR-C are depicted as grey boxes. Regions that have been shown to undergo changes in DNA methylation are indicated by black lollipops. **(B)** Schematic of the *Cd274* gene, which encodes PD-L1, showing the known transcription factor binding sites. Positions relative to the TSS in bp are indicated. **(C)** The *Pdcd1lg2* gene, encoding PD-L2, has two promoters that drive expression of cell-specific isoforms. Oct1/2 binding sites are shown with position in bp from the promoter located near exon 2.

Figure 1-1

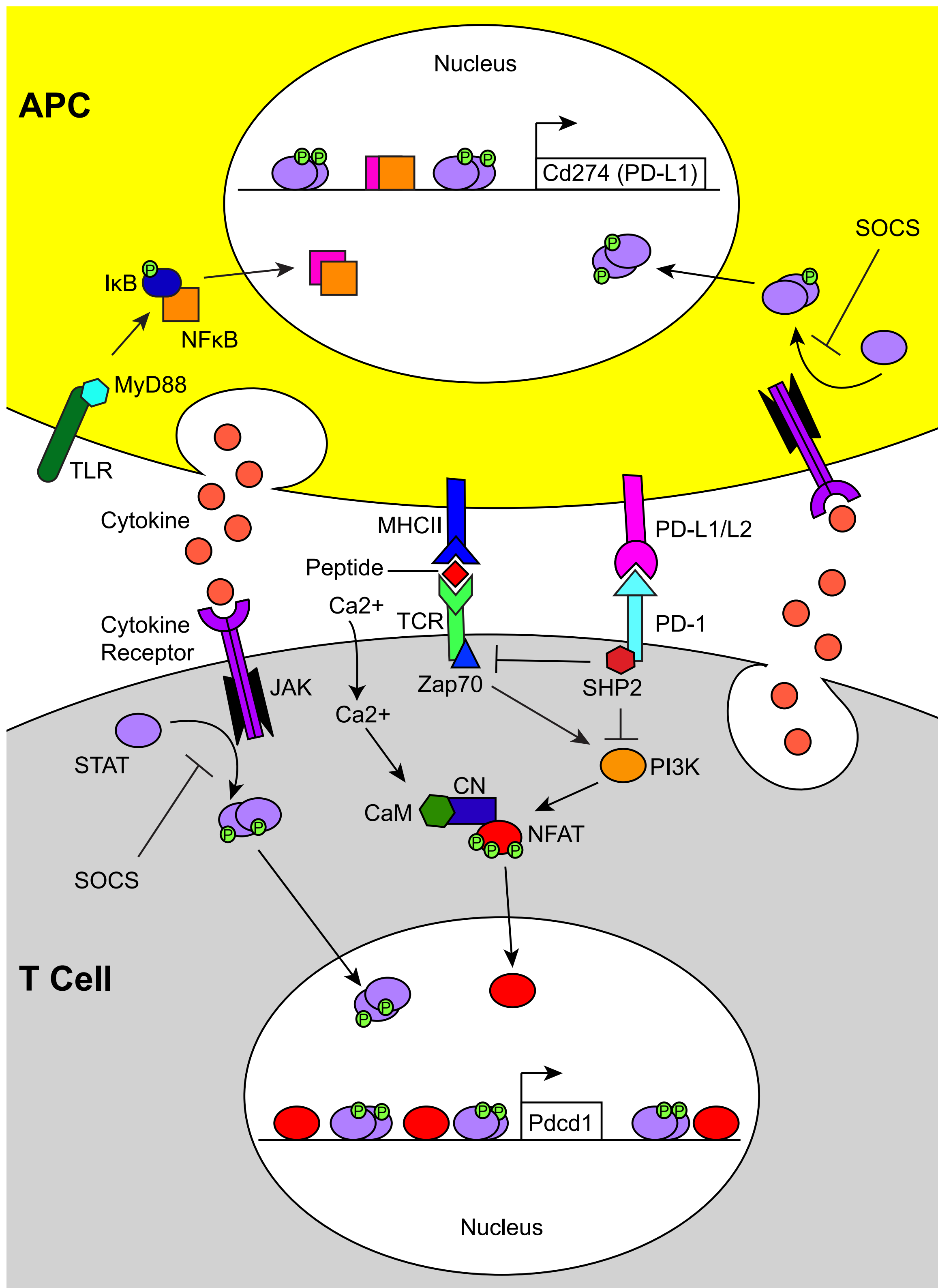


Figure 1-2

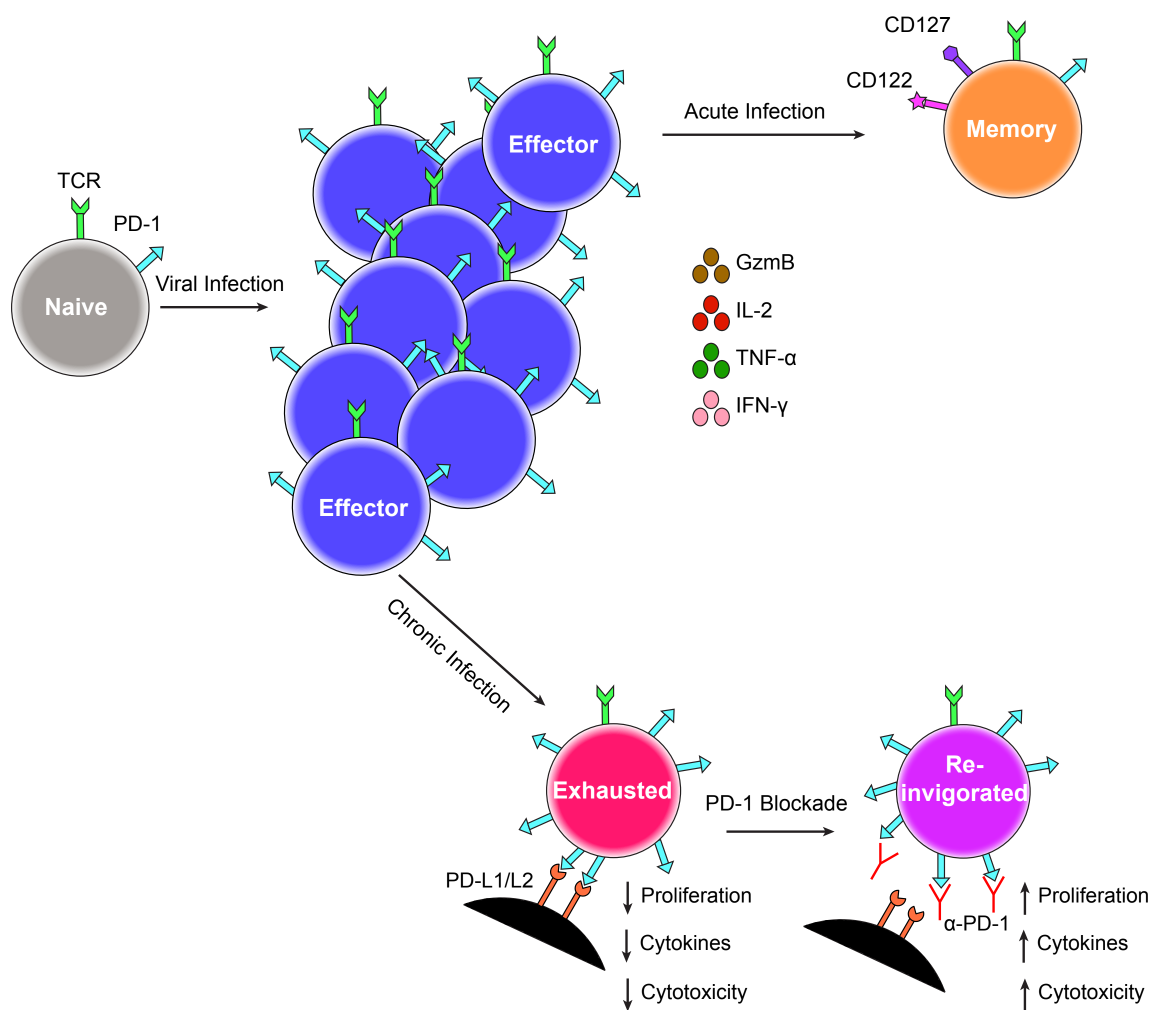
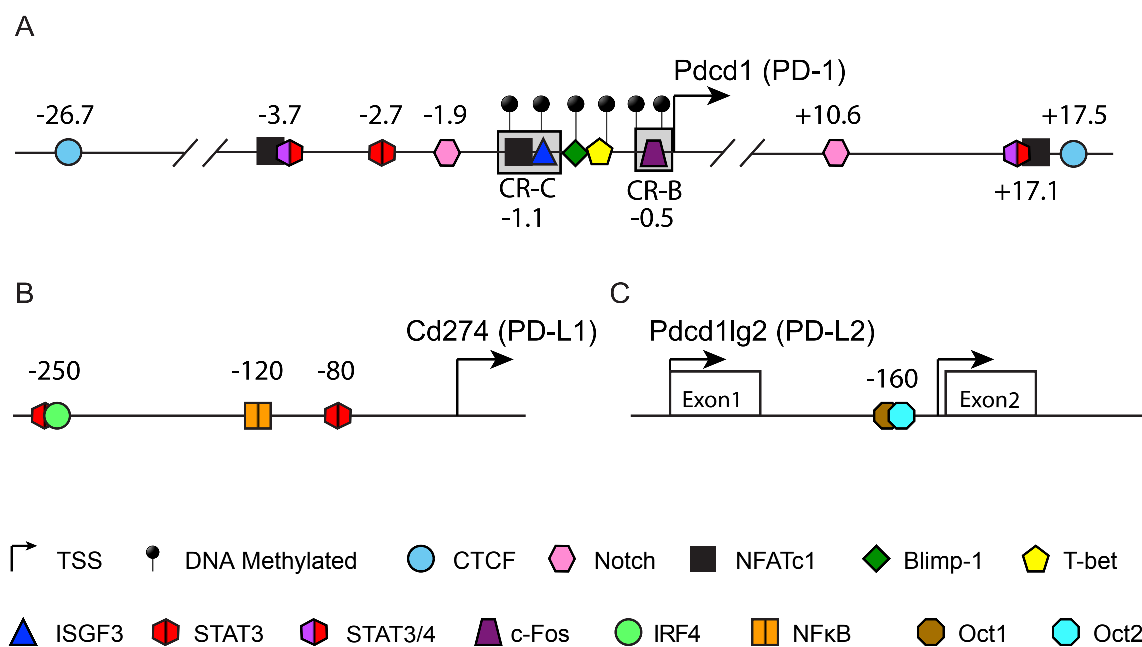


Figure 1-3



Chapter 2: STAT3, STAT4, NFATc1, and CTCF regulate Pdcd1 through multiple novel regulatory regions in murine T cells

James W. Austin, Peiyuan Lu, Parimal Majumder, Rafi Ahmed, and Jeremy M. Boss

Cell isolations and stimulations, DNase I hypersensitivity assays, qRT-PCR, and luciferase assays were performed by JWA.

ChIP assays were performed by JWA and PL with cells provided by JWA

3C assays were performed by PM with cells provided by JWA

This chapter was written by JWA and JMB.

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Abstract

Programmed cell death-1 (PD-1) is a crucial negative regulator of CD8 T cell development and function, yet the mechanisms that control its expression are not fully understood. Through a non-biased DNase I hypersensitivity assay, four novel regulatory regions within the *Pdcd1* locus were identified. Two of these elements flank the locus, bind the transcriptional insulator protein CTCF, and interacted with each other, creating a potential regulatory compartmentalization of the locus. In response to T cell activation signaling, NFATc1 bound to two of the novel regions that function as independent regulatory elements. STAT binding sites were identified in these elements as well. In splenic CD8 T cells, TCR-induced PD-1 expression was augmented by interleukin 6 and 12, inducers of STAT3 and STAT4 activity, respectively. IL-6 or IL-12 on its own did not induce PD-1. Importantly, STAT3/4 and distinct chromatin modifications were associated with the novel regulatory regions following cytokine stimulation. The NFATc1/STAT regulatory regions were found to interact with the promoter region of the *Pdcd1* gene providing a mechanism for their action. Together these data add multiple novel distal regulatory regions and pathways to the control of PD-1 expression and provide a molecular mechanism by which proinflammatory cytokines, such as IL-6 or IL-12 can augment PD-1 expression.

Introduction

Programmed Death 1 (PD-1), encoded by *Pdcd1,* is a transmembrane protein that is highly expressed on the surface of immune cells during chronic immune activation and in a variety of cancers (Akbay et al., 2013; Day et al., 2006; Fourcade et al., 2010; Wherry et al., 2007). Following engagement with its ligands, PD-L1/L2, signaling through PD-1 leads to an exhaustive phenotype wherein T cells lose their effector functions and ability to proliferate

(Zajac et al., 1998). In both *in vitro* and *in vivo* settings, blockade of PD-1 — PD-L1/L2 interactions results in reinvigoration of CD8 T cell effector functions and reduced viral loads in experimental systems (Barber et al., 2006; Dyavar Shetty et al., 2012; Nakamoto et al., 2009; Porichis et al., 2011). Recently, PD-1/PD-L1 blockade has been shown to be an efficacious treatment for some late stage cancers (Brahmer et al., 2010; Brahmer et al., 2012; Topalian et al., 2012a). Despite its clear importance in immune function, the mechanisms by which PD-1 is regulated are still poorly understood.

The transient upregulation of PD-1 during acute viral infection has been attributed to the action of nuclear factor of activated T cells c1 (NFATc1 or NFAT2) binding to a conserved region located upstream of the *Pdcd1* promoter termed Conserved Region C (CR-C) (Oestreich et al., 2008). cFos was identified as a factor that binds to CR-B, a promoter proximal element that was necessary for maximal induction by NFATc1 (Xiao et al., 2012). Additionally, an interferon-stimulated regulatory element (ISRE), located in CR-C, was reported to enhance and prolong PD-1 transcription upon T cell and macrophage activation (Cho et al., 2008; Terawaki et al., 2011). In contrast to these factors, T-bet has been shown to negatively regulate PD-1 in CD8 T cells during LCMV infection (Kao et al., 2011). Other reports have also suggested a role for B lymphocyte-induced maturation protein-1 (Blimp-1) in modulating PD-1 expression, although no direct role for that factor has been reported (Shin et al., 2009).

DNA methylation, a transcriptionally repressive epigenetic modification, was found to be dynamically modulated in antigen-specific CD8 T cells and inversely correlated with PD-1 expression during effector (on) and memory (off) phases following an acute viral infection with LCMV (Youngblood et al., 2011). During chronic LCMV infection, exhausted CD8 T cells, which express high levels of PD-1, became and remained

hypomethylated at the CR-B and CR-C regions of *Pdcd1*, suggesting that the presence of antigen may drive expression and the control of DNA methylation (Youngblood et al., 2011). However, analysis of *Pdcd1* DNA methylation in antigen-specific CD8 T cells of HIV infected individuals showed that despite viral control through HAART or the patients' natural immune response (elite controllers) the *Pdcd1* locus remains demethylated (Youngblood et al., 2013). These observations suggest that early immune events may establish epigenetic modifications of the locus that are maintained irrespective of antigen levels.

Multiple cytokines have been shown to regulate PD-1, including several in the common γ-chain family (IL-2, IL-7, IL-15, and IL-21) and Type I IFNs (IFN-α and IFN-β) (Cho et al., 2008; Kinter et al., 2008; Terawaki et al., 2011). IL-6, which acts through STAT3, has been shown to predict antiviral responses in individuals coinfected with HIV and HCV where high levels of IL-6 in the serum correlate with non-responding individuals (Guzman-Fulgencio et al., 2012; Yu et al., 2009). STAT3 is critical for differentiation and function of CD4 T cell subsets including T_H17, T_H2, T follicular helper (T_{FH}), and T regulatory cells (T_{reg}), as well as memory formation of CD4 and CD8 T cells (Chaudhry et al., 2009; Cui et al., 2011; Eddahri et al., 2009; Stritesky et al., 2011; Yang et al., 2007). In addition to IL-6, the cytokines IL-10 and IL-21 signal through the JAK family of proteins culminating in STAT3 activation (O'Shea and Murray, 2008). IL-10 has been shown to directly inhibit CD4 responses and blockade of IL-10 signaling leads to clearance of chronic LCMV infection, suggesting that STAT3 plays a role in viral persistence (Brooks et al., 2006; Ejrnaes et al., 2006). The above reports suggest that multiple cytokines can regulate PD-1. However, with the exception of IFN-α inducing responses from an ISRE located in CR-C, no direct effect

of cytokine induced factors regulating *Pdcd1* gene expression has been shown (Cho et al., 2008; Terawaki et al., 2011).

All current known regulators of *Pdcd1* are located in or adjacent to the previously described CR-B and CR-C regulatory regions that reside within the first 1.2 kb upstream of the transcription start site (TSS) (Kao et al., 2011; Oestreich et al., 2008; Terawaki et al., 2011; Xiao et al., 2012; Youngblood et al., 2013; Youngblood et al., 2011). However, in many genes it is common that distal regulatory elements can be found more than 10 kb away from the TSS (Li et al., 2012a; McCord et al., 2011; Sagai et al., 2009). To determine if *Pdcd1* is regulated by distal elements, a nonbiased approach was employed across the murine *Pdcd1* locus. The results identified four novel distal regulatory regions. Two of these elements flanked the locus and bound the insulator protein CCCTC-binding factor (CTCF). The third element, located upstream of CR-C, bound NFATc1 and STAT3 or STAT4 in response to TCR and IL-6 or IL-12 signaling, respectively. The final region, located close to the downstream CTCF site also bound NFATc1 and STAT3 or STAT4. The chromatin structure of each regulatory region is altered in response to T cell activation and cytokine stimulation, with unique patterns arising in response to each stimulus. Each of the novel NFAT/STAT elements was found to interact with the *Pdcd1* promoter region in CD8 T cells, demonstrating that they are likely to contribute directly to *Pdcd1* transcription. Together, these results introduce new pathways that integrate multiple mechanisms to stimulate and regulate PD-1 in response to different environmental cues.

Materials and Methods

Cells and Culture

The murine T lymphoma cell line EL4 was obtained from the American Type Culture Collection. Primary splenic CD8 T cells were isolated from C57BL/6 mice by magnetic bead

separation (Miltenyi Biotech Inc., Auburn, CA) according to manufacturer's recommendations. All cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Sigma-Aldrich Co, St. Louis, MO), 5% bovine calf serum (Thermo Fisher Scientific Inc., Waltham, MA), 10mM HEPES, 1mM sodium pyruvate, 4.5 g/L glucose, and 100 U/ml penicillin/streptomycin. Cells were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 2 μM ionomycin (Io) (Sigma-Aldrich), 1 μg/ml cyclosporin A (CsA) (Sigma-Aldrich), 500U IFN-α (eBioscience Inc., San Diego, CA), 20 ng/mL IL-2 (Miltenyi) 10 ng/mL IL-4 (Miltenyi), 20 ng/mL IL-6 (Miltenyi), 20 ng/mL IL-12 (Miltenyi), 20 ng/mL IFN-γ (Miltenyi), or CD3/CD28 activation beads (Life Technologies Co, Grand Island, NY) according to manufacturer's protocol where indicated. All mouse experiments were conducted in accordance with approved Emory University institutional animal care and use protocols.

PCR based DNase I Hypersensitivity Assays

The protocol for PCR-based DNase I hypersensitivity screening was previously described (Oestreich et al., 2008). Briefly, nuclei were isolated and treated with increasing concentrations of DNase I (Worthington Biochemical Co, Lakewood, NJ). Concentration ranges for DNase I were determined empirically for each lot and cell type by titrating DNase I for its ability to digest CR-C as the positive control but not regions previously found to be resistant to DNase I (e.g., +6.3 region). Following purification of the digested DNA, PCR was performed across the *Pdcd1* locus using a set of 59 primer pairs (Supplemental Table 1). For conventional PCR assays, PCR amplicons were designed to span approximately 1kb of the *Pdcd1* locus. After visualization of the PCR products by agarose gel, band intensities were quantified using ImageJ (Rasband, 1997-2012). DNase I hypersensitivity was

determined by using band intensities across increasing DNase I concentrations and calculating a slope from a best-fit line to represent the loss of signal. Slopes were normalized to the known DNase I hypersensitive CR-C region. For a higher resolution DNase I hypersensitivity assay, real-time PCR amplicons were designed to be 400-600 bp. Following amplification, values were normalized to a known DNase I insensitive region located within the *Ciita* gene, which is not expressed in these cells (Oestreich et al., 2008; Yoon and Boss, 2010). DNase I hypersensitivity was again determined by calculating a slope using the normalized threshold values across increasing DNase I concentrations. All DNase I assay primers are listed in Supplemental Table 1. DNase I hypersensitivity assays were performed at least three times from independent cultures or preparations of splenic CD8 T cells from mice.

Gene Reporter Assays

DNA sequences corresponding to the DNase I hypersensitive sites were cloned into the firefly luciferase reporter vectors pGL3-Promoter or pGL3-Basic (Promega Co, Madison, WI) using *Nhe*I (New England Biolabs Inc., Ipswitch, MA). Plasmids were transiently transfected into EL4 cells by nucleofection using kit L protocols as described by Lonza Inc. (Allendale, NJ). At 16 hours post transfection, cells were treated with PMA/Io, IL-6, and/or IL-12 where indicated and incubated for 24 hours. For experiments using CsA, cells were treated for 2 hours with CsA before addition of PMA/Io. Lysates were collected 24 hours after PMA and ionomycin addition. The Dual-Luciferase Reporter Assay System (Promega) was used to quantify luciferase activity. Firefly luciferase was normalized to the co-transfected, constitutively expressing *Renilla* luciferase gene (pRLTK). The average fold

change in normalized luciferase activity was calculated from three independent transfections and is shown with standard deviation.

RNA isolation and RT-PCR analysis

The RNeasy kit (Qiagen Sciences Inc., Germantown, MD) was used to isolate total RNA. Reverse transcription assays were carried out using SuperScript II reverse transcriptase (Life Technologies) and 1 μg of RNA. Real-time PCR analyses on three independent RNA preparations were conducted to quantify *Pdcd1* mRNA. *Pdcd1* levels were normalized to *18s* ribosomal RNA.

Chromatin Immunoprecipitation Assays

Chromatin Immunoprecipitation (ChIP) assays were previously described (Beresford and Boss, 2001; Choi and Boss, 2012). Cells were cross-linked in 1% formaldehyde for 15 minutes. Chromatin was sonicated to an average size of 400-600 bp and then 2 μg was immunoprecipitated with antibodies raised against NFATc1 (Thermo Scientific), p-STAT1 (Cell Signaling Technologies Inc., Danvers, MA), p-STAT3 (Cell Signaling) p-STAT4 (Cell Signaling Technologies), p-STAT5 (Cell Signaling), p-STAT6 (Cell Signaling), RNA Polymerase II (EMD Millipore Co., Billerica, MA), p300 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), H3K27ac (EMD Millipore), H3K4me1 (EMD Millipore), H3K4me3 (EMD Millipore), or CTCF (Rockland Immunochemicals Inc., Gilbertsville, PA). A negative control IgG (EMD Millipore) was also used in parallel. Following immunoprecipitation (IP) and washing, DNA from each IP was quantified by real-time PCR using a standard curve from sonicated murine genomic DNA. Each ChIP was performed from three independently

isolated cell preparations and plotted as the average percent pulldown of input chromatin with standard deviation.

Chromatin Confirmation Capture (3C)

3C assays were performed from modified procedures that have previously been described (Majumder and Boss, 2010; Majumder et al., 2008). Briefly, 10^7 EL4 or 3 x 10^6 CD8 T cells were suspended in RPMI supplemented with 5% fetal bovine serum and 5% bovine calf serum. Formaldehyde was added to a final concentration of 1% and cells were incubated for 10 min at room temperature. Following incubation, glycine was added to a final concentration of 125 mM to quench the cross-linking reaction. Cross-linked nuclei were isolated and digested overnight with *Pst*I (New England Biolabs). After digestion, *Pst*I was heat inactivated and the reaction was diluted 1:40 in ligation buffer and ligated overnight with T4 DNA ligase (New England Biolabs). Following purification, quantitative PCR using primer sets (Supplemental Table 1) located adjacent to *Pst*I sites was used to identify 3C ligation products. To generate material for a 3C standard curve, the RP23-4L19 bacterial artificial chromosome (Children's Hospital Oakland Research Institute, Oakland, CA), which contains the entire *Pdcd1* locus, was *Pst*I digested and religated to generate all possible *Pst*I-*Pst*I end-joined fragments. This DNA was then used with the same 3C primer sets to provide a qPCR standard curve. 3C data is presented as the average cross-linking frequency with the standard deviation from three biological replicates.

Results

The *Pdcd1* locus contains multiple DNase I hypersensitive sites

The *Pdcd1* locus in the mouse is homologous to other mammals from ~-10kb upstream the transcription start site (TSS) through and downstream of the gene (Figure 2-1A). Surprisingly, whereas the mouse sequences further upstream (-10 to -50kb) are similar among rodents, these sequences diverge in the human where *PDCD1* is located on chromosome 2. There is no homology between mouse and humans from -10 to -31kb. From -31 to -50kb, the murine sequence is homologous to a region ~123-134kb upstream of the *CHD1* gene on human chromosome 5. This might suggest that common elements regulating *Pdcd1* gene expression would be contained only within the homologous regions of mouse chromosome 1 and human chromosome 2 that encode the *Pdcd1*/*PDCD1* loci. Chromatin sensitive to digestion by DNase I is indicative of potential regulatory regions (Gross and Garrard, 1988). Using this conceptual approach, we previously identified and showed that the CR-B and CR-C regulatory regions of *Pdcd1* were differentially sensitive to DNase I digestion in unstimulated and PMA/Io stimulated CD8 T cells and EL4 cells (Oestreich et al., 2008). To identify additional regulatory elements, a PCR-based strategy employing 59 amplicons, was used to scan ~70 kb of the *Pdcd1* locus for DNase I hypersensitive regions (Figure 2-1A). Amplicons of 0.9-1.3 kb in length covered all the unique, non-repetitive DNA sequences within the locus. Splenic CD8 T cells were isolated from C57BL/6 mice and immediately processed or treated with PMA/Io for 24h a time point in which PD-1 expression is induced to a high level *ex vivo* (Oestreich et al., 2008). Representative amplicons displaying hypersensitivity in response to PMA/Io (-3.5 and -1.1 (CR-C)), constitutive hypersensitivity (-26.2 and +17.0), and background levels of hypersensitivity (-31.8) to DNase I are shown in Figure 2-1B. A representative sample for each amplicon from the entire set is provided in Supplemental Figure 2-1A. To quantitate these relative sensitivities, amplicon intensity was measured using ImageJ and a slope was

generated to identify regions that lost signal with increasing DNase I concentrations (Figures 2-1B and C). Using this strategy more than 70 kb of the *Pdcd1* locus was scanned (Figure 2-1D). It should be noted that the regions not scanned contained repetitive element sequences (Smit et al., 1996-2010). Stimulation of splenic CD8 T cells with PMA/Io led to an increase in DNase I sensitivity throughout the locus (Figure 2-1D). Regions showing either a differential sensitivity between control and stimulated CD8 T cells greater than a 2-fold change or a greater relative hypersensitivity than CR-C were chosen for further characterization as these could indicate areas essential for *Pdcd1* expression and regulation.

To obtain a higher resolution profile of the potential regulatory elements, primers were designed to produce amplicons between 400-600 bp across each of the regions of interest determined from Figure 1D. Quantitative real-time PCR was performed on DNase I digested CD8 T cell DNA (Figure 2-1E). Analysis from these studies showed two peaks of DNase I sensitivity in control, unstimulated cells located at -26.7 kb and +17.0 kb from the TSS (Figure 1E, green bars, red arrows). The sensitivity of these regions to DNase I increased following PMA/Io stimulation, further supporting the possibility that these regions may be vital for PD-1 expression.

The murine EL4 T lymphoma cell line has been previously used to characterize constitutive PD-1 expression and regulation (Oestreich et al., 2008). DNase I hypersensitivity was also assessed in EL4 cells by the same PCR-based DNase I hypersensitivity assay and ImageJ amplicon analysis (Figure 2-1F and Supplemental Figure 2-1B), as well as quantitated using real-time PCR (Figure 2-1G). Comparison of CD8 T cells stimulated with PMA/Io and EL4 cells showed similar DNase I hypersensitivity profiles. Five of these regions were hypersensitive to DNase I in both EL4 and CD8 T cells and contained sequence homology across multiple species (Black arrows in Figures 2-1E and G).

These regions were therefore chosen for further study. Thus, this DNase I hypersensitivity screen identified seven potential regulatory regions across the 70 kb encompassing the *Pdcd1* locus.

Two DNase I Hypersensitive sites act as calcineurin-dependent regulatory elements

To determine if any of the chosen hypersensitive sites have transcriptional regulatory activity, 300-600 bp sequences representing the selected DNase I hypersensitive regions were cloned into the pGL3-Promoter luciferase reporter vector (Figure 2-2A). The constructs were transiently transfected into EL4 cells, which constitutively express PD-1, and luciferase activity was determined. As with the previously described CR-B/C construct, two regions, -3.7 and +17.1, showed substantial increases in relative luciferase activity upon PMA/Io stimulation (Figure 2-2B). This suggests that these regions may play a role in regulating PD-1 during T cell activation. Regions -35.6 and -27.7, which did not display statistically different DNase I hypersensitivity and were chosen as negative controls, displayed no transcription stimulating activity. The two remaining regions, located at -23.8 and +3.5 did not display induced activation and were not characterized further. Previously, we showed that calcineurin-NFAT pathway is critical to PMA/Io induced PD-1 expression through CR-C (Oestreich et al., 2008).

To determine if the calcineurin pathway was also involved with the activity observed for the -3.7 and +17.1 regulatory regions, luciferase assays were carried out in the presence/absence of cyclosporine A (CsA). CsA blocks calcineurin-dependent dephosphorylation of NFAT and subsequent translocation into the nucleus (Flanagan et al., 1991; Fruman et al., 1992). Here, CsA inhibited/reduced the PMA/Io-dependent activation of both regulatory regions, as well as the previously defined CR-B/C region, suggesting a

role for an NFAT family member in the activity of these regions (Figure 2-2C).

IL-6 and IL-12 leads to increased expression and a change in the chromatin structure of *Pdcd1*.

In silico analysis using the JASPAR database identified several putative STAT binding sites within -3.7 and +17.1, suggesting that STATs could play a role in the regulation of PD-1 (Bryne et al., 2008). Analysis of ChIP-seq data on CD4 T cell subsets identified CR-C, -3.7, and +17.1, as being bound by multiple STAT family members (Durant et al., 2010; Wei et al., 2010). Therefore, to test which if any STATs could stimulate PD-1 expression, CD8 T cells were isolated from C57BL/6 mice and stimulated *ex vivo* with or without CD3/CD28 beads in the presence or absence of IFN-α (STAT1/2), IFN-γ (STAT1), IL-2 (STAT5), IL-4 (STAT6), IL-6 (STAT3), or IL-12 (STAT4) (Figure 2-3A). On their own, none of these cytokines induced *Pdcd1* gene expression. However, when coupled with CD3/CD28 bead stimulation, only IFN-α, IL-6 and IL-12 enhanced *Pdcd1* gene expression (Figure 2-3A). While IFN-α was shown previously to enhance PD-1 expression in both macrophages and T cells through binding of the ISGF3 complex to an ISRE located in CR-C (Cho et al., 2008; Terawaki et al., 2011), IL-6 and IL-12 enhancement of *Pdcd1* suggests a potential role for STAT3 and STAT4 in augmenting PD-1 expression.

An increase in *Pdcd1* expression suggests the chromatin architecture of the locus could be changed by IL-6 and IL-12 treatment. To determine if this is the case, DNase I hypersensitivity assays were conducted on primary splenic CD8s stimulated with CD3/CD28 beads and/or IL-6 and IL-12. Both IL-6 and IL-12 stimulation of activated CD8 T cells led to an increase in the DNase I hypersensitivity of CR-C, -3.7, and +17.1, but not of a control region (Figure 2-3B). IL-6 and IL-12 treatment had no effect on their own.

Enhancer regions are marked by H3K4me1 and when combined with H3K27ac, denote active enhancers (Creyghton et al., 2010; Heintzman et al., 2007). In addition, H3K4me3 marks the promoters of active genes. Chromatin immunoprecipitation assays (ChIP) were used to determine the presence of each of these marks in splenic CD8 T cells cultured with CD3/CD28 beads, IL-6, and/or IL-12. CR-B and CR-C were marked with H3K27ac in response to T cell activation, irrespective of cytokine treatment (Figure 2-3C). CR-B was also marked by high levels of H3K4me3 in response to T cell activation, correlating with active gene expression. The -3.7 and +17.1 regions acquired H3K27ac in response to T cell activation and cytokine stimulation, but not with either alone. In contrast, H3K4me1 was only present at -3.7 and +17.1 in response to cytokine stimulation, irrespective of whether the T cells were activated. Some active enhancers have also been shown to bind RNA polymerase II (PolII) (Zentner et al., 2011). Here, PolII binding was only observed at CR-B (a region that is close to the TSS) in response to CD3/CD28 stimulation and not at the other regulatory regions or a control upstream region. A control IgG ChIP at each of the sites showed no reactivity and demonstrates specificity for the assay. In some cases, active enhancers have been reported to express short transcripts known as eRNAs (Watanabe-Fukunaga et al., 1992). The presence of such transcripts was assessed in CD8 T cells stimulated with CD3/CD28 beads with and without IL-6 or IL-12. No significant levels of transcripts were observed irrespective of the treatment (data not shown).

NFATc1, STAT3, STAT4, and p300 bind to the -3.7 and +17.1 regions

To demonstrate that STAT3, STAT4, and NFAT bind to the regulatory regions, ChIP was performed using antibodies to NFATc1, STAT3, and STAT4. For these experiments, splenic

CD8 T cells were cultured for 24 hours with or without CD3/CD28 beads and in the presence/absence of IL-6 or IL-12. NFATc1 was bound to CR-C, -3.7, and +17.1 in CD3/CD28 bead activated cells, irrespective of cytokine stimulation (Figure 2-4A). STAT3 and STAT4 bound only to -3.7 and +17.1 in cells stimulated with IL-6 or IL-12, respectively. STAT binding was not dependent on T cell activation. The transcription factor p300, a histone acetyltransferase that can catalyze H3K27 acetylation (Tie et al., 2009; Visel et al., 2009), is often found at active enhancers. Using ChIP, p300 was found at CR-B and CR-C in response to CD3/CD28 stimulation (Figure 2-4A). The -3.7 and +17.1 elements were also bound by p300, but only in response to both CD3/CD28 and cytokine treatment. A control sequence or control IgG antibody showed only background levels of binding. Thus, NFATc1 binds in response to T cell activation, while STAT binding is induced through cytokine stimulation. p300 binding correlates with H3K27ac and is differentially recruited to the regulatory regions through either T cell activation at CR-B and CR-C, or through T cell activation combined with cytokine signaling at -3.7 and +17.1.

CTCF binding sites flank the *Pdcd1* gene

CTCF, the only known mammalian insulator protein, has previously been shown to be essential for formation of regulatory loops that segregate commonly regulated genes from other nearby genes (Majumder and Boss, 2010; Majumder et al., 2008). ChIP-seq experiments from whole thymus identified two regions within the *Pdcd1* locus (Consortium, 2011), -26.7 and +17.5, which were identified in our initial screen as being sensitive to DNase I in the absence of T cell activation (Figure 2-1). To generate a consensus CTCF logo, sites bound by CTCF in mouse whole thymus mapped by the ENCODE Consortium corresponding to the central 100 bp of each peak were analyzed using the MEME-ChIP

software package (Fortner and Budd, 2005). The CTCF site depicted in Figure 2-4B was the top scoring motif (p-value = 4.73e-243). Conventional qPCR-based ChIP using CTCF antibodies confirmed that CTCF was bound to both these sites in primary CD8 T cells, as well as in EL4 cells (Figure 2-4C). Moreover, CTCF binding levels did not change in response to PMA/Io, CD3/CD28 beads, and/or IL-6 treatment. It should be noted that CTCF binding to the +17.5 fragment is downstream of the DNA sequences that contain NFAT and STAT binding sites and was not included in the +17.1 reporter constructs used above. These data suggest that these CTCF sties may form the outer boundaries for *Pdcd1* regulatory regions.

STAT and NFAT binding sites are critical for regulatory function of the -3.7 and +17.1 regulatory regions

To assess whether the newly identified regulatory regions could respond to IL-6 or IL-12, luciferase reporter gene transfections were carried out in EL4 cells followed by IL-6, IL-12 and/or PMA/Io stimulation. The +17.1 region showed a significant increase in luciferase activity after addition of either IL-6 or IL-12 to PMA/Io treated EL4 cells (Figures 2-5A and B). In contrast, the -3.7 and CR-C regions did not display an increase in activity following cytokine treatment. Neither IL-6 nor IL-12 activated expression of the reporter vector without PMA/Io induction, suggesting that TCR signaling is required to initiate *Pdcd1* expression.

To confirm that the -3.7 and +17.1 regions function to promote *Pdcd1* expression, the two regions were cloned into the previously described CR-B reporter construct, which uses the *Pdcd1* promoter and CR-B instead of a heterologous promoter (Oestreich et al., 2008). In agreement with the above experiments, the -3.7/B and +17.1/B region-containing

vectors displayed a significant increase in luciferase activity in response to PMA/Io (Figure 2-5C). The +17.1/B vector also showed an additional increase in luciferase activity when costimulated with IL-6 or IL-12. These results confirm that the -3.7 and +17.1 regions are able to enhance the activity of the *Pdcd1* promoter.

To assess the role of both the NFAT and STAT sites in driving the activity of these regions, the putative binding sites were identified using JASPAR (Bryne et al., 2008) and then deleted in their respective pGL3-Promoter reporter constructs. The constructs were subsequently transfected into EL4 cells and stimulated with PMA/Io and/or IL-6. The -3.7 region contains two predicted STAT (S1 (chr1:95952803-95952809), S2 (chr1:95952814-95952820)) and two NFAT (N1 (chr1:95952661-95952667), N2 (chr1:95952686-95952691)) binding sites. Loss of either predicted NFAT site (ΔN1 or ΔN2) or STAT binding site (ΔS1 or ΔS2) led to a decrease in luciferase activity (Figure 2-5D). Deletion of both NFAT sites in concert (ΔN1ΔN2) resulted in less luciferase activity than deletion of either NFAT site alone. Unlike the NFAT sites, elimination of both STAT sites together (ΔS1ΔS2) showed no further loss of luciferase activity. Because the loss of luciferase activity was in response to PMA/Io, this suggests minimally that these sites are integral to the activity of this region in EL4 cells. A random control mutation (ΔCon (chr1:95952934-95952941)) showed a slight increase in luciferase activity, suggesting that not all mutations within the DNase I hypersensitive region negatively affect the reporter gene expression/activity.

Luciferase reporter vectors carrying the deletions of the STAT (ΔS (chr1:95932036-95932042)) or NFAT (ΔN (chr1:95932003-95932009)) binding sites in the +17.1 region showed a significant loss of luciferase activity compared to wild type, with the NFAT mutation showing a more dramatic loss of expression (Figure 2-5E). Importantly, there was no change when a control deletion (ΔCon (chr1:95932096-95932101)) is

introduced into another part of the region. A construct containing the double STAT and NFAT mutation (ΔNS) showed similar levels of luciferase to that of the single NFAT mutation (ΔN).

As shown above, deletion of the STAT binding sites in the -3.7 or +17.1 construct resulted in a decrease of luciferase activity in PMA/Io only treated cells (Figures 2-5D and E), suggesting that these sites were active in the absence of added cytokine. To determine if this was the case, ChIP of EL4 cells following PMA/Io stimulation was performed using antibodies to STAT1, STAT3, STAT4, STAT5, and STAT6. The results showed inducible binding of NFATc1 and STAT1 at -3.7 and +17.1 upon PMA/Io treatment, whereas NFATc1 but not STAT1 bound CR-C following PMA/Io stimulation (Supplemental Figure 2-2). There was no detectable binding of STAT3, STAT4, STAT5, or STAT6 compared to control IgG at any region queried. These data suggest that following PMA/Io stimulation of EL4 cells, STAT1 binding is activated and is able to augment expression of the reporter constructs described above. Thus, mutation of the STAT sites in -3.7 and +17.1 alter the PMA/Io stimulated activity.

Dynamic interactions between the -3.7 region and two insulator regions creates a unique chromatin architecture during *Pdcd1* activation

Distal enhancers can carry out their effects by interacting with promoters through long-range chromatin loops. (Kulaeva et al., 2012). Detection of these interactions can be accomplished using chromatin confirmation capture (3C) assays, which define spatial relationships formed by DNA bound factors (Dekker et al., 2002). To determine the organization of the *Pdcd1* locus and if that chromatin organization changes during *Pdcd1* expression, 3C assays were performed on both CD8 T and EL4 cells. A map of the *Pdcd1*

locus and the positions of the 3C fragments is shown in Figure 2-6A. In this 3C assay system, the restriction enzyme *Pst*I was chosen to provide the greatest resolution between regulatory elements. Cells were left untreated, activated (CD3/CD28 beads for CD8 T cells or PMA/Io for EL4), or activated and treated with IL-6 prior to preparation of 3C libraries. Five 3C anchor fragments, representing the flanking CTCF sites (Figure 2-6A, fragments 2 and 9), the +3.7 and -17.1 regulatory elements (fragments 5 and 8, respectively), and the *Pdcd1* promoter (fragment 6) were chosen to identify interactions between regulatory regions.

In CD8 T cells, an interaction between the flanking CTCF sites bound to *Pst*I fragments 2 (-26.7 region) and 9 (+17.5 region) was observed irrespective of CD3/CD28 stimulation or IL-6 treatment (Figure 2-6B). Using 3C anchor primers associated with the flanking CTCF sites, interactions between the CTCF sites (fragments 2 and 9) and the -3.7 region (fragment 5) were observed in unstimulated cells. Upon activation, the interaction frequency of CTCF sites and the -3.7 region increased ($p<0.001$), but addition of IL-6 had no effect on the interactions (Figure 2-6B). A similar set of interactions between the CTCF sites and the -3.7 region were constitutively observed in EL4 cells (Figure 2-6C). However, as these cells are already expressing PD-1, it was not surprising that no increase in CTCF–CTCF or CTCF – -3.7 interactions was observed in response to PMA/Io stimulation or IL-6 treatment (Figure 2-6C). When the promoter element was used as an anchor, interactions were not observed between the -3.7 or the +17.1 regions. However, when 3C anchors were placed on the opposite sides of the restriction fragments, reproducible interactions between -3.7 and +17.1 regions with the *Pdcd1* promoter fragment were readily detected. In CD8 T cells, a significant increase in the -3.7 – Promoter interaction upon T cell activation ($p<0.01$) that was unaffected by IL-6 was observed (Figure 2-6B). This interaction was constitutive and unchanged with respect to treatment in EL4 cells. Interactions between -17.1 and the

promoter region were constitutive in both CD8 T and EL4 cells. Because the change in anchor primers results in different DNA ligations being detected in the 3C assay, this indicates that there is a distinct spatial orientation of the regulatory elements and DNA fragment ends that could be readily available for 3C ligation. This possibility could provide an explanation for the inability to detect interactions between the 3C -3.7 anchor and the CTCF sites. No interactions were observed with randomly chosen fragments (1, 3, 4, 5, or 10) across the locus, demonstrating specificity of the assay. Experiments assessing the accessibility of the locus in crosslinked cells were performed and demonstrated that *Pst*I can access all restriction sites used with relatively equal efficiency (Supplemental Figure 2-3). Taken together, these results describe a unique chromatin architecture for the *Pdcd1* locus that is enhanced when *Pdcd1* is activated in primary CD8 T cells.

Discussion

The *cis*-acting elements that are known to control *Pdcd1* expression were limited to promoter proximal regulatory regions identified from sequence conservation and DNase I hypersensitivity assays a number of years ago (Oestreich et al., 2008). Using an unbiased DNase I hypersensitivity assay approach to discover potential regulatory regions (Gross and Garrard, 1988), a number of complex, distal regulatory elements located both upstream and downstream of the TSS were discovered. Two of the DNase I hypersensitive elements (-3.7kb and +17.1 kb) displayed regulatory activity when transfected into the PD-1 constitutive expressing T cell line EL4. Two additional elements were constitutively hypersensitive and bound the transcriptional insulator factor CTCF. The downstream elements showed a high degree of homology with human sequences, including consensus binding sequences and binding by ChIP-seq for STATs, NFATc1, and CTCF (Blanchette et

al., 2004; Consortium, 2011), suggesting that the conservation is important for the regulation of this locus. The -3.7 and -26.7 elements are well conserved in rat but not in humans. However, in humans, ChIP-seq data shows STAT and NFATc1 binding and H3K4me1 to an upstream region located approximately 4.8 kb upstream of the TSS, suggesting a conservation of regulatory mechanisms. Chip-seq experiments also suggest that CTCF binds approximately 7.5 kb upstream of the TSS of the human *PDCD1* gene (Consortium, 2011). Thus, the arrangement and functionality of elements appears to be conserved between species. Together with CR-B, CR-C, and the T-bet binding site, the new data presented here raises the total number of cis-elements that could potentially regulate *Pdcd1* to seven (Kao et al., 2011; Oestreich et al., 2008). As discussed below, the discovery of a role for STAT factors to directly regulate PD-1 expression broadens the overall mechanisms by which this gene can respond to cytokine stimulation and local immune environments.

Previously, NFATc1 was found to modulate PD-1 expression through CR-C (Oestreich et al., 2008). Here, through sensitivity to cyclosporine A and ChIP assays, NFATc1 was found to be a necessary, but not sufficient, mediator of the transcriptional activity of the -3.7 and +17.1 regulatory elements. While these elements may coordinate TCR-mediated induction of *Pdcd1* with CR-C, their full activity is unlikely to be realized until STATs are bound, as active enhancer modifications (p300, H3K27ac, and H3K4me1) did not appear unless both NFATc1 and the STATs were bound. The presence of multiple cis-elements bound by the same key regulatory factors is found in other systems (Corbo et al., 2010; Hong et al., 2008; O'Meara et al., 2009), and we propose that the multiple elements identified here could provide synergy and/or greater activation potential than a single element. Interactions between each of the new NFATc1 elements and the *Pdcd1* promoter

region fragment (which contained CR-C) were observed, supporting the concept that all three of these NFATc1 elements participate in activating transcription of *Pdcd1*.

An *in silico* search for transcription factor-binding sites at the -3.7 and +17.1 regulatory regions revealed a potential role for multiple STAT family proteins. ChIP-seq experiments on differentiated CD4 T cells suggested that STAT3 and STAT4 could be binding to these sites in CD8 T cells as well (Durant et al., 2010; Wei et al., 2010). Indeed, IL-6 and IL-12 stimulation of activated CD8 T cells led to STAT3 or STAT4 binding, respectively, at the -3.7 and +17.1 regulatory regions. Importantly, in *ex vivo* stimulated CD8 T cells, IL-6 or IL-12 stimulation led to an increase in *Pdcd1* gene expression over activated cells that received no cytokine. No effect of cytokine treatment alone was observed on *Pdcd1* expression, suggesting that NFATc1-mediated initiation of *Pdcd1* transcription is required for STAT3 and STAT4 to effect PD-1 levels. Thus, both -3.7 and 17.1 are complex regulatory elements that bind both NFATc1 and STAT3 or STAT4. Dissection of the -3.7 and +17.1 elements by site mutagenesis and reporter assays showed that both the NFAT and STAT elements were functional. Upon cytokine stimulation, the +17.1 reporter construct showed an increase in luciferase activity, although there was no increase from the -3.7 construct. The failure of -3.7 to respond to IL-6 and IL-12 stimulation may be due to the fact that upon PMA stimulation, EL4 cells produce IFN-γ, IL-2, and IL-4 (Farrar et al., 1983; Maeda and Shiraishi, 1996). Thus, it is possible that these cytokines, which activate STAT1, STAT5, and STAT6, respectively, could already be contributing to the expression of the -3.7-region reporter construct and that further stimulation by IL-6 or IL-12 could not be detected. Indeed, ChIP assays showed STAT1 binding to the -3.7 region in PMA/Io activated EL4 cells. Interestingly, +17.1 was also bound by STAT1 in these cells, however IL-6 stimulation augmented the reporter activity. The differences between the regulatory

elements could reflect the fact that -3.7 has two sites compared to a single site in +17.1 or the relative binding capacity of these elements *in vivo* for STAT proteins and, whether under the endogenous situation, the elements are designed to work in concert. The finding of STAT1 binding in EL4 cells would also suggest a potential role for interferons in regulating *Pdcd1* activity through these elements under specific conditions. Although a role for IFN-γ in the system used here was not observed, a role for type I interferon's, which utilize STAT1 was previously described to use an ISRE located in CR-C (Cho et al., 2008; Terawaki et al., 2011).

Although *Pdcd1* expression requires T cell activation, there is a change in the chromatin architecture at -3.7 and +17.1 regions in response to cytokine treatment alone as H3K4me1 was observed at these regions following just cytokine stimulation. This suggests that the -3.7 and +17.1 regions become "poised" enhancers in response to cytokine stimulation. The induced binding of p300 and concomitant accumulation of H3K27ac in the presence of TCR and cytokine signaling imply that p300 is part of the *Pdcd1* induction mechanism and likely is involved in activating these enhancers. These data suggest that the elements function differently with CR-C responding to only TCR/NFATc1 signaling and the distal elements (-3.7 and +17.1) functioning as the cytokine response elements. This may reflect a built-in protective feature for making sure that PD-1 is not expressed on cells that are exposed to cytokines but have not received an antigen-specific activation signal.

Analysis of published ChIP-seq data on CD4 T cell subsets stimulated with various cytokines to induce STAT3, STAT4, STAT5, and STAT6 identified seven sites within the *Pdcd1* locus that bound multiple STATs (Consortium, 2011; Durant et al., 2010; Wei et al., 2010). All of the peaks were enriched for STAT3 and STAT4, with four of these peaks represented by CR-C, -3.7, +17.1, and +17.5. The three remaining peaks were located at -

2.7 kb, the promoter region, and 1.2 kb downstream of the TSS. As shown here, quantitative ChIP in CD8 T cells stimulated with IL-6 and IL-12 for each of these sites confirmed binding of STAT3 and STAT4 to -3.7 and +17.1 (Figure 2-4). Additional ChIP assays showed binding to the -2.7 site (data not shown); however, no STAT binding was observed at CR-C or any of the other peaks (data not shown). This suggests that there is some background to the ChIP-seq datasets or that binding in those assays represents CD4 T cell subset-specific pattern. If the latter is the case, then a greater role for STAT factors in controlling PD-1 expression in other T cell subtypes is likely.

CTCF can function to block the activity of enhancers in *cis* and/or function as a chromatin boundary factor to prevent the spreading of heterochromatin (Bushey et al., 2008). CTCF has previously been shown to control the chromatin architecture and expression of genes in the major histocompatibility complex class II and interferon-γ loci, as well as V(D)J recombination (Guo et al., 2011; Majumder and Boss, 2010; Majumder et al., 2008; Seitan et al., 2011). Regulation of gene expression by CTCF is thought to be due to the formation of long-range interactions that bring enhancers and promoters together (Li et al., 2013; Majumder and Boss, 2010; Yoon and Boss, 2010). Here, two CTCF sites flank the *Pdcd1* gene and likely serve to isolate it from its neighboring genes. As such, the CTCF-CTCF interactions have the potential to create a regulatory domain that would insulate *Pdcd1* from external enhancers and/or focus the regulatory potential of the elements defined here on the *Pdcd1* promoter.

The 3C experiments produced a complex set of data suggesting that four different interactions are present in PD-1 expressing CD8 T cells. The first set reflects the constitutive CTCF–CTCF site interactions mentioned above (Figure 2-7, red lines). A second set of interactions occurs between either CTCF site and the -3.7 region (Figure 2-7,

black lines). In CD8 T cells, these interactions increase upon NFAT stimulation. Curiously, reciprocal interactions between these sites were not observed when the anchors were placed on the opposing sides of the restriction fragments. This suggests that there are spatial constraints established by the elements that firmly establish the orientation of the free DNA ends, restricting the ability of the ends to be ligated, and ultimately detected in the 3C assay. The third and fourth set of interactions occurs between the -3.7 region and the promoter and the +17.1 region and the promoter (blue lines). As above, these interactions were orientation specific, suggesting that the interactions between the elements, promoter, and CTCF sites define a critical structure that might be required to control *Pdcd1* expression.

Taken together, our findings have expanded the role of NFATc1 and added STAT3, STAT4, and CTCF to the list of factors contributing to PD-1 expression. Intriguingly, STAT3 or STAT4 could explain how PD-1 is expressed at high levels in chronic infections, a condition in which NFATc1 translocation to the nucleus is impaired (Agnellini et al., 2007). The use of STAT family members to regulate PD-1 allows for environmental cues to regulate PD-1 expression. This suggests that regulation of PD-1 on CD8 T cells could be accomplished by targeting other cell types that produce cytokines that signal CD8 T cells. Previously, it has been shown that IL-2 inhibits IL-17 expression through STAT5 binding to the IL-17 locus and this repression could be reversed by the addition of IL-6, which induces STAT3 binding at the same sites (Yang et al., 2011). Thus, a similar system may occur where cytokine stimulation leads to different STAT members binding to the same sites to control PD-1 expression. Such control would be envisioned to reflect cell type accessibility to the different sites, as well as cell type expression of the various cytokine receptors.

Figure Legends

Figure 2-1. The *Pdcd1* locus contains multiple inducible DNase I hypersensitive sites. (A) Schematic of the PD-1 locus showing relative positions to the TSS, conservation of sequences among mammals defined by MULTIZ alignment, and the human chain alignment of chromosome 2 (green) and 5 (red) (Blanchette et al., 2004; Chang et al., 2000). Amplicons (Black boxes) used for conventional PCR-based DNase I hypersensitivity analysis are shown as is the previously defined CR-B/C regulatory region (B/C, blue). Using increasing amounts of DNase I, each of the 59 amplicons were used to assess the DNase I hypersensitivity of splenic CD8 T cells (control or stimulated with PMA and ionomycin (+P/I) ex vivo for 24h). Each PCR amplicon is between 0.9-1.3 kb in length. **(B)** Select examples of the 59 amplicons that were evaluated by conventional PCR are shown and their position from the TSS is indicated. **(C)** Each of the 59 amplicons was quantitated using ImageJ software. The bands from the +17.0 PCR shown in B were used as an example with slopes determined by linear regression. **(D)** Relative DNase I hypersensitivity of CD8 T cells for unstimulated (green) and PMA/Io cultured cells (black). DNase I sensitivity was calculated by taking the negative value of the slope following ImageJ quantitation and normalizing to the previously known hypersensitive region CR-C. Amplicon locations are shown below. The amplicon representing CR-C is shown with a blue C. **(E)** A higher resolution DNase I hypersensitivity map was constructed using real-time PCR on PMA/Io stimulated CD8 T cells. Asterisks above bars indicate regions that were chosen for further analyses that showed a statistically significant ($p < 0.05$) increase in DNase I hypersensitivity over control samples. Black and red arrows denote regions chosen for further study and the location of these regions with respect to the TSS is indicated. The DNase I sensitivity for each of the regions indicated by the arrows was statistically significant ($p < 0.05$). Amplicons are displayed as blue boxes along the bottom. **(F)** Relative hypersensitivity of the *Pdcd1* locus

in the murine EL4 T cell line using the same methodology as in CD8 T cells from D**.** (**G**). Quantitative real-time PCR analysis of DNase I hypersensitivity in EL4 cells using the methodology from E. The data from these experiments were averaged from three independent cell preparations.

Figure 2-2. Two novel regulatory regions respond to T cell activation. (A) DNase I hypersensitive sites selected from Figures 1E and 1G were cloned into the pGL3-Promoter firefly luciferase reporter vector. **(B)** Luciferase assays from EL4 cells transiently transfected with the construct indicated. Following transfection cells were cultured for 16h then stimulated with PMA/Io (P/I) for 24h. Firefly luciferase activity was quantitated, normalized to the cotransfected *Renilla* luciferase plasmid, and plotted relative to the empty pGL3-promoter (pGL3-Pro) vector. Data shown are the average of three independent experiments with standard deviation. Two-way ANOVA statistical tests comparing each stimulated sample to the empty vector were performed with *** representing $P<0.001$. **(C)** Constructs responding to PMA/Io were tested for their ability to respond to cyclosporine A (CsA) in transfection assays performed as above except that some cultures were treated for 2 hours with CsA prior to PMA/Io stimulation. The averages of three independent luciferase transfections were plotted. Two-way ANOVA comparing PMA/Io stimulated samples -/+ CsA were performed. ***$P<0.001$, *$P<0.05$.

Figure 2-3. IL-6 and IL-12 stimulation induces distinct chromatin modifications at *Pdcd1* regulatory regions. (A) *Pdcd1* mRNA expression from splenic CD8 T cells that were cultured *ex vivo* with CD3/CD28 bead stimulation in the presence or absence of IFN-a, IFN-γ, IL-2, IL-4, IL-6, or IL-12 for 24h was measured by real-time RT-PCR. Data is

presented as the relative *Pdcd1* expression normalized to *18s* rRNA from three biological replicates with standard deviation. The student's t-test was used to determine significance of activated vs. activated and cytokine treated CD8 T cells. **(B)** Primary splenic CD8 T cells were cultured for 24h in the presence or absence of CD3/CD28 beads, IL-6, and/or IL-12 as indicated and subsequently analyzed for DNase I hypersensitivity at -3.7, +17.1, CR-C and a control (+18.2) region. The significance of activated vs. cytokine treated, activated CD8 T cells was determined by student's t-test. **(C)** Quantitative ChIP assays using antibodies against H3K27ac, H3K4me1, H3K4me3, Pol II, and a control IgG were performed from splenic CD8 T cells cultured for 24h with and without CD3/CD28 beads, IL-6, and/or IL-12 as indicated. Data were presented as the average percent chromatin input from three independent experiments. Error bars represent standard deviation. Student's t test comparisons between control and IL-6 or IL-12 treated samples showing a significance of $p<0.001$ are indicated by gray shading across the ChIP samples. In the other panels p values are represented as follows: *, $P<0.05$; ***, $P<0.001$

Figure 2-4. NFATc1, STAT3, STAT4, p300, and CTCF bind to the *Pdcd1* locus. Quantitative ChIP for NFATc1, STAT3, STAT4, p300 and CTCF binding to the regions indicated was performed. All ChIP data is presented as average percent input from three independent experiments with standard deviation. An IgG control antiserum was used as a specificity control for antibody binding. **(A)** ChIP for NFATc1, STAT3, STAT4, p300, or a control IgG from splenic CD8 T cells stimulated for 24h with CD3/CD28 beads, IL-6, and/or IL-12 as indicated. **(B)** Consensus CTCF binding sequence in logo format aligned with the DNA sequences from -3.7 and +17.1 is shown. **(C)** ChIP for CTCF or a control

IgG from CD8 T cells stimulated with CD3/CD28 beads, or beads and IL-6 and EL4 cells stimulated with PMA/Io or PMA/Io (P/I) and IL-6.

Figure 2-5. NFATc1 and STAT3 binding sites are necessary for regulatory activity of the -3.7 and +17.1 regulatory regions. (A and B) EL4 cells were transfected with the indicated construct as in Figure 2 and stimulated simultaneously with PMA/Io (P/I) and/or the cytokine indicated. At 24h post stimulation, luciferase activity was determined and normalized to the cotransfected Renilla plasmid. **(C)** The -3.7 and +17.1 regions were cloned into the previously described *Pdcd1* promoter–CR-B–pGL3-Basic–luciferase reporter expression vector (Oestreich et al., 2008). **(D and E)** NFAT and STAT binding sites identified *in silico* (indicated as N or S, respectively), were deleted in the pGL3-Promoter based constructs. The resulting constructs were subsequently transfected into EL4 cells and stimulated with PMA/Io and/or IL-6 as indicated. Δ indicates which sequence was deleted. ΔCon represents a random deletion and serves as a negative control. Data from three independent experiments were averaged and plotted as relative luciferase to pGL3-Promoter (pGL3-Pro) or to pGL3-Basic with standard deviation. Two-way ANOVA tests were used to determine the significance between samples. In **A** and **B**, the statistical tests compared PMA/Io stimulation to PMA/Io and IL-6; or PMA/Io stimulation to PMA/Io and IL-12. In **C**, PMA/Io stimulated cultures were compared to unstimulated, PMA/Io +IL-6, and PMA/Io +IL-12 cultures. PMA/Io stimulated cultures of -3.7, +17.1, and CR-B/C constructs were also compared to the PMA/Io stimulated CR-B construct. Statistical differences in **D** and **E** were assessed between the wild-type construct and the deletion constructs stimulated with PMA/Io. Additional analysis between the single and double

deletion constructs were also assessed as indicated. *, P<0.05; **, P<0.01; and ***, P<0.001.

Figure 2-6. Multiple long-range interactions occur across the *Pdcd1* locus. (A) Schematic of the *Pdcd1* locus with CTCF, NFATc1, and STAT3 binding sites indicated. Vertical lines denote *Pst*I sites. Arrowheads denote locations of anchors (gray) or fragment test primers (black) used in the 3C assays. **(B)** 3C assays of splenic CD8 T cells unstimulated or activated with CD3/CD28 beads for 24h in the presence or absence of IL-6. Anchor primers are indicated at the top of each column and fragment test primers are indicated in the abscissa. The region used as the anchor is shaded in gray. Crosslinked and non-crosslinked samples are indicated by black and white bars, respectively. **(C)** 3C assays were performed on EL4 cells untreated, activated with PMA/Io, or PMA/Io and IL-6 for 24h. Relative crosslinking frequency is defined as the average of the real-time PCR based values for each 3C amplicon divided by a non-specific control amplicon. Data is presented as the average relative crosslinking efficiency from three experiments plus standard deviation. Two-way ANOVA comparing crosslinked and non-crosslinked samples was performed to determine significant changes in crosslinking frequency. ***, P<0.001; **, P<.01; and *, P<0.05.

Figure 2-7. Schematic of long-range cis-element interactions at the *Pdcd1* locus that occur following CD8 T cell activation. CTCF, NFATc1, and STAT3/4 binding sites are shown. *Pst*I restriction enzyme sites are represented by vertical black lines. CTCF—CTCF

(red lines), -3.7—CTCF (black lines), -3.7—Promoter (blue lines) and +17.1—Promoter (blue lines) interactions are shown with the relative strength represented by line thickness.

Supplemental Figure 2-1. T cell activation leads to a multiple DNase I hypersensitive sites in CD8 T cells. DNase I hypersensitivity assay of **(A)** CD8 T cells or **(B)** EL4 cells unstimulated or cultured for 24h with PMA/Io (P/I). Shown is one representative PCR for each cell type using all 59 primer sets. Each PCR is between 0.9-1.3 kb in length. Band intensities were determined and used to determine a slope representing loss of signal intensity. Slopes were normalized to a fragment containing the known hypersensitive CR-C region (-1.1) and used to determine relative hypersensitivity (Figures 2-1D and F).

Supplemental Figure 2-2. NFATc1 and STAT1 bind to the -3.7 and +17.1 regulatory regions in PMA/Ionomycin stimulated EL4 cells. Quantitative ChIP for the binding of STAT1, STAT3, STAT4, STAT5, STAT6, NFATc1, and a control IgG was performed at the indicated regions from EL4 cells stimulated with PMA/Io for 24h. Data is presented as the average percent input of three independent experiments. Error bars represent the standard deviation.

Supplemental Figure 2-3. All *Pst*I sites are equally accessible during 3C library preparation. (A) Schematic of the *Pdcd1* locus with all *Pst*I sites denoted as vertical lines. NFATc1, STAT3, and CTCF binding sites are also shown, while primer locations are denoted as arrows. **(B and C)** PCR of undigested or *Pst*I digested chromatin from **(B)** CD8

T or **(C)** EL4 cells using the primer pairs indicated. Cells were treated as indicated for 24h prior to cross-linking and chromatin isolation.

Figure 2-1

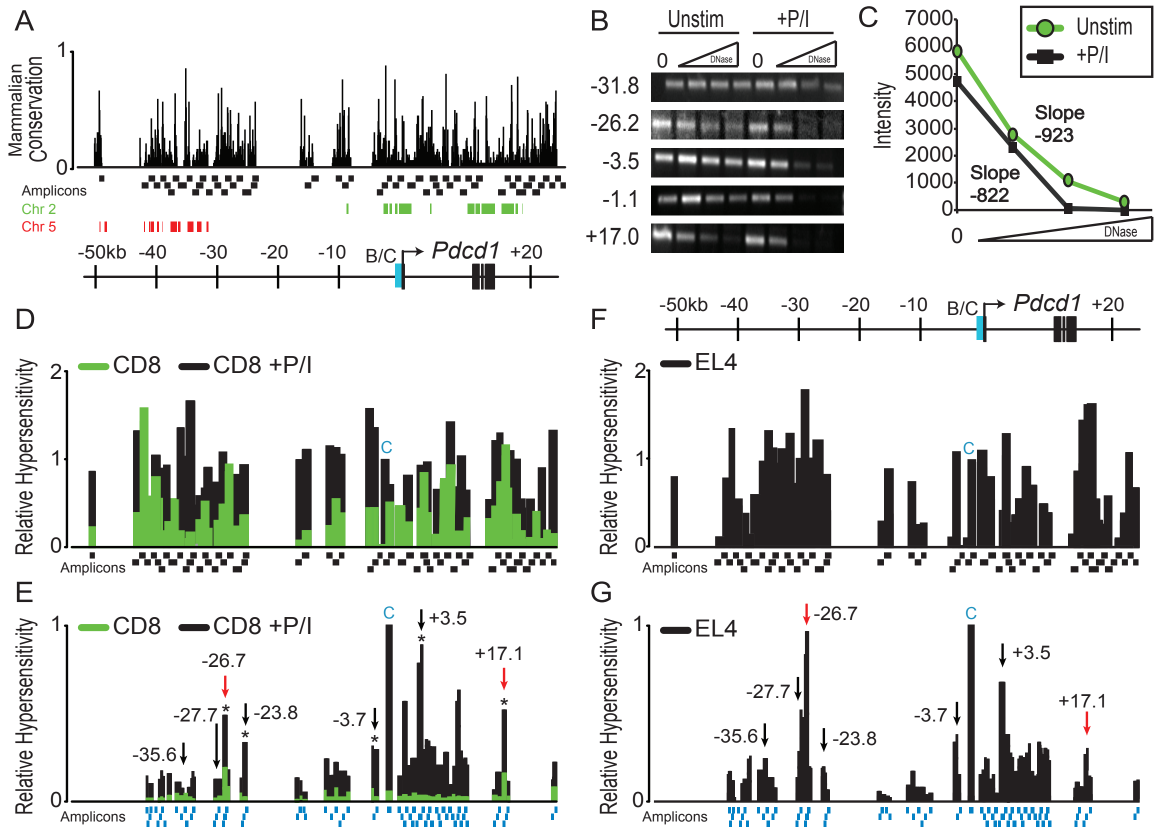


Figure 2-2

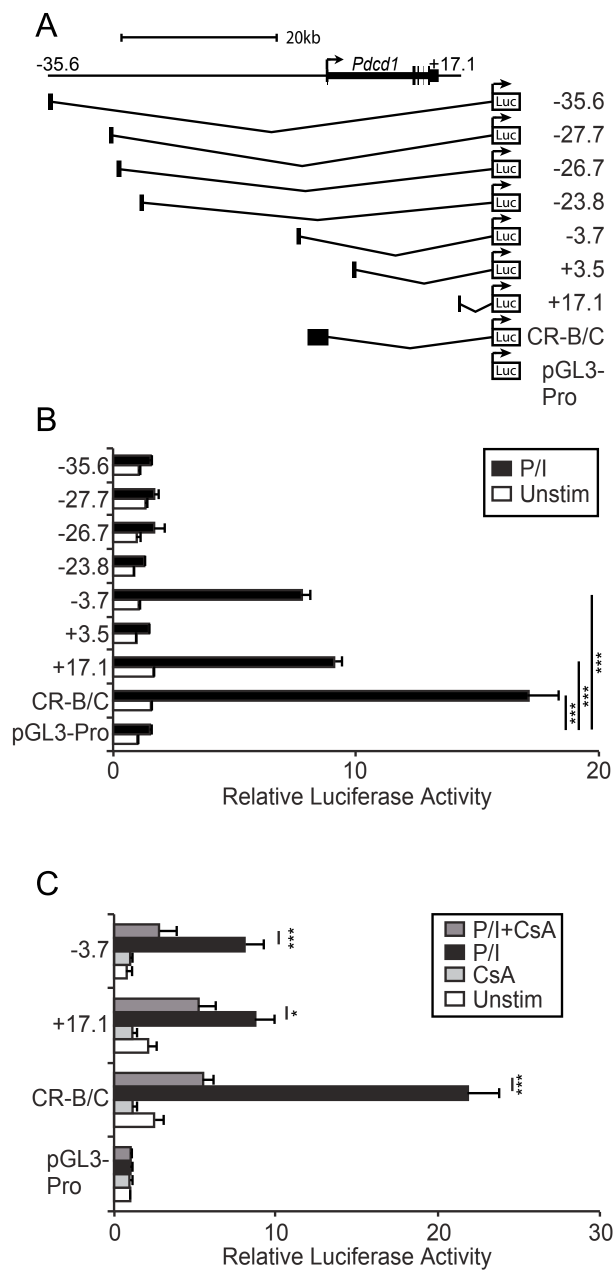


Figure 2-3

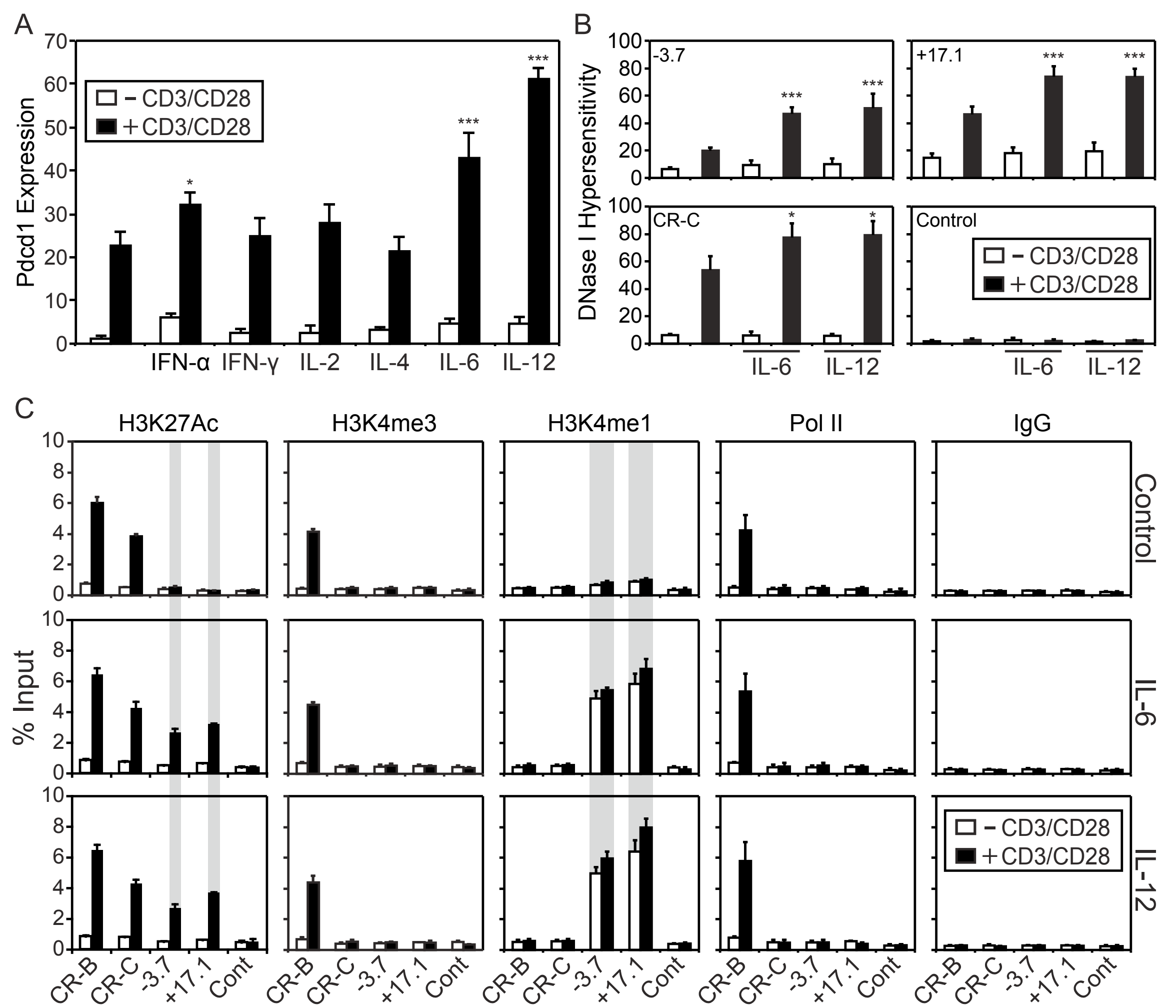
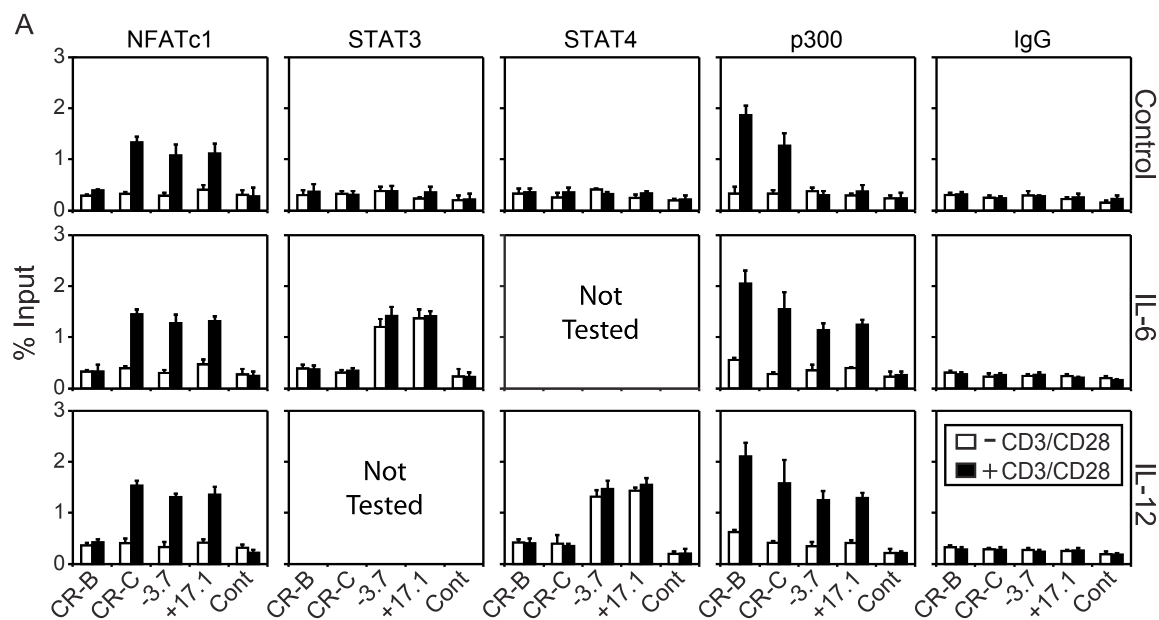
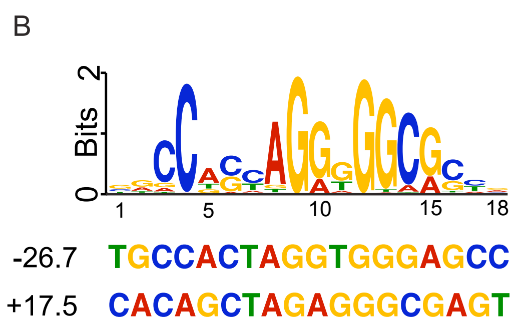


Figure 2-4





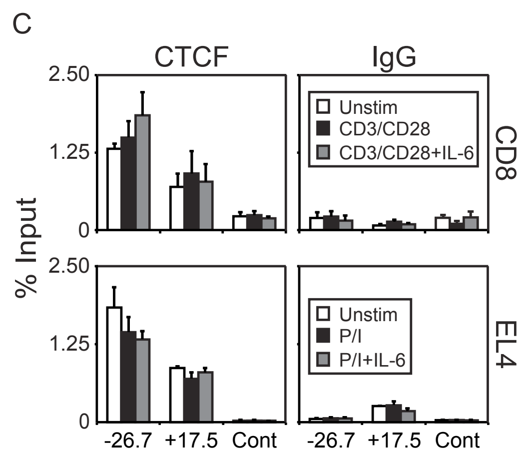


Figure 2-5

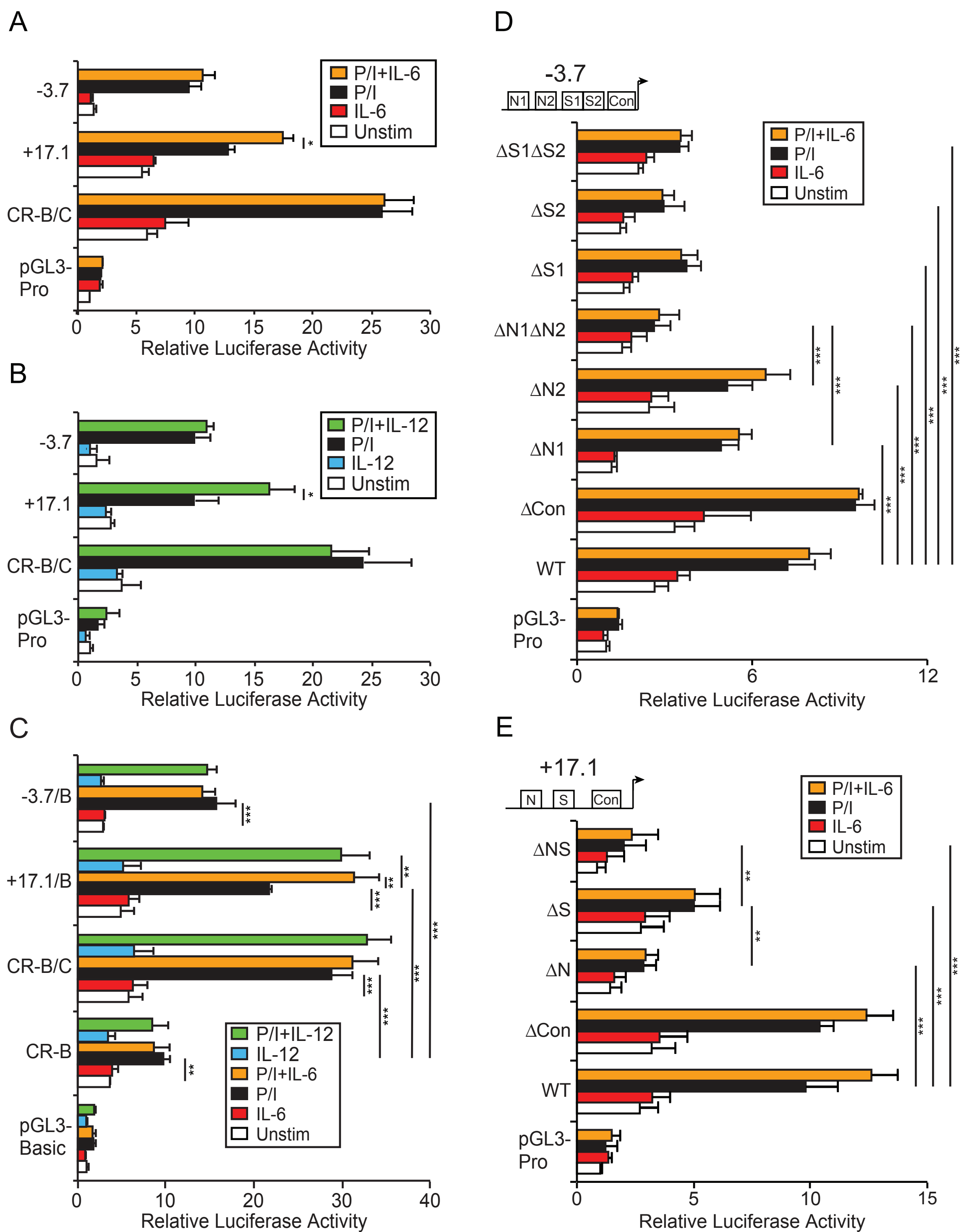


Figure 2-6

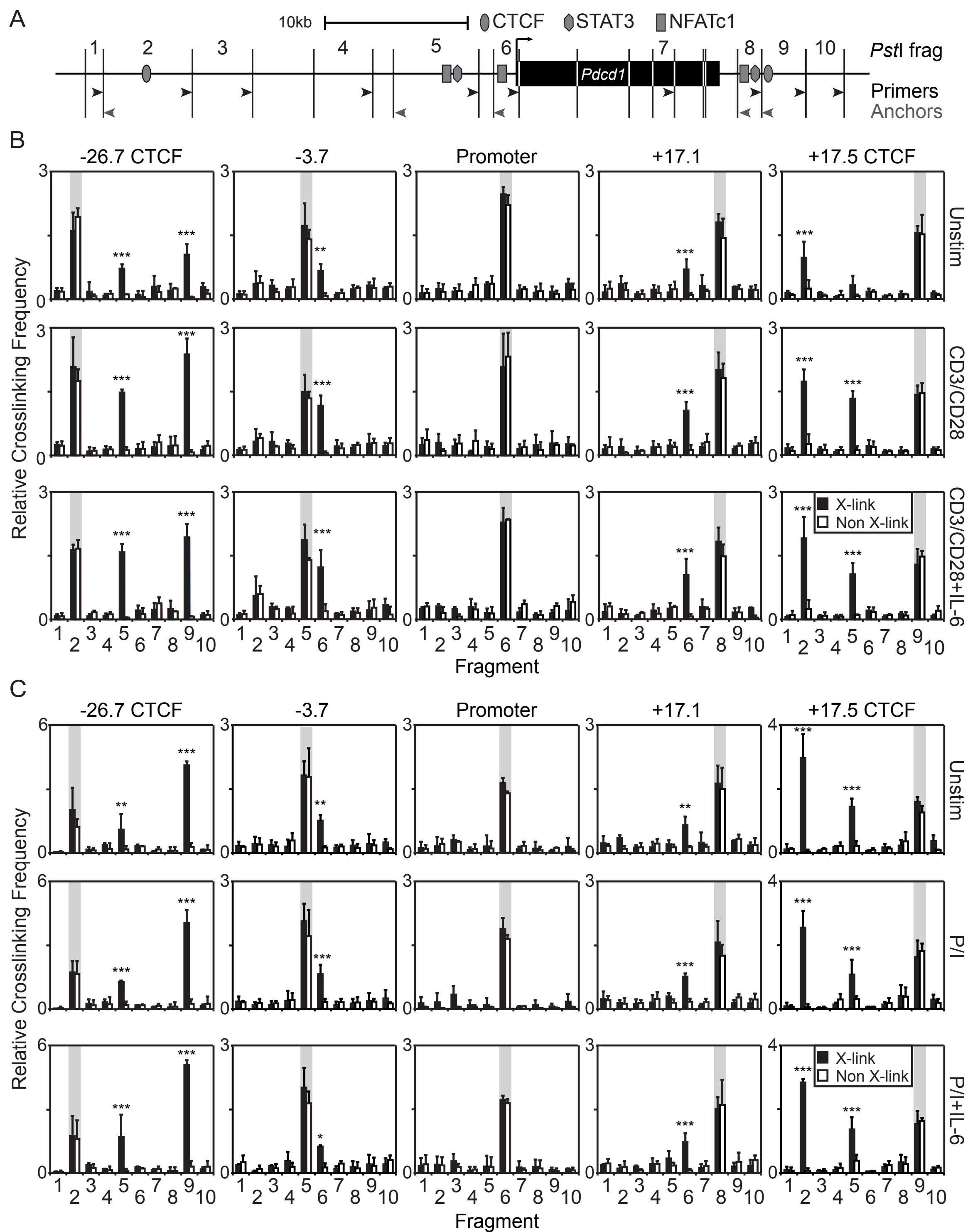
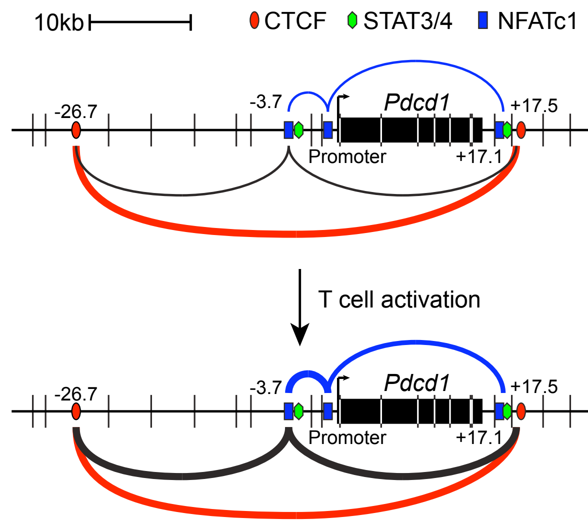
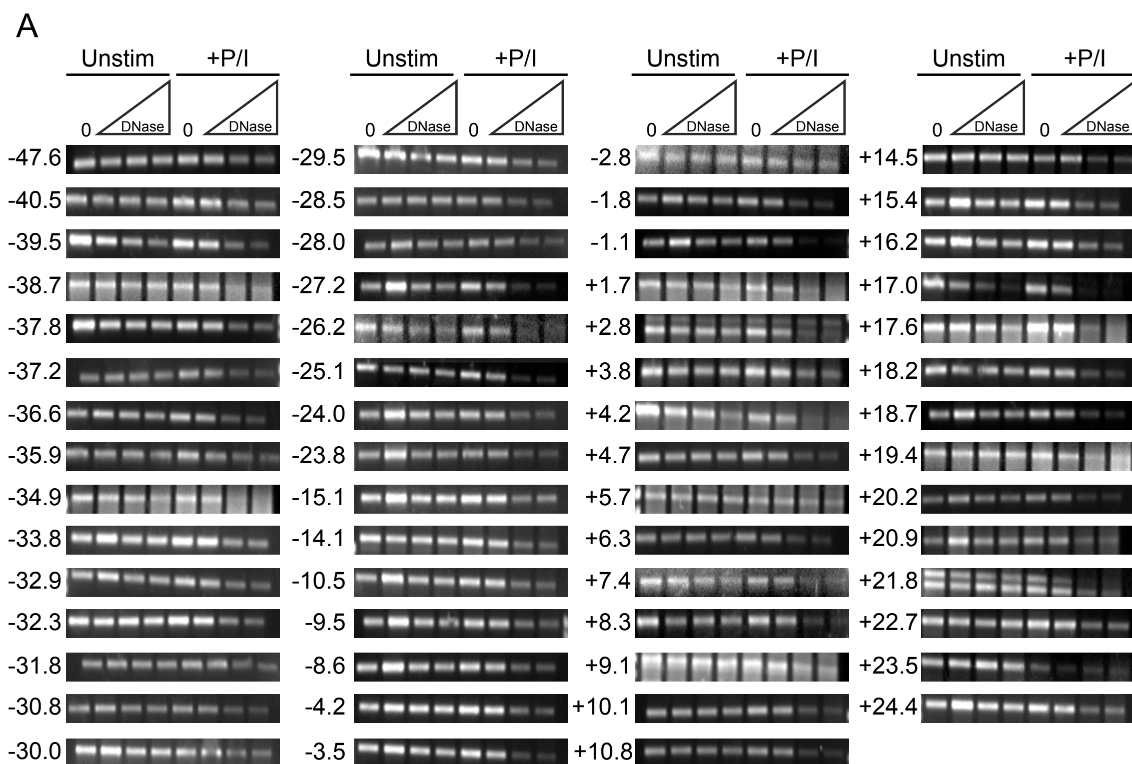
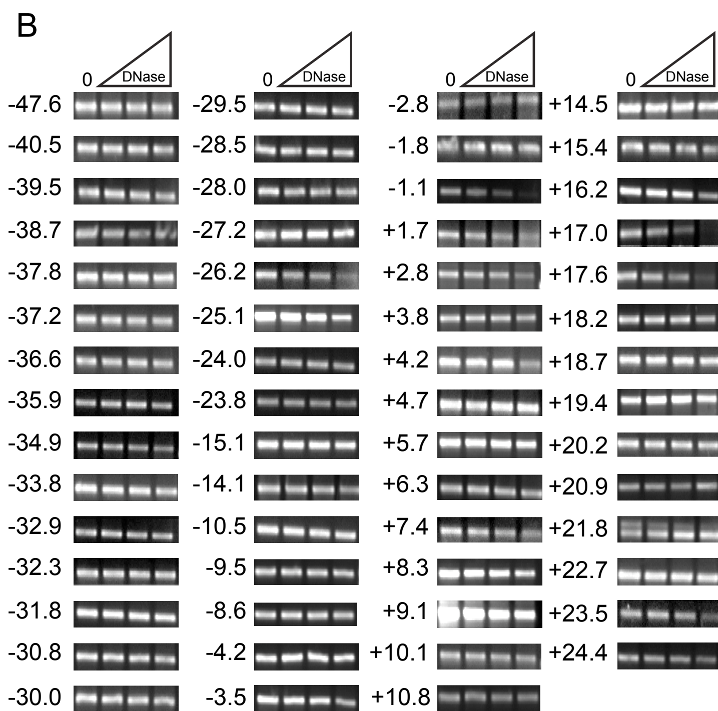


Figure 2-7

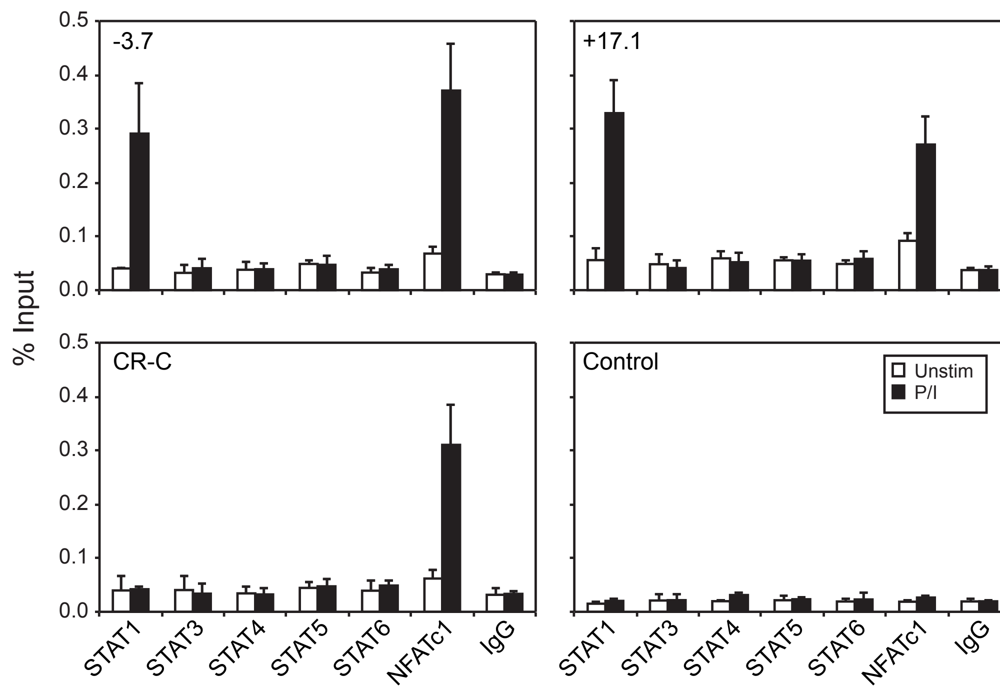


Supplemental Figure 2-1

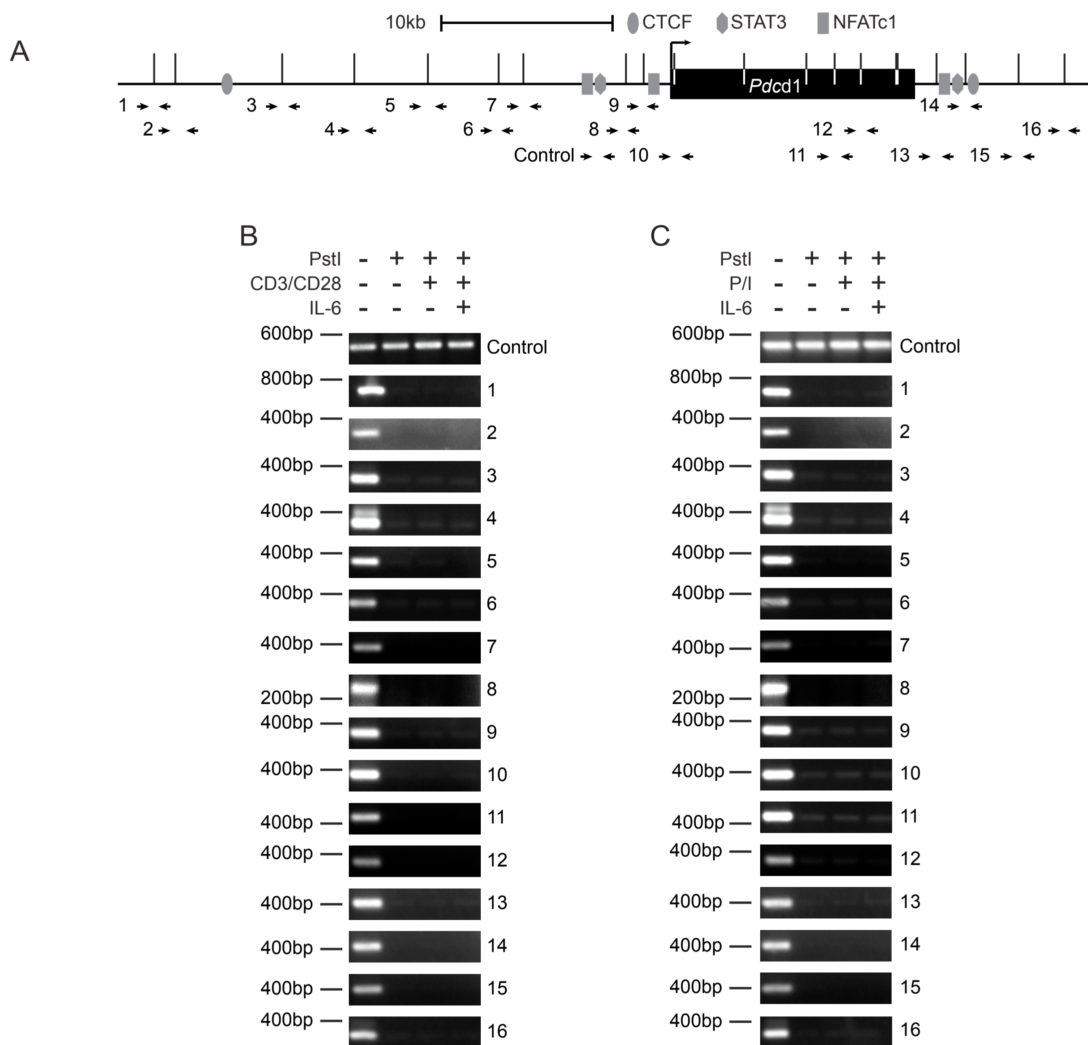




Supplemental Figure 2-2



Supplemental Figure 2-3



Supplemental Table 2-1: List of the oligonucleotide primers used in this study.

Conventional PCR DNase I Hypersensitivity Primers

-47.6 Forward	CTGCTCCGATTCCCTTTGTA	-2.8 Forward	TACCCACACCCTGGAGGTAA
-47.6 Reverse	ACAGGATGATCTGGCTGGAC	-2.8 Reverse	CCAGGGGAGTCATCTTTTCA
-40.5 Forward	ATGCATGCAAGATGGGTTTC	-1.8 Forward	CAAGACCCCTGTCAAGGCTA
-40.5 Reverse	TCCCCAATCTTCTCTCTTGTG	-1.8 Reverse	CTGTCCAGAAGGAAGGATGG
-39.5 Forward	GCTGGTTGTACCAATGAGCA	-1.1 Forward	CCTCTCCAGACACCCCTGTA
-39.5 Reverse	GCATCTGTGCAATCCAGAAA	-1.1 Reverse	CAGCAGAAGGGGAAAAGAGA
-37.8 Reverse	AACTTGTGCCAAACCAAAGG	+1.7 Forward	TTGGCAGTTTTGACAGATGG
-38.7 Forward	CCTTTGGTTTGGCACAAGTT	+1.7 Reverse	TTGCTGTCAAGACTGGATGG
-37.8 Forward	CGGTGCTGGATTCCCTTACT	+2.8 Forward	ATGCTTCCTAAAGCCCCTCT
-37.8 Reverse	AGGCCAGGCAGTTACAAGAA	+2.8 Reverse	GCCTTGGCCATGAGATTTAC
-37.2 Forward	ATATTCACCCCGCTCCCTAA	+3.8 Forward	TGACAGGGAGCTTCTTGGTT
-37.2 Reverse	AGTGAGTGTATTTAACCTCCCATAA	+3.8 Reverse	TCACCGAGTACCACAGTTGG
-36.6 Forward	TTGGGAAAGGAATGTTGAGC	+4.2 Forward	GCGGGTTTGTAAGACTGAGC
-36.6 Reverse	CACAGGTTCAGGGACACAAG	+4.2 Reverse	CTGGGGTAAAGATTCCAGCA
-35.9 Forward	CAAAACAAACAAAACCAACCAA	+4.7 Forward	GAGCAGCCTGACTTTTGCTT
-35.9 Reverse	CAGGAAGTTATTTGTCTGCTGGT	+4.7 Reverse	ATTGTGTGCGTCTGTGTGGT
-34.9 Forward	GGGGCTGTTGCTTATGGAAG	+5.7 Forward	CCCTTACCTCCCCATACGAT
-34.9 Reverse	TTGGTTGGTTTTGTTTGTTTTG	+5.7 Reverse	AAGCAAAAGTCAGGCTGCTC
-33.8 Forward	GGCCTTTGGAAAATCAGATG	+6.3 Forward	ATGGCCAGTCATTTTGGATG
-33.8 Reverse	CAGCCTCAGAACCTCCTTGT	+6.3 Reverse	AGGGTTTCCACCCTCTGTCT
-32.9 Forward	AAATGAAAATTTGGAAGTCTTTTAGC	+7.4 Forward	CAGCTAGAGGGGTCAGCACT
-32.9 Reverse	AGTTCAGCAAAGCACCATCC	+7.4 Reverse	CATCCTGTCTGCCCGTCTAT
-32.3 Forward	CCCAGTTCCAGGAAACAGAA	+8.3 Forward	GAAACCTGGGGTCCCTTTAC
-32.3 Reverse	GGCACACCTCTAATCCCAGA	+8.3 Reverse	TTATGTTCCACAGGCATCCA
-31.8 Forward	AACATGCATGGATCGTGTCT	+9.1 Forward	AAAACACCTGGCAAACAAGG
-31.8 Reverse	GCAATGGCTTCATTAAGATGG	+9.1 Reverse	ACTGGACAGAGGTGCCTAGC
-30.8 Forward	ATTATTTGCCTGCCCTGTCA	+10.1 Forward	GTGGGAGTGTAGGGTTTCCA
-30.8 Reverse	CGATCCATGCATGTTAGACC	+10.1 Reverse	ATGGATGTCTCTGGGTTTGC
-30.0 Forward	TCCAGGAGAGCAGAGAGACC	+10.8 Forward	ATGGCCCCACAGAGGTAGAT
-30.0 Reverse	GTGTGGCTCCTGTCCTGTCT	+10.8 Reverse	TTTTGAGTGCTGGTGGAATG
-29.5 Forward	CACAAAACATCGGTTGTCCA	+14.5 Forward	CCTCTAACCCTGCTTGTCCA
-29.5 Reverse	GGTGCAAGCTCAAGGCTATT	+14.5 Reverse	GTTGACAGCAGGGAGGAAAG
-28.5 Forward	ATGTAGTCACCACCGGCATT	+15.4 Forward	GAGTTGTGGCTTTTGCCTTC
-28.5 Reverse	TGTGGACAACCGATGTTTTG	+15.4 Reverse	TCTCCGACCTCTGTCTGGAT
-28.0 Forward	AATGCCTTTGCTCTGCTGTT	+16.2 Forward	GCACAACCCTAGCTTGCTTC

-28.0 Reverse	TGATGATGACCGTGGAGAAA	+16.2 Reverse	AGATTCCCACCACATGCAAT
-27.2 Forward	AACCAAACAGAGCGAGCAAT	+17.0 Forward	CCTCTAGTCCTCACCGTGGA
-27.2 Reverse	GCTCAGGTGTTAGCGCTTTT	+17.0 Reverse	CACAGGGCTGTCAGTGCTAA
-26.2 Forward	ATGCAATCGCACTAGGGTCT	+17.6 Forward	TGTAACAGGCAGGTCTGTGG
-26.2 Reverse	ATTGCTCGCTCTGTTTGGTT	+17.6 Reverse	GGACCAGTCAGCACTTCACA
-25.1 Forward	CTGCCCATGTGTTTGTTTTG	+18.2 Forward	GACTCGCCACCTCTGACATT
-25.1 Reverse	GCCCCATCAATCAAATGTGT	+18.2 Reverse	TGCCACTGCTTCCAGTGTAG
-24.0 Forward	GGACTTACCAAACCCCGAGT	+18.7 Forward	GAAGCCAAGGACCATGTTGT
-24.0 Reverse	GTTCAGGCGAGAGGAGAGAA	+18.7 Reverse	GTCAATGGCTGCTTCACAAA
-23.8 Forward	CTACCAGTCGGTTCCTGCTC	+19.4 Forward	CAGGGAAAACCTCCCCTTTA
-23.8 Reverse	AAAGGCAGGTTGTGCTGAGT	+19.4 Reverse	CTGGCACTTAATGGGGTCAG
-15.1 Forward	CACAATGACAGGACATTTGGA	+20.2 Forward	CAGAGGCTTTTCCCCCTATC
-15.1 Reverse	TTCCATGCTCTGTTTCATGC	+20.2 Reverse	CAGCCTAGCCTGTGGTTGTT
-14.1 Forward	TGCTCCCTTCTGGAGTGTCT	+20.9 Forward	CTGTCTGTGTTTGTGAGTGGTG
-14.1 Reverse	TGGAATCCAAATGTCCTGTC	+20.9 Reverse	AGAGTGCGGCTTACATCAGG
-10.5 Forward	CTGCCCTATTCCCTTGGTTT	+21.8 Forward	AAGTCATTGGTTCACCAAGTTAGA
-10.5 Reverse	TGGAAGCTCTTAAGCAACCAC	+21.8 Reverse	GCTCATAAAATAGAAAGACACCACT
-9.5 Forward	CATGTGGGTGTGGTGAGGTA	+22.7 Forward	CTAACCCCAACCCCAACTTC
-9.5 Reverse	AACCAAGGGAATAGGGCAGT	+22.7 Reverse	ACCCCCAAACCAAGAAAACT
-8.6 Forward	TTATGATCCCACCCCAGCTA	+23.5 Forward	GCTCCAAGCACAAACCAAAT
-8.6 Reverse	TGCCACCACAATCTTCAGTT	+23.5 Reverse	TGGCTGCAATTGGTGTATTC
-4.2 Forward	CAGGCTGCTCAGCTCTTAGG	+24.4 Forward	AGGACAAATTGGCTCCACTG
-4.2 Reverse	TCTTTCACCGGGCTGATAAC	+24.4 Reverse	TGTCTAAAGAGCCATCCAGGT
-3.5 Forward	TTGTCACTTTGGCACAGAGG		
-3.5 Reverse	TCCACTCACAAGTCAATCAACC		

Real Time DNase I Hypersensitivity Primers

-39.0 Forward	TTCCACCCACACGGAATTAT	-3.2 Forward	ACTTTGGCACAGAGGGCTTCATCT
-39.0 Reverse	AGGCCAGGCAGTTACAAGAA	-3.2 Reverse	TCATCAAAGGAAGTCAGGGCAGGA
-38.9 Forward	AAACCCCACACATTCCAGAG	-1.2 Forward	CGACTTGTGTGCATGCATAGTACC
-38.9 Reverse	GACGACCCCAAATGGTAGAA	-1.2 Reverse	GAGGTCCTTTCACTCTCCACG
-38.6 Forward	GCTTCCCCTTCTCTGACCTT	+0.4 Forward	AGAAATGCTAGCACGGGAAG
-38.6 Reverse	CTTCTGCTTCCTCCCTCTCA	+0.4 Reverse	CAATCAGGGTGGCTTCTAGG
-38.4 Forward	TCCTCCTACTTCTGCCTCCT	+0.7 Forward	ACTGGGACGTCATATGAGCAAAGG
-38.4 Reverse	TCCAATGAAAACCATTGGCTA	+0.7 Reverse	TCAGCTGTCTACCCTCATGGACAA
-37.0 Forward	TCCCTAAGCTATGTCCCTGCTTGT	+1.2 Forward	GGTGTCCCACGTTGAGCTAT
-37.0 Reverse	CCATCATGAAGTGAACACACACAGGC	+1.2 Reverse	ATGACGTCCCAGTCACCTTC
-36.7 Forward	TGTTGATTCCACTTTGATGAGTG	+1.4 Forward	TGGGCACGATCCACTATGGCTGT
-36.7 Reverse	ACAGCAGTAGCCAGGGAAGA	+1.4 Reverse	CCCACGGAGCAAAGTCGGAAGG
-36.3 Forward	TTGGGAAAGGAATGTTGAGC	+2.0 Forward	CTCATGGCCAAGGCAATACT

-36.3 Reverse	TTTGAAACTGGCAAAGTTGTG	+2.0 Reverse	ATGGTGATCTTGGGCTTGTC
-36.0 Forward	ATGTTGGGCTGAAGAGATGG	+2.4 Forward	TGTGAACAGAGGGAACACCA
-36.0 Reverse	ATGGTCCTGGTCCCTTTGTA	+2.4 Reverse	TGGGTGCTGGAAATTGAATC
-35.6 Forward	TGGAGTGGATGTAGCACAAAA	+3.6 Forward	TGTCAGAGCTCTTGCTGCAT
-35.6 Reverse	CCATCTCTTCAGCCCAACAT	+3.6 Reverse	TGGATGGTTGTCACCGAGTA
-33.9 Forward	CACATCTGCCCGTTTCCTAA	+4.0 Forward	TGCTAGGGCCCAATCATAAC
-33.9 Reverse	CTTGAGTCACATTGCCTGTAGA	+4.0 Reverse	CCCACTTTCTCCCATCACTG
-33.6 Forward	CAGGGTCACAAGTGCTAAGT	+4.4 Forward	CAGAGGCCACTCTTGACTCC
-33.6 Reverse	CACACTAACCCAGCAGACAA	+4.4 Reverse	CAGGTGGACATGGGGAATAC
-33.1 Forward	CACTGGACTCGTTAAGCTACAG	+4.7 Forward	TGCCCAACCTATCTCAGGAC
-33.1 Reverse	ACCCTGCCTCCAATTTCATTA	+4.7 Reverse	AAGGCTCCCTGGAGGAGATA
-32.7 Forward	TGCAGTTACCCTGCTTGTT	+5.0 Forward	ACGCACAGACACTCCTTTCA
-32.7 Reverse	GCACACCTCTAATCCCAGATAC	+5.0 Reverse	CTGTGAGTGGCTTTCCAGTG
-32.3 Forward	AGTGTTGGGCTGAAAGGTATG	+5.3 Forward	ATCATCCTCTGGCCCCATGGACAT
-32.3 Reverse	CATCCCTGCATGGCTATTGT	+5.3 Reverse	AGGCTGCTTCTGGGTGCATTCC
-32.0 Forward	AGGTCTAGTCTCTTGACTCCTG	+6.0 Forward	GCTTGTCTGGGTCAGTGGAG
-32.0 Reverse	GCAATGGCTTCATTAAGATGGG	+6.0 Reverse	AGGGTTTCCACCCTCTGTCT
-28.3 Forward	CGGTTTCAAGCCCTAGCTTA	+6.4 Forward	GTAGCCCTGGTTGTCCTGAA
-28.3 Reverse	CGGCTCCATTCATCCATCTA	+6.4 Reverse	TGTGTGTGTTTGTCCCTAGCA
-28.0 Forward	ACATAAGTGTCGCCCAGAGG	+6.6 Forward	CATGGGGACCTAAGTTTGGA
-28.0 Reverse	GGAGAGTGCAGGGAACAAGA	+6.6 Reverse	GGGTCTGGACCAACACCTAA
-27.7 Forward	AATGCCTTTGCTCTGCTGTT	+7.6 Forward	GCTACTTCAGTGGCCGAGAC
-27.7 Reverse	TAACCATTTCGCTCCTCTGG	+7.6 Reverse	CAAGCTCTGTGGGGTACTGG
-27.0 Forward	AGAGTCCCAGGTTAGGGTTATGCT	+8.1 Forward	TGACTTCCCAGGTCTCTGTCCTTT
-27.0 Reverse	GGTCTAAGTCCTTCTAGGAAACCA	+8.1 Reverse	TCCACAGCATGAAAGGGAGACTCA
-26.8 Forward	ATTGCCCAGGTGAAGGTGGCTATT	+8.5 Forward	CCCCAATCTCCAGAGTCTCA
-26.8 Reverse	TAGCGTTGGGCAGTGTTGACTAGA	+8.5 Reverse	GCAAAACCACCAGGACCTAA
-26.5 Forward	TCTCTGGGTGTTCAAGCTCAAGGG	+8.9 Forward	CCACGATGGGGACTTCTAAA
-26.5 Reverse	CTGCCACTAGGTGGGAGCCTCT	+8.9 Reverse	ACTGGACAGAGGTGCCTAGC
-24.4 Forward	CTCCCAATCTGGGTGAGTGT	+9.3 Forward	CCGCACCATGTAGCCTTGCCC
-24.4 Reverse	GACTCTCTGCTGGCAGGTTC	+9.3 Reverse	AGAAGTCCCCATCGTGGTTGTGT
-24.2 Forward	CAGGCTGTGTAGTTGCTCCA	+9.5 Forward	GGTTATGGGCTCAGAAAGCA
-24.2 Reverse	GGGGATCTTTGATGAGGTGA	+9.5 Reverse	CTGACACCGCTGGTTCTTCT
-23.8 Forward	GGACTTACCAAACCCCGAGT	+9.8 Forward	GCTCTGGCCTCAACTCTGTC
-23.8 Reverse	GGGCAGCTCAACAGATTGAA	+9.8 Reverse	GGTTTGCCTCTGCTGAAGTC
-15.6 Forward	ATCCAAAGCCTGCCTATAGCACCA	+10.2 Forward	CCAGATCAGAGGTCACAGCA
-15.6 Reverse	TTTAGCAGTTGGCTATGGCTCCGT	+10.2 Reverse	TTCTCTACGAGCCCCAGTTG
-14.9 Forward	TTTGTTTATGCTTGGCCCAGGGAG	+10.5 Forward	CCTTCCCACTCCTGTTCAAG
-14.9 Reverse	TGCTGGGATTTGAACTCTGGACCT	+10.5 Reverse	TTTTGAGTGCTGGTGGAATG
-14.4 Forward	GCAGAATTCAGAGGGCAAACAGCA	+10.8 Forward	CCATTGGGGACCTCTGAAAT
-14.4 Reverse	ACAGCTGGTTGTGAGCCACTATGT	+10.8 Reverse	TCCCTGGGACAAAATACTGC

-14.1 Forward	AGCATGCAGAGGTCCTGAGTTCAA	+15.1 Forward	AGGAAGGTTGAGGACCACTG
-14.1 Reverse	TCTTCTGCCTCTCTGTGTGTCTGT	+15.1 Reverse	TCTCCGACCTCTGTCTGGAT
-10.5 Forward	GCCATTTGATGTGGATGCTATG	+15.4 Forward	ACCGTGAGCAGCACTCTGAT
-10.5 Reverse	GTCTAGCTCTGGGACATGATTG	+15.4 Reverse	GTCTGGTCGCACAGCTCATA
-10.2 Forward	AAGGACATACACTGCCCTATTC	+15.8 Forward	GAGTTGTGGCTTTTGCCTTC
-10.2 Reverse	CCTCAGAACTCCTGCCTATTC	+15.8 Reverse	GCAGGAGAAGCAGGGTACAG
-9.9 Forward	ATCTACCTCTCTTCAGCTCACT	+16.7 Forward	GGTTCAGAGACCCGTTGAGA
-9.9 Reverse	CGACTCTATGCTTCTCCCATTT	+16.7 Reverse	CACAGGGCTGTCAGTGCTAA
-9.4 Forward	AGTGATCTGAGGCAGGAGATAG	+17.1 Forward	TGGAGAGGAGAGAGCAAAGG
-9.4 Reverse	GTGAGCTGAAGAGAGGTAGATAGA	+17.1 Reverse	TCAACGGGTCTCTGAACCTC
-9.0 Forward	CACCTGGACAGTGGCTATTT	+17.4 Forward	AAGAGGCCTCACCCACATC
-9.0 Reverse	CAGCATCTCTTCCTCTCATCTTT	+17.4 Reverse	CTCCCACTGTGGTGTCCTTA
-8.7 Forward	GGCCACTCACTCATTTCCTTA	+24.2 Forward	CAGAGCTTGGGTCTCTCATTT
-8.7 Reverse	CTATTCCCTGCCACAACTAGAC	+24.2 Reverse	CAG GTG GAC AGC CTT GTA TT
-3.7 Forward	TCCTGCCCTGACTTCCTTTGATGA	+24.7 Forward	ACAGTCACATGCCAACTACC
-3.7 Reverse	AAAGCCTGTATCGAGCTGTGCTGA	+24.7 Reverse	GCTTCTCCACTACACCACTTAC
Luciferase Reporter Cloning Primers		**ChIP Primers**	
-35.6 Forward	CTGTGCTAGCGCTTTTGGGCCTGTCATGTGGGT	-26.7 Forward	TCAAGACACACAGCGTACAGAC
-35.6 Reverse	TCTTGCTAGCCACCAGAAGAGGGCATCGAATCCC	-26.7 Reverse	ACAAGCCATTGCTCGCTCTGTT
-27.7 Forward	ATGAGCTAGCAATGCCTTTGCTCTGCTGTTTGAGA	-3.7 Forward	AGGGTGAGCAGGCGAGAGCAA
-27.7 Reverse	AGGTGCTAGCAGGGTGCACAGGCTCCTCAGT	-3.7 Reverse	AGCACAGGGAAGAAGCTGTTGGG
-26.7 Forward	TCTGGCTAGCTAGCGTTGGGCAGTGTTGACTAGAGA	CR-C Forward	CCTCACCTCCTGCTTGTCTCTC
-26.7 Reverse	TGTCGCTAGCTGTGTGTGAGACTTCAGGAGCCTATT	CR-C Reverse	GTGAGACCCACACATCTCATTGC
-23.8 Forward	TGTCGCTAGCCTCCTAAGGGACTTACCAAACCCCG	CR-B Forward	CTCTGACTAGCTGTCCTTGCCTC
-23.8 Reverse	CCAAGCTAGCCCCCAAAGCAGGTGAAGGCTCA	CR-B Reverse	CTCGACACCCACCCTCCAAAG
-3.7 Forward	GTGGGCTAGCCTATCCCTGGCCTGGTGGTCCT	+17.1 Forward	GGAGGGGATAGGCGCTGGGT
-3.7 Reverse	AAAGGCTAGCTCGAGCTGTGCTGATGGACACCC	+17.1 Reverse	TCTGGGCCAAGCCATCCGGT
+3.5 Forward	TCACGCTAGCGGGAGAAAGTGGGAGATGGGGGAT	+17.5 Forward	ATACAAAGAGGCCTCACCCACA
+3.5 Reverse	GTCAGCTAGCACCACAGTTGGCGGAGAGGGG	+17.5 Reverse	TGGAAGGCAGAATTGGCACCTA
+17.1 Forward	TGTCGCTAGCTGGTTTGTGGTGGAGTAGCCACAT	Control Forward	CAGAGGCCACTCTTGACTCC
+17.1 Reverse	TCTGGCTAGCAGTGGTCATACTCTCAACGGGTCT	Control Reverse	AAGGCTCCCTGGAGGAGATA
-3.7 ΔNFAT1 Forward	GATGATAAACAGTGATAGTAAGCCAAATAAACC		
-3.7 ΔNFAT1 Reverse	GGTTTATTTGGCTTACTATCACTGTTTATCATC		
-3.7 ΔNFAT2 Forward	GTAAGCCAAATAAACCTTTCACCGCCTTGGGTT	**3C Primers**	
-3.7 ΔNFAT2 Reverse	AACCCAAGGCGGTGAAAGGTTTATTTGGCTTAC	Fragment 2 Anchor	TCAGGATGCTATGGCTGAAAG
-3.7 ΔStat1 Forward	AGGGCTTGGGCATCTTGGCTGAAAGTACA	Fragment 5 Anchor	TTAGTGCACAAGTGTGGTGT

	G		
-3.7 ΔStat1 Reverse	CTGTACTTTCAGCCAAGATGCCCAAGCCCT	Fragment 6 Anchor	GCTGTCAGCACATCCCTATT
-3.7 ΔStat2 Forward	ATCTTGCCAGATGGCACAGAAGTTGACCT G	Fragment 8 Anchor	CACCATGCTTGTGGTATGGA
-3.7 ΔStat2 Reverse	CAGGTCAACTTCTGTGCCATCTGGCAAGAT	Fragment 9 Anchor	CTCTGGTTTGTGGTGGAGTAG
-3.7 ΔStat1ΔStat2 Forward	AGGGCTTGGGCATCTACAGAAGTTGACCT G	Fragment 1 Reverse	TGACCTTGAACCTGCCATATT
-3.7 ΔStat1ΔStat2 Reverse	CAGGTCAACTTCTGTAGATGCCCAAGCCCT	Fragment 2 Reverse	ACCCTGATCTCAGTTAAGCAAG
-3.7 ΔControl Forward	GGACAGGATTCCCATCCCTCCTAAAGCTTG TCCTCAA	Fragment 3 Reverse	CCAATGTCCCTTCACACTGA
-3.7 ΔControl Reverse	TTGAGGACAAGCTTTAGGAGGGATGGGA ATCCTGTCC	Fragment 4 Reverse	CGGAGAAAGTGAACCTGTTTAGA
+17.1 ΔNFAT Forward	GGCAGAGCGAGCGGAAATGAGTTCAGGG AG	Fragment 5 Reverse	CAGGGCAGAACAGAGAGTTT
+17.1 ΔNFAT Reverse	CTCCCTGAACTCATTTCCGCTCGCTCTGCC	Fragment 6 Reverse	GAAGAGGAGACTGCTACTGAAG
+17.1 ΔStat Forward	GGAGGCGCAGCTGCTGCTTTACCGGATGT GAAGTGCT	Fragment 7 Reverse	AGGTCCCTCACCTTCTACC
+17.1 ΔStat Reverse	AGCACTTCACATCCGGTAAAGCAGCAGCT GCGCCTCC	Fragment 8 Reverse	TGAGACCCAGCGCCTAT
+17.1 ΔControl Forward	CTCTCTCTGTTCTTATAGGGGCTGCCACTA C	Fragment 9 Reverse	TGTTCCTCCTCCCACTTGA
+17.1 ΔControl Reverse	GTAGTGGCAGCCCCTATAAGAACAGAGAG AG	Fragment 10 Reverse	TAGGTAATCATGCCTGCTAAGG

RT-PCR Primers

PD-1 Forward	CGTCCCTCAGTCAAGAGGAG	18S Forward	GTAACCCGTTGAACCCCATT
PD-1 Reverse	GTCCCTAGAAGTGCCCAACA	18S Reverse	CCATCCAATCGGTAGTAGCCG

Restriction Enzyme Accessibility

Site 1 Forward	TCTTCGTGTCTTGTGTGTTAGT	Site 9 Forward	CCTGTCAAGGCTAGGTGATTAG
Site 1 Reverse	GTGACAGGGCAGGCAAATA	Site 9 Reverse	GGGAGGAAAGGAGAAAGTAAGG
Site 2 Forward	TGTGGGCAAAGCCTGTAAT	Site 10 Forward	CATAGAGAAAGGCCAAGGATACC
Site 2 Reverse	GGCAGCTAAGATCGCAGAATA	Site 10 Reverse	AGGGTGGCTTCTAGGTATGT
Site 3 Forward	TCACCCAGTAAACCAACTTCTC	Site 11 Forward	GGAGGGAATTCTACCCGATTAC
Site 3 Reverse	CCTCAGCATGTGTCTGCTATT	Site 11 Reverse	TGAGAACATCAAGGAGGGAAAG
Site 4 Forward	AGTAAGACCTGTGGAGGATACA	Site 12 Forward	TGCCCACGTAGGTTCTACTA
Site 4 Reverse	ACCAGAGGTAGTGTGAAAGAAAG	Site 12 Reverse	AGTGTCAGAGGGAGCAAATG
Site 5 Forward	AAACTTTGTGAGCTGCTGTTT	Site 13 Forward	ACCGTGAGCAGCACTCTGAT
Site 5 Reverse	GTTTCTTCTGCCTCTCTGTGT	Site 13 Reverse	GTCTGGTCGCACAGCTCATA
Site 6 Forward	TCTACCTCTCTTCAGCTCACTT	Site 14 Forward	TGGAGAGGAGAGAGCAAAGG
Site 6 Reverse	GGTGGAGAAACCAAGGGAATAG	Site 14 Reverse	TCAACGGGTCTCTGAACCTC
Site 7 Forward	GGTGCATAGGACTTGGATTGA	Site 15 Forward	GTCAGTGAGGACACTCAACAG
Site 7 Reverse	TACTGGGCTCTGTGGAATAGA	Site 15 Reverse	GCTATGAGCTTGTTGGGAAATG
Site 8 Forward	GTTCTGAGGTTGAAACAGGAAATG	Site 16 Forward	CCAGCCAGAGAACAGATGAATA
Site 8 Reverse	GAAACAGGAGTTAGGGATCACAT	Site 16 Reverse	TCTCTTCCCAGAAACCATTCAC

Chapter 3. Dynamic DNA Methylation Of PD-1 Regulatory Regions

James W. Austin, Alexander P.R. Bally, Benjamin A. Youngblood, Kenneth J. Oestreich, Alessandra Noto, Filippos Porichis, Karen Fortner, Ralph Budd, Rafi Ahmed, Rafick-Pierre Sékaly, Daniel E. Kaufmann, and Jeremy M. Boss

Bisulfite sequencing presented in Figure 3-1 was performed by BAY.

Bisulfite sequencing presented in Figure 3-2 was performed by JWA and BAY.

Bisulfite sequencing presented in Figures 3-3, 3-4, 3-5, and 3-6 was performed by JWA.

DNase I hypersensitivity assays were performed by JWA and KJO.

HIV-specific T cell isolations were performed by AN and FP

LCMV infections and LCMV-specific T cell isolations were performed by APRB and BAY.

Cell isolation and flow cytometry from lpr mice was performed by KF.

This chapter was written by JWA.

This chapter contains data from multiple published papers. The paper containing the original figure is cited in the figure legend.

Abstract

DNA methylation has been shown to regulate multiple immune response genes. PD-1 is a negative regulator of T cell function that is epigenetically regulated by histone modifications; however, the role of DNA methylation in PD-1 regulation has not been established. Here, DNA methylation of multiple regulatory regions of PD-1 is shown to negatively correlate with PD-1 expression during acute viral infection, but in chronic viral settings the PD-1 gene is irreversibly demethylated. Through the use of conditional knockout mice, two factors, Blimp-1 and LSD1, are shown regulate the remethylation of the PD-1 regulatory regions following acute viral clearance. Loss of the histone demethylase LSD1 delays remethylation of PD-1 in the late effector stage, while Blimp-1 is critical for the proper remethylation of memory CD8 T cells following acute viral clearance. Using the lpr mouse system, we show that double negative (DN) T cells express high levels of PD-1 without a complete loss of DNA methylation at a *cis*-regulatory region. Together this work indicates that the epigenetic program of virus-specific CD8 T cells is different in acute and chronic infection. In addition, it establishes that the epigenetic program is stably inherited irrespective of antigen levels.

Introduction

DNA methylation plays a crucial role in X-chromosome inactivation, genomic imprinting, transposable element silencing, and maintenance of pluripotency (Bird, 2002; Goll and Bestor, 2005; Howard et al., 2008). In cancer, aberrant DNA methylation, including global and oncogene hypomethylation as well as hypermethylation of tumor suppressors, has been well established (Baylin and Jones, 2011; Portela and Esteller, 2010; Robertson, 2005). In the immune system, dynamic DNA methylation has been shown to regulate genes in multiple cell types. For instance, DC-SIGN (CD209), the gene encoding an important

protein for establishing DC-T cell interactions, is demethylated during differentiation (Bullwinkel et al., 2011). During Th2 differentiation, DNA methylation has been shown to directly inhibit IFN-γ expression (Jones and Chen, 2006), whereas during Th1 differentiation the IFN-γ locus is demethylated, correlating with gene activation (Schoenborn et al., 2007). A hallmark of Tregs is the expression of Foxp3. A necessary regulatory region for maintenance of Foxp3 expression, the Treg cell-specific demethylation region (TSDR), is only demethylated in Tregs and plays a role in activating Foxp3 expression (Baron et al., 2007; Lal et al., 2009). Recently, the global pattern of DNA methylation of CD8 T cells during LCMV infection was determined (Scharer et al., 2013). This study showed that dynamic DNA methylation at promoters and *cis*-regulatory regions inversely correlated with gene expression, further supporting the role for DNA methylation in regulating CD8 T cell gene expression.

DNA methylation is deposited onto cytosines at CpG dinucleotides by the DNA methyltransferases (DNMTs). There are currently 5 known DNMTs: DNMT1, 2, 3a, 3b, and 3L. DNMT2, also known as tRNA aspartic acid methyltransferase 1 (TRDMT1), was shown to methylate tRNA and not DNA (Goll et al., 2006). DNMT1 is mostly associated with the maintenance of DNA methylation during DNA replication. Through an interaction with ubiquitin-like with PHD and ring finger domains 1 (UHRF1, known as Np95 in mice), DNMT1 is targeted to the replication fork during S phase where it methylates hemimethylated CpG dinucleotides (Avvakumov et al., 2008; Sharif et al., 2007). Although DNMT1 has a preference for hemimethylated DNA, it has also been connected to *de novo* methylation of single stranded targets through interactions with DNMT3a (Christman et al., 1995; Fatemi et al., 2002; Jair et al., 2006). The DNMT3 family, responsible for most *de novo* methylation, contains two members with catalytic activity (DNMT3a and 3b) and one

without catalytic activity (DNMT3L). There is no preference of DNMT3a or 3b for hemimethylated versus unmethylated DNA (Gowher and Jeltsch, 2001; Okano et al., 1998); however, studies suggest that DNMT3a and 3b have sequence preferences (Handa and Jeltsch, 2005; Okano et al., 1999). While DNMT3L has no catalytic activity on its own, it is vital for proper *de novo* methylation by acting as a cofactor for DNMT3a and 3b (Chedin et al., 2002; Chen et al., 2005; Suetake et al., 2004). The DMNT3 family has been shown to have some maintenance DNA methylation activity at heterochromatic regions (Chen et al., 2003a; Kim et al., 2002; Liang et al., 2002).

DNMT1 prefers hemimethylated DNA, allowing for a template-dependent DNA methylation mechanism. In contrast, *de novo* DNA methylation by DNMT3a or 3b requires a different targeting mechanism. This is accomplished by recognition of histone H3K4 by DNMT3L and the lysine's modification state. Binding of the DNMT3s is disrupted by tri- (me3) or di-methylation (me2) of H3K4, with even H3K4me1 drastically reducing DNMT3L's binding affinity (Hu et al., 2009; Ooi et al., 2007; Otani et al., 2009; Zhang et al., 2010). Genome-wide studies have shown that a lack of H3K4 methylation correlates with DNA methylation (Edwards et al., 2010; Meissner et al., 2008). In addition, the H3K4 demethylase lysine-specific demethylase 1B (KDM1B) is necessary for proper formation of maternally imprinted DNA methyl marks (Ciccone et al., 2009). Together this establishes the role of H3K4 methylation in regulating *de novo* DNA methylation. At least two enzymes are required to fully demethylate H3K4me3. Members of the Jarid1 family are able to recognize H3K4me3 and demethylate the residue to the me2 state (Iwase et al., 2007). H3K4me2 is then a target for LSD1, which is able to remove one or both methyl groups (Shi et al., 2004). Thus, LSD1 is able to convert H3K4me2 to unmethylated (me0) H3K4.

Originally identified as a repressor of IFN-β expression (Keller and Maniatis, 1991), B lymphocyte-induced maturation protein-1 (Blimp-1) has subsequently been identified as the master regulator of plasma cell differentiation (Turner et al., 1994), a regulator of T cell activation and homeostasis (Kallies et al., 2006; Martins et al., 2006), and critical for macrophage differentiation (Chang et al., 2000). Recently, Blimp-1 has been shown to directly repress PD-1 by binding to a site between CR-B and CR-C (Lu et al., In press). Blimp-1 interacts with multiple histone modifying enzymes that can lead to a repressive chromatin state such as HDAC1 and 2 (Yu et al., 2000), G9a (Gyory et al., 2004), and LSD1 (Su et al., 2009). Indeed, Blimp-1 binding at the PD-1 locus correlates with a replacement of active histone marks (e.g. H3K9ac, H3K4me3, H3K14ac) with repressive marks (e.g. H3K27me3, H3K9me3) and DNA methylation (Lu et al., In press). As mentioned above, LSD1 can lead to H3K4me0, a target for DNMT3L. Therefore, Blimp-1 is hypothesized to target DNA methylation through recruitment of LSD1. If true, Blimp-1 deficiency could lead to a loss or delay in remethylation of PD-1 regulatory regions.

Identified over 20 years ago as a retrotransposon insertion mediated disruption of the *Fas* gene, the lymphoproliferation (lpr) mutation leads to accumulation of multiple types of T cells (Watanabe-Fukunaga et al., 1992). In addition to an increase in $CD44^{hi}$ $CD4^{+}$ and $CD8^{+}$ T cells, there is an increase in polyclonal $CD4^{-}$ $CD8^{-}$ TCR^{+} CD45R (B220) T cells (Cohen and Eisenberg, 1991). These $CD4^{-}$ $CD8^{-}$ cells, known as double negative cells (DN), are distinct from DN thymocytes that are developing in the thymus. For one, DN cells from lpr mice express the B cell-specific marker B220. In addition, despite being $CD4^{-}$ $CD8^{-}$, reports suggest that DN cells arise from $CD8^{+}$ T cells (Landolfi et al., 1993; Mixter et al., 1995; Takahashi et al., 1994). Interestingly, there seems to be little to no defect in negative selection of developing thymocytes in lpr mice, suggesting that the lymphadenopathy is not

due to an increase in thymic output (Kotzin et al., 1988; Mountz et al., 1990; Singer et al., 1989). Recent work by Fortner et al. has suggested that the increase in DN cells is due to a failure of Fas mediated death of $CD8^{+}$ T cells undergoing chronic TCR stimulation during homeostatic proliferation (Fortner et al., 2010; Fortner and Budd, 2005). Why CD8 expression is lost is still not understood. DN cells proliferate poorly and do not produce cytokines *ex vivo*, but their *in vivo* functionality is still not well characterized (Davidson et al., 1991). Despite this lack of effector function, NFATc1, an activator of multiple effector genes (Campbell et al., 1996; Rooney et al., 1995; Weiss et al., 1996), is overexpressed in DN cells in both the cytoplasm and nucleus (Kyttaris et al., 2011).

This chapter will explore various aspects of PD-1 DNA methylation that I have been involved with. The experiments presented here have contributed to multiple studies of PD-1, some of which have been published. The papers containing the published figures are referenced in the figure legends and in the text.

Materials And Methods

Cells and Culture

The murine T lymphoma EL4 and B sarcoma A20 cell lines were obtained from the American Type Culture Collection. Both cell lines were grown in RPMI 1640 supplemented with 5% bovine calf serum (Thermo Fisher Scientific Inc., Waltham, MA), 5% fetal bovine serum (Sigma-Aldrich Co, St. Louis, MO), 4.5 g/L glucose, 10mM HEPES, 1mM sodium pyruvate, and 100 U/ml penicillin/streptomycin.

Murine Cell Isolation

For experiments using WT cells, recipient C57BL/6 mice were injected with LCMV-specific

P14 transgenic CD8 T cells and subsequently infected with LCMV Armstrong at 2 x 10^5 pfu or clone 13 at 2 x 10^6 pfu (Matloubian et al., 1990; Wherry et al., 2003). Chronically infected (clone 13) mice were also CD4 depleted using 500 μg of the anti-CD4 GK1.5 antibody that was injected the day prior and day of infection. Thy1.1$^+$ P14 cells were FACS sorted from mouse spleens at day 4 (D4) and day 8 (D8) post infection as previously described (Tolhuis et al., 2002). Naïve P14 cells were used as a control to compare D4 and D8 effector cells.

Blimp-1$^{fl/fl}$ (Rutishauser et al., 2009) and LSD-1$^{fl/fl}$ (Kerenyi et al., 2013) mice were bred with the GranzymeB (GzmB)-*cre* transgenic mouse (Jacob and Baltimore, 1999) to generate conditional knockout mice (cKO). Blimp-1 and LSD1 cKO mice were infected with LCMV Armstrong at 2 x 10^5 pfu. Cells were isolated at the indicated day by the following procedure. A single cell suspension of whole splenocytes was enriched for total CD8 T cells by MACS (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. Following enrichment, cells were FACS sorted via the H2b-gp33 tetramer to obtain LCMV-specific cells. Naïve CD8 T cells and LCMV-specific cells from GzmB-*cre*$^-$ littermates were used as controls. Experiments with C57BL/6, P14 transgenic, Blimp-1$^{fl/fl}$, and LSD-1$^{fl/fl}$ mice were conducted in accordance with approved Emory University institutional animal care and use protocols.

In collaboration with Ralph Budd's group, isolation and characterization of DN cells from *lpr* mice was performed as previously described (Fortner et al., 2010; Fortner and Budd, 2005). DN cells (CD4$^-$ CD8$^-$ TCRβ$^+$) and naïve CD8 T cells (CD8$^+$ CD44^{lo}) were FACS sorted from a combined preparation of splenic and lymph node cells. Anti-CD4, anti-CD8, and anti-CD44 antibodies were purchased from Invitrogen/Caltag Laboratories (Carlsbad, CA). Anti-TCRβ antibody was purchased from BD Biosciences (San Jose, CA). Following isolation, cell pellets were shipped to Emory for bisulfite sequencing analysis. Experiments

conducted with C57BL/6 *lpr* mice were conducted in accordance with the University of Vermont's Animal Care and Use Committee.

Isolation of HIV-specific cells

Total CD8 T cells were isolated from PBMCs by magnetic bead separation (Stem Cell Technologies Inc., Vancouver, BC, Canada) according to manufacture's instructions. CD8 T cells were then FACS sorted for naïve ($CD8^{+}CD45RA^{+}CCR7^{+}CD27^{+}$) and HIV-specific ($CD8^{+}CD3^{+}tetramer^{+}$) cells on a BD ARIAII flow cytometer (BD Biosciences). Antibodies for CD3, CD8, CD45RA, and CCR7 were purchased from BD Biosciences. CD27 was purchased from Life Technologies Co (Grand Island, NY). This research followed the ethical guidelines established by the ethics committee of Massachusetts General Hospital, University of Montreal Health Center, and Vaccine and Gene Therapy Institute Florida Institutional Review Board. The University of Montreal Health Center Ethics Review Board and Partners Human Research Committee of the Massachusetts General Hospital received written, informed consent from study participants before enrollment in the study.

Bisulfite Sequencing

The protocol for bisulfite sequencing was adapted from Youngblood et al. (Youngblood et al., 2011). Briefly, genomic DNA from virus-specific, DN T cells, or naïve CD8 T cells was isolated and bisulfite converted using the EpiTecht Bisulfite Kit (Qiagen Sciences Inc., Germantown, MD). Bisulfite converted DNA was amplified via PCR and cloned using the TOPO TA cloning kit (Life Technologies). Primers used for cloning are listed in Table 3-1. Individual colonies were selected and sequenced. Following compilation of the data, a Fischer's exact test was used to determine the statistical significance between samples.

DNase I Hypersensitivity

PCR based DNase I hypersensitivity assays were adapted from those previously described (Oestreich et al., 2008). P14 cells were adoptively transferred into recipient C57BL/6 mice as described above. At the days indicated, 5 x 10^5 LCMV-specific cells were isolated by streptavidin magnetic bead separation (Miltenyi Biotech) after incubation with a Thy1.1-biotin antibody (BD Biosciences). Cells were then resuspended in DNase I buffer (10 mM HEPES pH 8.0, 50 mM KCl, 5 mM MgCl2, 3 mM CaCl2, 0.1% NP40, 8%glycerol, 1 mM DTT) and incubated on ice for 5 min. Following incubation, 5 units of DNase I (Worthington Biochemical Co, Lakewood, NJ) was added and samples incubated for 3 min at room temperature. DNase I stop buffer (20 mM EGTA and 1% SDS) was then added to quench the reaction. Samples were phenol-chloroform extracted and ethanol precipitated. PCR was then performed using the locus-specific primers listed in Table 3-1.

Results

DNA methylation at CR-B and CR-C is altered during LCMV viral infection

The CR-B and CR-C regulatory regions were originally discovered through DNase I hypersensitivity assays using the PD-1 expressing EL4 and non-PD-1 expressing A20 cell lines (Oestreich et al., 2008). CR-B and CR-C were found to be sensitive to DNase I only in EL4 cells. In addition, the active histone marks H3K9ac, H3K18ac, and H3K4me3 were present in EL4 but not A20 cells. Differential DNase I sensitivity and histone modifications support the hypothesis that PD-1 is epigenetically regulated. To test if another epigenetic regulatory mechanism, DNA methylation, also regulates PD-1, A20 and EL4 cells were bisulfite sequenced. As expected, EL4 cells were almost completely unmethylated (Figure 3-

1A). In contrast, A20 cells were extensively methylated at CR-B, and partially methylated at CR-C.

To ascertain if DNA methylation plays a role in PD-1 regulation *in vivo*, the DNA methylation status at CR-B and CR-C was determined in CD8 T cells from LCMV infected mice. CD8 T cells from P14 TCR transgenic mice were isolated and adoptively transferred into recipient C57Bl/6 mice. Following adoptive transfer, recipient mice were infected with either acute (Armstrong) or chronic (clone 13) strains of LCMV. Adoptively transferred P14 cells were isolated at various days and DNA methylation was determined by bisulfite sequencing. Naïve CD8 T cells isolated from uninfected P14 mice contained extensive DNA methylation at CR-B and partial methylation at CR-C (Figure 3-1B). During acute LCMV infection, DNA methylation at both regions was lost by day 4, a time when PD-1 was highly expressed. As PD-1 expression decreased (day 8), DNA methylation increased. Memory cells formed following viral clearance contained comparable levels of DNA methylation as naïve cells at CR-B. Unlike CR-B, CR-C was less methylated in memory cells compared to naïve cells. During chronic viral infection, DNA methylation was lost and did not return. These observations suggest a possible mechanism for sustained PD-1 levels during chronic infections.

DNase I hypersensitivity of LCMV-specific cells is indicative of PD-1 expression

Previously, it was shown that DNase I hypersensitivity of the A20 and EL4 cell lines correlated with PD-1 expression (Oestreich et al., 2008). To determine the *in vivo* DNase I sensitivity of these regions, the same P14 adoptive transfer system described above was utilized. Naïve CD8 T cells have a low basal level of DNase I sensitivity that was significantly increased following LCMV infection at CR-C but not CR-B (Figure 3-1C).

DNase I sensitivity decreased as the acute infection was resolved (day 8), returning to naïve levels in memory cells. DNase I sensitivity was increased in LCMV-specific CD8 T cells 9 days post chronic infection, correlating with high PD-1 expression. With the DNA methylation data from above, this suggests that the epigenetic modifications at CR-B and CR-C contribute to the regulation of PD-1 *in vivo*. In addition, the differences in DNA methylation and DNase I sensitivity between LCMV-specific cells in acute and chronic infection suggest a possible mechanism for PD-1 overexpression during chronic infection, that is, the failure to return chromatin to the naïve state. The bisulfite sequencing and DNase I hypersensitivity of LCMV-specific CD8 T cells was published in collaboration with the Ahmed group in Immunity in 2011 (Youngblood et al., 2011).

Loss of DNA methylation is irrespective of PD-1 expression during HIV infection

PD-1 expression on HIV-specific T cells correlates with disease progression and functional exhaustion (Day et al., 2006; Trautmann et al., 2006; Zhang et al., 2007). To determine if the epigenetic regulation of PD-1 is similar between HIV and chronic LCMV infection, the DNA methylation status of the putative CR-C regulatory region of human PD-1 was bisulfite sequenced (Figure 3-2A). Consistent with the previous studies in mice, naïve CD8 T cells contained a partially methylated regulatory region (Figure 3-2B). Irrespective of the stage of infection (i.e. acute vs. chronic), HIV-specific T cells lacked DNA methylation, which was also consistent with the murine studies of chronic infection.

Persistent TCR stimulation is known to contribute to the overexpression of PD-1 during chronic viral infection (Barber et al., 2006; Wherry et al., 2007). Following highly active antiretroviral therapy (HAART) treatment, viral loads of HIV can decrease to undetectable levels (<50 copies per mL), thereby attenuating TCR stimulation. Therefore,

we wanted to know if HAART treatment led to a change in the epigenetic state of PD-1. Interestingly, HIV-specific CD8 T cells maintain their demethylated state even after extended periods of viral control through HAART (Figure 3-2C). A population of HIV patients, known as elite controllers, initially has increased viremia, but soon after infection, viral loads are controlled even in the absence of anti-retroviral therapy (Miura et al., 2010). PD-1 expression on HIV-specific CD8 T cells from elite controllers is significantly lower than HIV progressors, although PD-1 expression is still significantly increased compared to bulk CD8 T cells (Salisch et al., 2010; Zhang et al., 2007). Analysis of HIV-specific CD8 T cells from elite controllers revealed the same pattern as before, where DNA methylation was absent from the putative PD-1 regulatory region (Figure 3-2D). Combined, the data suggest that the DNA methylation state of PD-1 during HIV infection is set at an early point of infection, and that TCR stimulation is not necessary to maintain the demethylated state. This work was published in collaboration with the Ahmed, Sekaly, and Kaufmann labs in the Journal of Immunology in 2013 (Youngblood et al., 2013).

T cell activation leads to a decrease in DNA methylation at the -3.7 regulatory region.

As discussed above, DNA methylation at CR-B and CR-C was shown to correlate with PD-1 expression during acute infection (Youngblood et al., 2011), but a chronic infection sets up an epigenetic program of DNA methylation at the CR-B and CR-C regions that is maintained upon reduction in antigen levels (Youngblood et al., 2013). To determine if the DNA methylation state of the +17.1 and -3.7 regulatory regions is also dynamic, bisulfite sequencing of LCMV (Armstrong)-specific CD8 T cells was performed. The +17.1 region is essentially unmethylated in naïve and antigen-specific cells at day 4 or day 8 post infection

(Figure 3-3A). The -3.7 region has a small number of CpGs, which are moderately methylated in naïve CD8 T cells. A significant decrease in DNA methylation ($P<0.01$, Fisher's exact test) across the -3.7 region was observed in the LCMV-specific CD8 T cells 4 days post infection with no remethylation at day 8 post infection (Figure 3-3B). These results suggest that DNA methylation may contribute to repressing the regulatory potential of the -3.7 region prior to T cell activation. Although no remethylation was observed at day 8, it is possible that the -3.7 region could become remethylated at later timepoints such as in memory cells.

Blimp-1 and LSD-1 are required for the proper remethylation of PD-1

Blimp-1 directly represses PD-1 through a feed-forward repressive circuit, and an exchange of active for repressive histone marks is seen following Blimp-1 binding (Lu et al., In press). The exchange of repressive for active marks is seen at day 6 post LCMV Armstrong infection. At day 8 post infection PD-1 is becoming remethylated (Figure 3-1B). The timing of these events imply that Blimp-1 and/or T-bet could be critical to direct the remethylation of PD-1. Blimp-1 has been shown to interact with the H3K4 demethylase LSD-1, but there is no currently known interaction between T-bet and a chromatin-modifying enzyme that negatively regulates gene expression. To test if Blimp-1 is necessary for the remethylation of PD-1, Blimp-1 conditional knockout (cKO) mice were infected with LCMV Armstrong, virus-specific CD8 T cells isolated, and bisulfite sequencing performed. Naïve CD8 T cells from uninfected Blimp-1 cKO mice have the same pattern of DNA methylation as seen in WT B6 mice (Figure 3-4A). There was no statistical difference in the DNA methylation levels of GzmB-*cre*$^{+}$ compared to GzmB-*cre*$^{-}$ littermate controls at day 8. In contrast to the experiments at day 8, when LCMV-specific T cells were isolated 30 days post infection from

either WT or Blimp-1 cKO mice there was a significant decrease in DNA methylation at CR-B but not CR-C in Blimp-1 cKO mice (p= 2.2e-16) (Figure 3-4B).

As described above, Blimp-1 has been shown to interact with the H3K4 demethylase LSD1 (Shi et al., 2004; Su et al., 2009). LSD1 is capable of demethylating the lysine residue to an unmethylated state, which is required for recruitment of DNMT3L and subsequent de novo methylation (Hu et al., 2009; Otani et al., 2009; Zhang et al., 2010). To determine if LSD1 is necessary for the proper remethylation of PD-1, LSD1 cKO mice were infected with LCMV and virus-specific CD8 T cells were isolated 8 days post infection. As in the Blimp-1 cKO mice, naïve CD8 T cells from uninfected animals have similar levels of DNA methylation as WT B6 animals (Figure 3-5A). LSD1$^{fl/fl}$;GzmB-*cre*$^{+}$ animals have significantly less methylation at CR-B than GzmB-*cre*$^{-}$ littermates (p= 3.64e-07). There was no difference in methylation at CR-C. Using the same setup, LCMV-specific CD8 T cells were isolated at days 8 and 10 post infection. Once again, LSD1$^{fl/fl}$;GzmB-*cre*$^{+}$ mice have less DNA methylation at day 8 compared to GzmB-*cre*$^{-}$ littermates (Figure 3-5B). However, at day 10 there was no statistical difference in the amount of DNA methylation at CR-B between GzmB-*cre*$^{+}$ and GzmB-*cre*$^{-}$ littermates. Although loss of LSD1 delayed remethylation of the PD-1 locus, CR-B is eventually remethylated, which could indicate redundant H3K4 demethylation pathways. Studies using the two strains of cKO mice imply that both Blimp-1 and LSD1 contribute to the remethylation of PD-1 following acute viral infection, but are not essential.

A change in DNA methylation patterns accompanies increased PD-1 expression in DN T cells from lpr mice

DN T cells in *lpr* mice are thought to arise from CD8 T cells that are stimulated through their TCR to undergo homeostatic proliferation (Fortner et al., 2010; Fortner and Budd, 2005). This suggests that some activation-induced genes, such as PD-1, may become active. Indeed, DN T cells had high surface expression of PD-1 compared to naïve (CD44lo) memory (CD44int) or activated (CD44hi) CD8 T cells (Figure 3-6A). To determine if the increase in PD-1 expression is accompanied by a change in the chromatin structure of the locus, the DNA methylation status of CR-B and CR-C was assayed. Both CR-B ($p< 2.2e-16$) and CR-C ($p= 2.924e-07$) were less methylated in DN T cells than naïve CD8 T cells (Figure 3-6B). Interestingly, although PD-1 was expressed at high levels in DN T cells, CR-B maintained substantial amounts of methylation with a pattern that resembled a late effector stage (Figure 3-1B).

Discussion

DNA methylation is an important negative epigenetic regulatory mechanism. Consistent with this, there is a strong inverse correlation between PD-1 expression and DNA methylation during acute LCMV infection. The timepoints collected during this work represent naïve, early effector, late effector, and memory T cell stages. Naïve cells contain the most DNA methylation while day 4 virus-specific cells lack almost all methylation. How DNA methylation is lost during this timeframe is not understood. T cells undergo a 10,000 fold expansion following activation, allowing for the possibility of a passive loss of methylation. However, in studies of IFN-γ expression upon restimulation of memory T cells, DNA methylation was lost prior to cell division, suggesting an active mechanism (Kersh et al., 2006). An active process of DNA demethylation likely occurs through a process involving the formation of hydroxymethylcytosine from methylcytosine by the Tet enzymes

(Shen and Zhang, 2013). Hydroxymethylcytosine can then be converted into formylcytosine and subsequently carboxylcytosine (He et al., 2011; Ito et al., 2011; Maiti and Drohat, 2011). Formylcytosine and carboxylcytosine are targets for thymine DNA glycosylase (TDG). Removal of formyl- and caroxylcytosines by TDG creates an abasic site, which can be repaired through the base excision repair pathway, resulting in an unmodified cytosine base. Determining if PD-1 is demethylated through an active or passive process is worth exploring. An active mechanism would present targets for molecular therapies designed to inhibit the demethylation process, thereby inhibiting PD-1 expression.

Binding of DNMT3L requires an unmethylated H3K4 residue (Hu et al., 2009; Otani et al., 2009; Zhang et al., 2010). Like most genes, PD-1 expression is accompanied by H3K4me3 (Oestreich et al., 2008), therefore the lysine needs to be demethylated before DNA methylation can occur. The H3K4 demethylase LSD1 has been shown to interact with PD-1 repressor Blimp-1, implying LSD1 and Blimp-1 could be critical for remethylation of PD-1 (Lu et al., In press; Shi et al., 2004; Su et al., 2009). Conditional deletion of LSD1 decreased the rate of remethylation, but did not abolish the ability of the region to be remethylated. Loss of Blimp-1 had no effect on methylation levels at day 8, however at day 30, Blimp-1-deficient cells contain less DNA methylation at CR-B than Blimp-1-sufficient cells. Neither Blimp-1 nor LSD1 deletion led to a complete inability to remethylate. Clones displaying a methylated phenotype could represent cells that did not efficiently delete their target gene. Alternatively, another repressor of PD-1 could recruit histone-modifying enzymes, allowing for some remethylation of the locus. One possible factor is T-bet, the only other known repressor of PD-1 expression, although no molecular interaction of T-bet with LSD-1 or correlation with DNA methylation has been found to date. Another possibility is the dilution of H3K4me3 through replacement of histones containing

unmodified H3K4 residues following DNA replication during cell division. Conditional deletion of both Blimp-1 and LSD1 in the same mouse may result in a more severe phenotype and is worth further study.

Blimp-1^{lo} memory cells respond faster and proliferate to a greater extent than Blimp-1^{hi} memory cells (Rutishauser et al., 2009). In addition to increased recall responses, effector CD8 T cells lacking Blimp-1 have increased proliferative capacity, polyfunctionality, and differentiate into central memory cells in greater numbers. The lack of Blimp-1 maintains the PD-1 locus in an active chromatin state during acute LCMV infection (Lu et al., In press), and as presented here, Blimp-1 deletion led to a loss of methylation in memory CD8 T cells. This could set up a heritable chromatin architecture that allows for a more rapid PD-1 response following restimulation. PD-1 expression was not determined following restimulation of Blimp-1-deficient memory T cells in this study. Also not determined was the methylation status of LSD1-deficient memory CD8 T cells. It will be important to establish if loss of LSD1 leads to a defect in memory cell formation or in the DNA methylation pattern of memory cells, thereby suggesting a change in the epigenetic program of PD-1.

During chronic viral infections, DNA methylation is lost and does not return, even at late stages of infection. PD-1 and DNA methylation are inversely correlated during acute viral infection, but during HIV infection the inverse correlation is lost. Long-term progressor T cells express high levels of PD-1 on their surface, thus it is unsurprising that CR-C is demethylated. In contrast, HIV-specific T cells from patients on HAART or elite controllers still lack DNA methylation despite low viral loads. This uncoupling of the inverse relationship between DNA methylation and PD-1 expression says that the epigenetic state of the locus is set early in infection and antigen is not needed to maintain the active state. How lower PD-1 expression is maintained despite the lack of epigenetic silencing following

HAART treatment or in elite controllers is not understood. Two known repressors of PD-1 in mouse, Blimp-1 and T-bet, have not been examined in HIV, thus Blimp-1 and T-bet could be mediating the repression of PD-1 in low viral load settings. Alternatively, the activator NFATc1 may not be bound to CR-C in low expressing cells, but present in high expressing cells. The histone modifications associated with PD-1 have not been studied in HIV-specific cells. It is possible that the putative distal regulatory regions that bind the STAT factors may be active in high expressing cells, but inactive in low expressing cells.

DN T cells from *lpr* mice have increased PD-1 expression and less DNA methylation than naïve CD8 T cells. These cells are thought to arise due to weak TCR stimulation of CD8 T cells delivered during homeostatic proliferation (Fortner et al., 2010; Fortner and Budd, 2005). TCR stimulation activates the calcium-signaling pathway, leading to NFAT translocation and expression of target genes. In agreement with the hypothesis that TCR signaling induces DN cell formation, NFAT is activated in DN T cells (Kyttaris et al., 2011). Activation of NFAT is the likely cause of increased PD-1 in these cells. Interestingly, although DN cells are less methylated than naïve CD8 T cells, there is still a substantial amount of methylation present. DN T cells proliferate poorly and lack expression of many effector genes (Davidson et al., 1991). This suggests that NFAT is able to induce high levels of PD-1 but other factors that aren't induced/recruited may be necessary to change the chromatin architecture. Another possibility is that passive loss of DNA methylation does not occur due to the lack of proliferation.

Combined, these studies of DNA methylation provide insight into the chromatin architecture of the PD-1 locus during the immune response. There is a clear difference in the epigenetic state of the locus between acute and chronic infection. Further, it appears that early events in infection establish the epigenetic fate of virus-specific cells. Determining the

mechanism for this establishment is important to develop molecular therapies to reverse exhaustion. In addition to DNA methylation, further characterization of histone modifications and regulatory region accessibility is needed to fully understand the complex interaction of epigenetic factors regulating PD-1.

Figure Legends

Figure 3-1. DNA methylation at CR-B and CR-C inversely correlates with PD-1 expression. (A and B) Bisulfite sequencing of CR-B and CR-C with each line representing one clone. Open circles represent unmethylated while filled circles represent methylated CpG dinucleotides. **(A)** A20 and EL4 cells lines. **(B)** LCMV-specific CD8 T cells during acute and chronic viral infection at the day indicated. **(C)** Relative DNase I hypersensitivity of LCMV-specific CD8 T cells during acute and chronic infection. P values were calculated using the Student's T test. This data was published in a paper in collaboration with the Ahmed group in Immunity in 2011 (Youngblood et al., 2011).

Figure 3-2. HIV-specific T cells are irreversibly demethylated at CR-B and CR-C. (A) Schematic of the CR-C regulatory region of PD-1 that was assayed. Relative positions to the transcriptional start site are indicated. Primers are represented by red arrows. **(B, C, and D)** Bisulfite sequencing of the CR-C putative regulatory region. Each line denotes a clone sequenced with filled circles being methylated and open circles being unmethylated. Viral load and the HIV epitope used to sort cells is indicated. Naïve CD8 T cells were used as controls. **(B)** HIV-specific CD8 T cells from patients in the acute and chronic phase of infection. **(C)** HIV-specific CD8 T cells from patients either pre or post HAART treatment.

(D) HIV-specific CD8 T cells from an elite controller. This figure was published in a paper in the Journal of Immunology in collaboration with the Ahmed, Sekaly, and Kaufmann groups (Youngblood et al., 2013).

Figure 3-3. T cell activation leads to a decrease in DNA methylation at the -3.7 regulatory region. (A and B) Bisulfite sequencing of LCMV-specific CD8 T cells during acute LCMV infection at day 4 and day 8 post infection at **(A)** the +17.1 region and **(B)** the -3.7 region. Naïve antigen-specific cells are also shown as a control. Each line represents one clone with open circles denoting unmethylated CpG dinucleotides while filled circles represent methylated CpG dinucleotides.

Figure 3-4. Blimp-1 deficiency results in a loss of DNA methylation at CR-B following clearance of acute LCMV. (A and B) Bisulfite sequencing of the CR-B regulatory region. Filled circles represent methylated cytosines and open circles represent unmethylated cytosines. Each line is an individual clone sequenced. **(A)** LCMV-specific CD8 T cells from Blimp-1 cKO mice 8 days post LCMV Armstrong infection that either carry the GranzymeB-*cre* recombinase transgene (cre+) or cre- littermates. Naïve CD8 T cells are shown as a control. **(B)** LCMV-specific CD8 T cells from WT or Blimp-1 cKO mice 30 days post LCMV Armstrong infection. All cKO mice expressed cre recombinase.

Figure 3-5. Loss of LSD1 leads to a defect in the remethylation of LCMV-specific CD8 T cells following LCMV Armstrong infection. (A and B) Bisulfite sequencing of LCMV-specific CD8 T cells from LSD1 cKO mice. Each line represents an individual clone sequenced with open unmethylated CpGs being denoted by white circles and methylated

CpGs being denoted by black circles. Cells from mice carrying the GzmB-*cre* transgene (cre+) or those that lack the transgene (cre-) are shown from **(A)** day 8 and **(B)** day 10 post acute LCMV infection.

Figure 3-6. Loss of DNA methylation at CR-B and CR-C correlates with PD-1 expression in DN cells from lpr mice. (A) Freshly isolated wild-type and *lpr* lymph node cells were stained for CD4, CD8, TCR-β, CD44, and PD-1. Shown is the expression of PD-1 on $CD8^+$ T cells separated by CD44 expression (low, intermediate, and high) and $CD4^-CD8^-TCR\alpha\beta^+$ T cells (DN). **(B)** Bisulfite sequencing of CR-B and CR-C from naïve CD8 (CD44 low) or double negative (DN) T cells from lpr mice. Each clone sequenced is presented as a single line with methylated cytosines represented as filled circles and unmethylated cytosines represented as open circles.

Figure 3-1

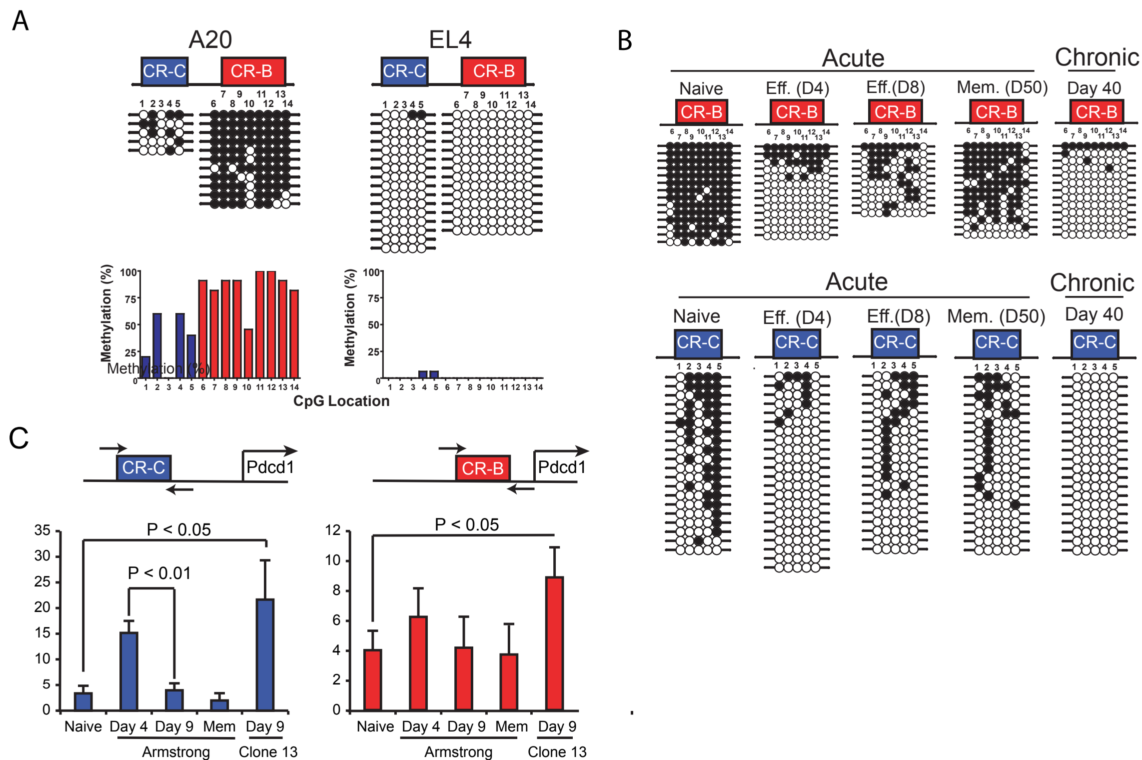


Figure 3-2

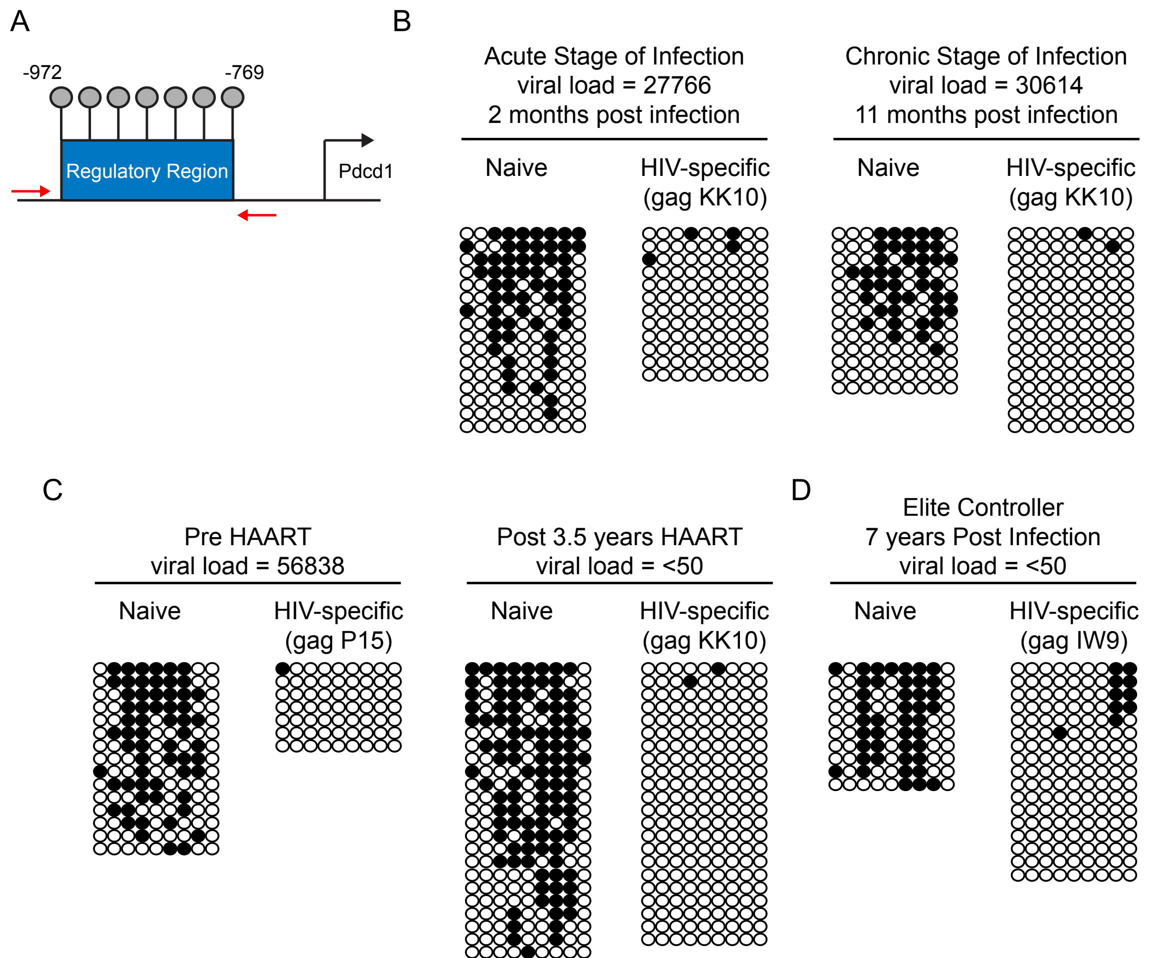


Figure 3-3

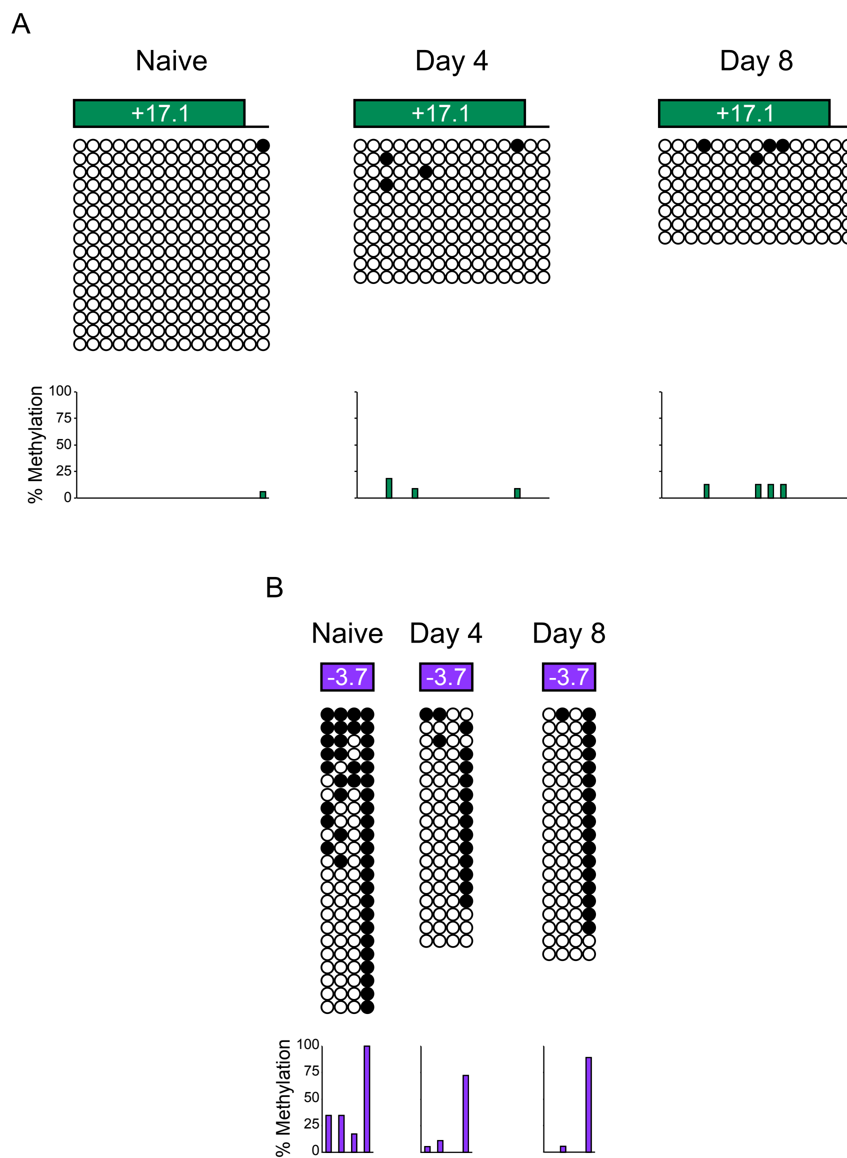


Figure 3-4

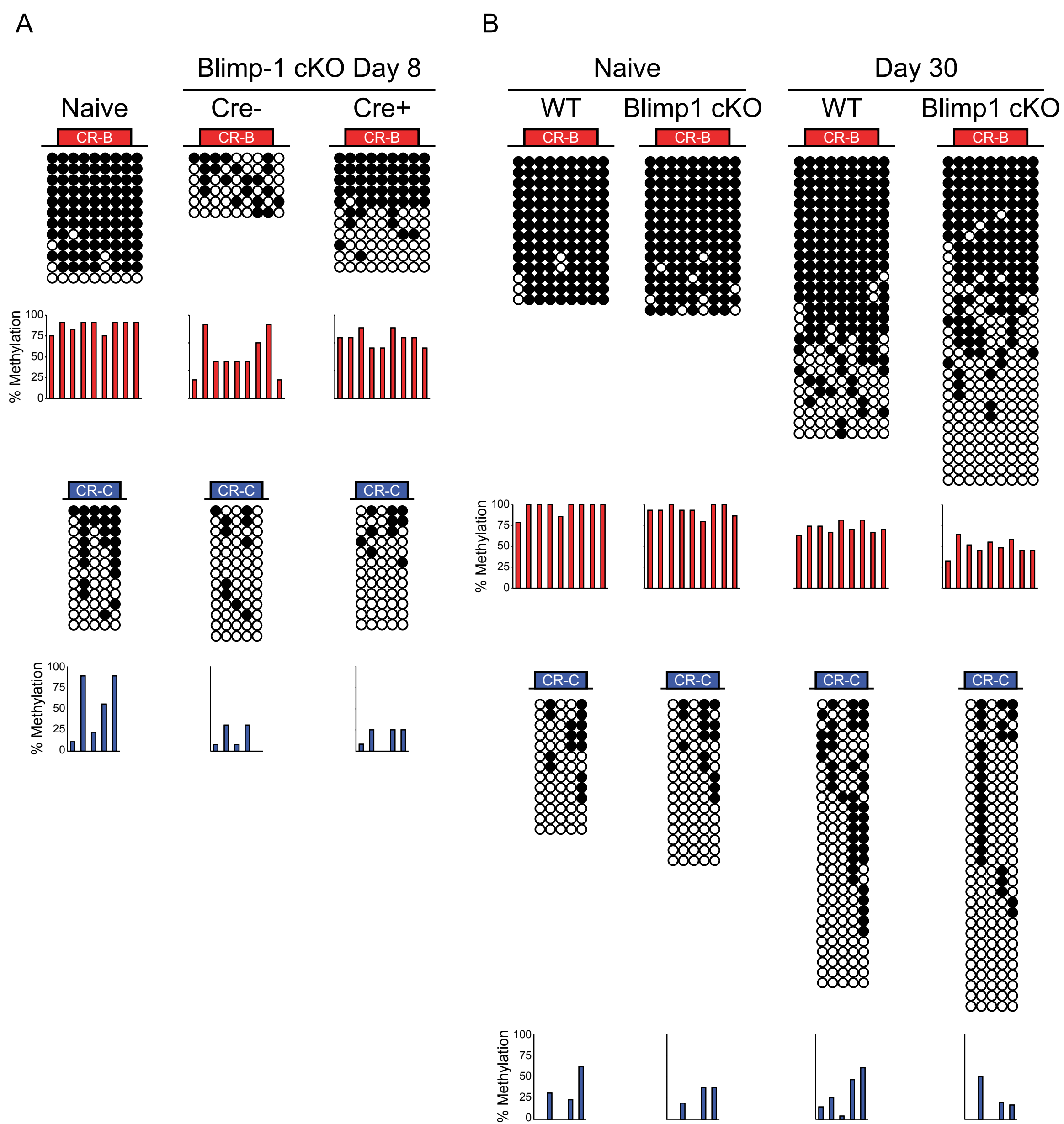


Figure 3-5

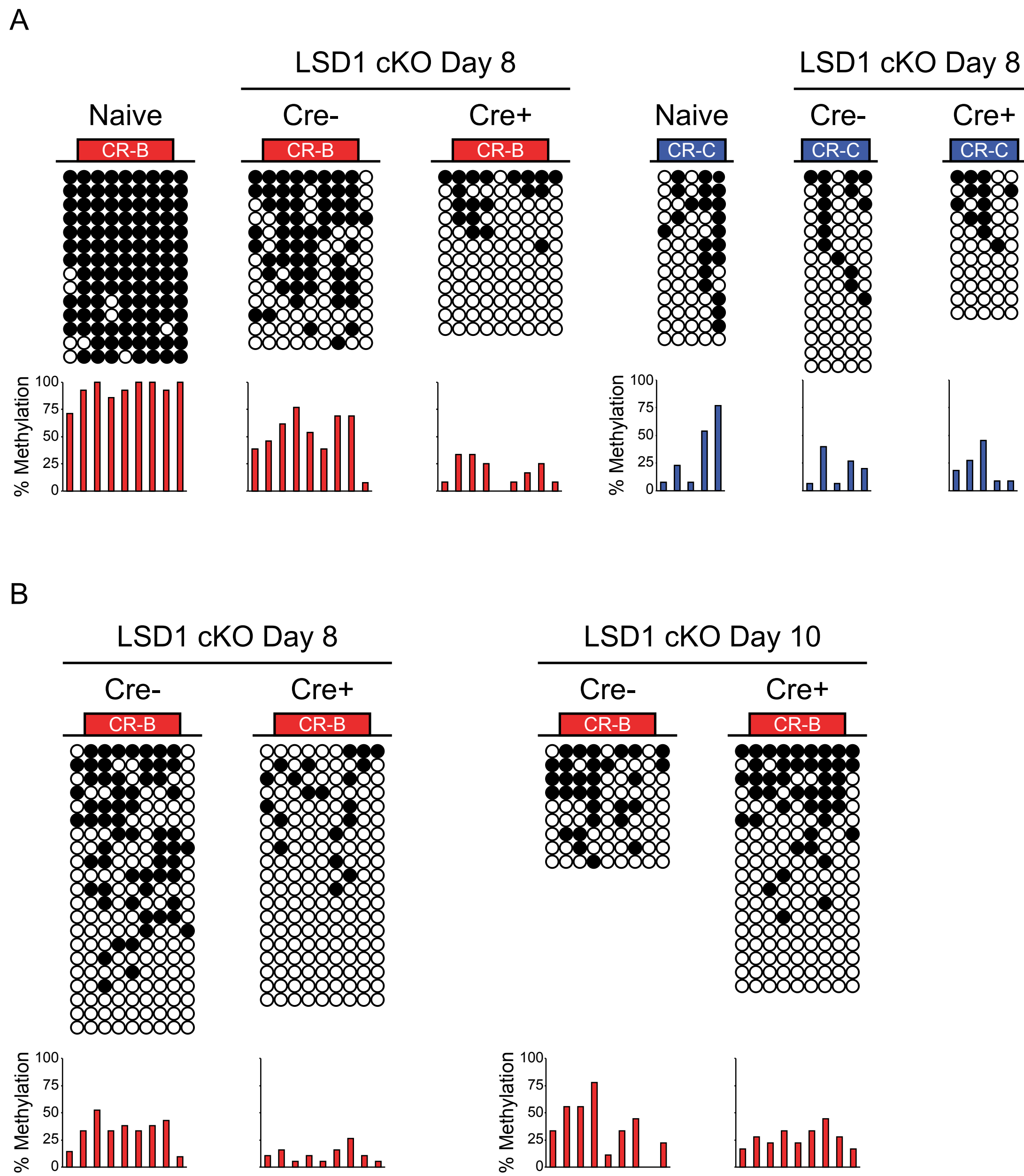


Figure 3-6

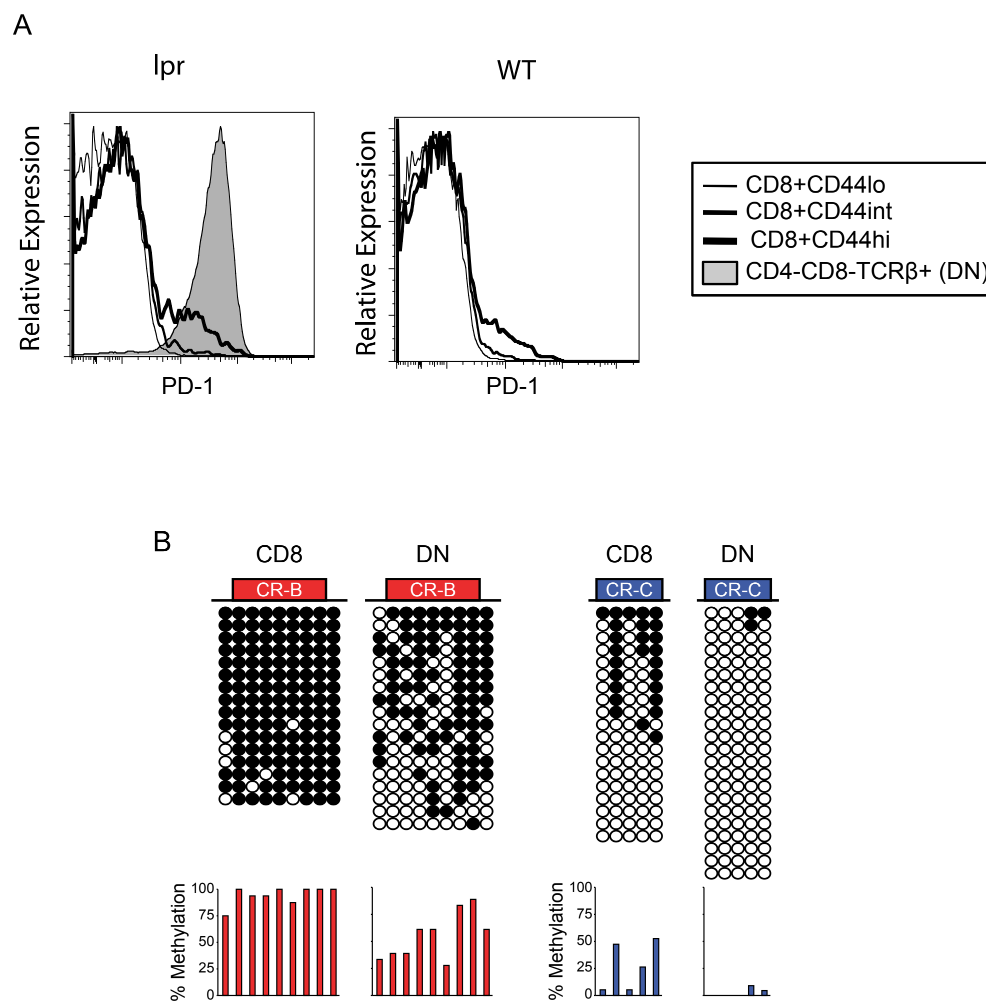


Table 3-1: Primers used in this study	
Bisulfite Sequencing Primers	
Mouse CR-B Forward	5'-GGTGGGTTTTATTTTTTAGGGATTGAGG-3'
Mouse CR-B Reverse	5'-CTAAAACTAAAACCAAACTCTTATCCC-3'
Mouse CR-C Forward	5'-ATTTTTTTAGTTTTGTTTATAGGTTTTAT-3'
Mouse CR-C Reverse	5'-TTCTCTTCCCCTAAAAAAAACCTAACACCA-3'
-3.7 Forward	5'-AAATAGGTTGAGTAAGTTATGGGGAG-3'
-3.7 Reverse	5'-TAAATAAAAACTATACCTACCAAAAACC-3'
+17.1 Forward	5'-GGTGGAGTAGTTATATTAAGAGTTGTAG-3'
+17.1 Reverse	5'-TCCACTTTAAATATACTATTACAAAAAC-3'
Human CR-C Forward	5'-AAGTTATTTATATAGTTTTATATTTTTGAG-3'
Human CR-C Reverse	5'-CACACCATAACCACAATTCCAAATCTTTCC-3'
DNase I Hypersensitivity Primers	
CR-C Forward	5'-CGACTTGTGTGCATGCATAGTACC-3'
CR-C Reverse	5'-GAGGTCCTTTCACTCTCCACG-3'
CR-B Forward	5'-GGCAGTGTCGCCTTCAGTAGC-3'
CR-B Reverse	5'-CTCGACACCCACCCTCCAAG'3'
Control Forward	5'-TAGCACATAACAGCGGTAGATTAC-3'
Control Reverse	5'-CCCAAGTTTGAGACAAGCAGAC-3'

Chapter 4. Multiple STATs Prolong PD-1 Expression In CD8 T Cells

James W. Austin, Peiyuan Lu, Benjamin G. Barwick, Christopher D. Scharer, and Jeremy M. Boss

Luciferase assays were performed by JWA

ChIP assays and qRT-PCR were performed by PL.

ChIP-seq analysis was performed by CDS and BGB.

This chapter was written by JWA.

Abstract

STAT3 and STAT4 enhance PD-1 expression from activated CD8 T cells by binding to two distal regulatory regions flanking the gene. STAT binding is induced by the appropriate cytokine signal and is not depending on TCR induction. Multiple *cis*-regulatory regions have been discovered when CD8 T cells are activated through the TCR pathway *ex vivo*; however, as STATs are able to bind in the absence of TCR signaling some regulatory elements may have been missed. Using available ChIP-seq data, multiple putative STAT binding sites were identified in differentiated, activated CD4 T cells. Validation of the putative sites determined that STAT3 bound to the previously identified -3.7 and +17.1 regulatory regions as well as a novel site, -2.7, in IL-6 stimulated CD8 T cells. STAT binding at PD-1 has only been determined during T cell activation. STATs are known to bind to the PD-1 locus and enhance PD-1 expression, but it is uknown how long the enhancement lasts. To determine if STATs play a role in PD-1 expression past the initial activation stage, multiple cytokines were used to stimulate CD8 T cells and PD-1 expression was determined over time. Two cytokines, IL-12 and IL-6 prolonged PD-1 expression in CD8 T cells. In addition, IL-6 mediated STAT3 binding at the -3.7, -2.7, and +17.1 STAT binding sites was stable over the length of the experiment. Together this data indicates that multiple cytokines are able to enhance and prolong PD-1 expression through induced STAT binding to at least three sites in the PD-1 locus.

Introduction

Cytokine driven gene expression changes are mediated through the action of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. With about 40 members, the type I and II cytokine receptors include receptors for interleukins,

interferons, and hormones (e.g. growth hormone, erythropoietin, and granulocyte-colony stimulating factor) (Boulay et al., 2003). The intracellular domains of the cytokine receptors associate with members of the JAK family, of which there are four. Jak1, Jak2, and Tyk2 bind to multiple receptors, while Jak3 binds only to the common gamma chain of the IL-2 receptor family. Upon recognition of its cognate cytokine, the cytoplasmic domain of the receptor is tyrosine phosphorylated by the associated JAKs (Shuai and Liu, 2003). The phosphorylated domains recruit proteins with Src homology 2 (SH2) domains, including members of the STAT family. The seven members of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) are tyrosine and serine phosphorylated by the JAKs. The phosphorylated STATs dimerize and subsequently translocate into the nucleus where they bind to target genes. Mutations in *Jak3* or *Stat5a* and *Stat5b* cause specific primary immunodeficiency syndromes designated severe combined immunodeficiency (SCID), while mutations in *Tyk2* lead to autosomal-recessive hyperimmunoglobulin E syndrome (AR-HIES), showing the importance of JAK-STAT signaling in the immune response (Minegishi et al., 2007; Notarangelo et al., 2001; Yao et al., 2006).

STATs are important for both development and differentiation of T cells. IL-12, an activator of STAT4, drives Th1 differentiation (Jacobson et al., 1995), whereas IL-4, an activator of STAT6, drives differentiation to Th2 (Kaplan et al., 1996). Loss of STAT6 or STAT4 leads to impaired Th1 or Th2 function, respectively (O'Garra and Arai, 2000). Despite early reports, more recent studies have shown that Th2 responses can occur in the absence of STAT6 (van Panhuys et al., 2008), suggesting that there is some redundancy in the pathways and processes. STAT5 has roles in the development of Th1, Th2, and Treg cells. STAT5 activated by IL-2 binds to the IL4 receptor alpha gene to activate expression, promoting Th2 differentiation (Liao et al., 2008). However, STAT5 can also promote Th1

differentiation by activating T-bet and IL-12 receptor beta 2 (Liao et al., 2011). STAT5a and STAT5b are required for Treg differentiation (Yao et al., 2007a) by inducing Foxp3, the master regulatory of Treg differentiation (Burchill et al., 2008).

IL-6 activation of STAT3 is necessary for the formation of Th17 cells (McGeachy and Cua, 2008), whereas IL-2 inhibits the formation of Th17 in a STAT5-dependent manner (Laurence et al., 2007). A defining characteristic of Th17 cells is the production of IL-17 (Park et al., 2005). STAT5 and STAT3 directly compete for binding at the IL-17 locus, with STAT3 activating and STAT5 repressing IL-17 expression (Yang et al., 2011). The reciprocal actions of STAT3 and STAT5 are also seen in the formation of Tfh cells. IL-6 and IL-21 signaling through STAT3 leads to expression of Bcl6, a critical factor in Tfh formation. When induced by IL-2, STAT5 directly competes with STAT3 binding at Bcl6 and also induces Blimp-1 expression, a negative regulator of Bcl6 (Johnston et al., 2012; Oestreich et al., 2012). STAT4 induced expression of IL-21 and Bcl6 by IL-12 also drives Tfh formation (Nakayamada et al., 2011; Schmitt et al., 2009). Thus, cytokine signaling through the STATs can modulate/restrict cell differentiation and skew responses in one direction or another.

STAT5 is essential for survival and memory formation of CD8 T cells through stimulation by IL-7 and IL-15 (Hand et al., 2010; Tripathi et al., 2010). The effect of STAT5 on survival is specific for CD8 T cells as there is no effect on CD4 T cells when STAT5 expression is ablated. IL-10 and IL-21 signaling through STAT3 is also essential for proper memory formation in CD8 T cells (Cui et al., 2011). Many of the known regulators of PD-1 such as T-bet and Blimp-1 have also been shown to regulate memory formation of CD8 T cells (Rao et al., 2012; Rutishauser et al., 2009), suggesting that other regulators of memory formation may regulate PD-1. The action of STATs is complex, and a potential role for the proteins in PD-1 expression in CD8 T cells had not been explored prior to this work. As

shown in Chapter 2, STAT3 and STAT4 bind to at least two regions in the PD-1 locus. The experiments presented in this chapter were aimed at determining how many additional STAT binding sites are located within the PD-1 locus and to determine if the cytokines that induce STAT binding have the ability to prolong PD-1 expression.

Materials and Methods

Mice and Cell Culture

The EL4 T cell lymphoma line was obtained from the American Type Culture Collection. Total splenic CD8 T cells were isolated from C57Bl/6 mice using MACS (Miltenyi Biotech, Auburn, CA) per the manufacturer's protocol. Following isolation, cells were cultured in RPMI 1640 supplemented with 4.5 g/L glucose, 10mM HEPES, 1mM sodium pyruvate, 100 U/ml penicillin/streptomycin, 5% bovine calf serum (Thermo Fisher Scientific Inc., Waltham, MA), and 5% fetal bovine serum (Sigma-Aldrich Co, St. Louis, MO). Where indicated cells were stimulated with anti-CD3/CD28 beads (Life Technologies Co, Grand Island, NY) according to the manufacturer's protocol, 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 2 μM ionomycin (Io) (Sigma-Aldrich), 20 ng/mL IL-2, 10 ng/mL IL-4, 20 ng/mL IL-6, 20 ng/mL IL-12, and/or 20 ng/mL IFN-γ. All cytokines were purchased from Miltenyi Biotech. Cytokine stimulation was added at the same time as anti-CD3/CD28 stimulation and again every 48 hours for the duration of the culture. All mouse experiments were conducted in accordance with approved Emory University institutional animal care and use protocols.

RNA Isolation and RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen Sciences Inc., Germantown, MD). 1 μg of RNA was used for a reverse transcription reaction using Superscript II (Life Technologies Corp.) with oligo dT and random hexamer primers (Integrated DNA Technologies, Coralville, Iowa). Quantitative real-time PCR was used to determine relative PD-1 mRNA levels after normalizing to 18S mRNA expression. All experiments were conducted at least three times from independent cell cultures and statistical significance was determined by Student's t-test. PD-1 and 18S primers are listed in Table 4-1.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations (ChIP) were performed as previously described (Lu et al., In press; Oestreich et al., 2008). Briefly, CD8 T cells were crosslinked in 1% formaldehyde for 15 minutes followed by chromatin isolation. Following sonication of the chromatin to an average of 400-600 bp, 10 μg was immunoprecipitated with anti-phosporylated-STAT3 (Cell Signaling Technologies Inc., Danvers, MA) or a negative control IgG (EMD Millipore Co, Billerica, MA). DNA from each immunoprecipitation was quantified using real-time PCR as compared to a standard curve created from sonicated murine genomic DNA. Three independent cell preparations and immunoprecipitations were performed. The data is presented as the average percent pulldown of input DNA plus standard deviation.

Luciferase Reporter Assays

The -2.7 region was cloned into the firefly luciferase reporter vector pGL3-Promoter (Promega Co, Madison, WI) using *Nhe*I (New England Biolabs Inc., Ipswitch, MA). Luciferase reporter vectors described in Chapter 2 and the novel -2.7 vector were nucleofected into EL4 cells using Kit L protocols from Lonza Inc. (Allendale, NJ). 16 hours

post transfection, cells were stimulated with PMA/Io, IL-6, and/or IL-12 as indicated and incubated for 24 hours. Following incubation, lysates were collected and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The co-transfected Renilla luciferase gene (pRLTK), was used to normalize firefly luciferase activity.

Results

ChIP-seq identifies multiple STAT binding sites in the PD-1 locus

In Chapter 2, STAT3 and STAT4 were shown to bind to the -3.7 and +17.1 regions of PD-1 in CD8 T cells stimulated with IL-6 and IL-12, respectively. However, ChIP-seq data from a variety of CD4 differentiated T cells suggest that there are more STAT binding sites contained within the flanking CTCF sites of the PD-1 locus (Figure 4-1) (Consortium, 2011; Majumder et al., In press; Wei et al., 2010). Furthermore, the datasets also suggest that multiple members of the STAT family bind to the PD-1 locus. Most sites are shared among the multiple STAT members, including those at -3.7, CR-C, and +17.1 (green boxes, gray shading). DNase I hypersensitive sites (black boxes) determined in Chapter 2 that were cloned into reporter vectors but had no activity (Figure 2-2B) did not overlap with predicted STAT binding sites, suggesting PMA/Io stimulation of CD8 T cells is not sufficient to induce STAT binding at the PD-1 locus.

IL-6 and IL-12 prolong PD-1 expression in activated T cells

To determine if multiple STATs regulate PD-1 in CD8 T cells, splenic CD8 T cells were isolated and stimulated *ex vivo* with anti-CD3/CD28 beads and either IL-2 (STAT5), IL-4 (STAT 6), IL-6 (STAT3), IL-12 (STAT4), or IFN-γ (STAT1). There was an increase in PD-1

expression from CD8 T cells stimulated for 24 hours with beads and IL-6 or beads and IL-12 compared to beads alone (Figure 4-2A). In addition, IL-6 and IL-12 treatment of activated splenic CD8 T cells led to prolonged PD-1 expression, with IL-12 having a more dramatic effect. IL-2, IL-4, and IFN-γ had no effect on PD-1 expression. Furthermore, cytokine stimulation alone had no effect on PD-1 expression (data not shown), suggesting that T cell activation is a requirement for cytokine (STAT) mediated PD-1 expression.

STAT3 binds to 3 sites in the PD-1 locus

In Chapter 2 STAT3 was shown to bind to two sites, -3.7 and +17.1, following IL-6 treatment for 24 hours with or without TCR stimulation. To determine if the other predicted STAT binding sites from the ChIP-seq data sets were bound in CD8 T cells, ChIP was performed on CD8 T cells stimulated *ex vivo* for 1 or 5 days with anti-CD3/CD28 beads and IL-6 or beads alone. PCR primers were designed to test each significant peak of binding found between CTCF sites (Figure 4-1 red bars). As before, STAT3 binds to -3.7 and +17.1 in activated CD8 T cells only in the presence of CD3/CD28 beads and IL-6 but not beads alone (Figure 4-2B). STAT3 was also found to bind to another region, located 2.7 kb upstream of the TSS (-2.7 kb), but not at CR-C (Figure 4-2B) or any other region tested (data not shown). STAT3 binding is maintained even after 5 days in culture, suggesting that the maintenance of PD-1 expression by IL-6 is due to STAT3 binding at the PD-1 locus. We were unable to test cells stimulated with only IL-6 at day 5 because CD8 T cells stimulated with only IL-6 do not survive beyond 24 hours in culture.

The -2.7 region does not have regulatory activity

The binding of STAT3 to the -2.7 region suggests that this region could positively regulate PD-1 expression. To test this hypothesis, the -2.7 region was cloned into the pGL3-promoter luciferase reporter vector and transiently transfected into EL4 cells (Figure 4-3A). Unlike -3.7, CR-C, or +17.1, activation of the EL4 cells with PMA/Io did not lead to an increase in luciferase activity from the -2.7 reporter construct (Figure 4-3B). In addition, IL-6 had no effect on the -2.7 construct, despite the ability of STAT3 to bind the region. Together these data suggest that the -2.7 region is not sufficient to regulate PD-1 expression.

Discussion

Published ChIP-seq data suggests that in addition to STAT3 and STAT4, other STAT family members bind to the PD-1 locus (Consortium, 2011; Wei et al., 2010). The data also suggest that there are multiple STAT binding sites across the locus. Not all of the STAT binding sites identified could be validated, with only the -3.7, -2.7, and +17.1 regions being bound by STAT3 following IL-6 treatment of CD8 T cells. The ChIP-seqs were performed on CD4 T cells that had been differentiated in culture using cytokines that induce the given STAT. Moreover, 2 hours before cross-linking, additional cytokine stimulation and cell activation via plate bound anti-CD3/CD28 was added to induce maximal STAT binding. Thus, the ChIP-seq experimental protocol may have artificially induced more binding than is seen under physiological conditions. Most of the STAT binding sites overlap between the various CD4 subsets; however, CD8 T cells may have a different pattern, which would indicate there are cell type-specific regulatory regions of PD-1. This hypothesis could help explain why PD-1 expression is variable between subsets of lymphocytes.

When tested for regulatory activity, the -2.7 region was unable to induce luciferase activity upon stimulation with PMA/Io and/or IL-6. This is unique among the STAT

binding sites as both the -3.7 and +17.1 regions induce luciferase upon PMA/Io stimulation, and the activity of +17.1 is enhanced when IL-6 is added. The -2.7 region lacks a canonical NFAT binding site, explaining the lack of induction by PMA/Io. A lack of NFAT binding could also explain why IL-6 stimulation does not increase luciferase activity as in the other regions NFATc1 is critical for regulatory activity. Alternatively, the site may already be bound by another STAT family member, thereby making it unable to be induced by IL-6, much like the -3.7 region. Cytokine stimulation is not enough to induce PD-1 expression by itself. This is similar to the studies of PD-1 regulation that identified Notch signaling, AP-1, and the common γ-chain cytokines as regulators of PD-1 (Kinter et al., 2008; Mathieu et al., 2013; Xiao et al., 2012). Combined with the original studies showing that cyclosporine A inhibits PD-1 induction (Oestreich et al., 2008), these data suggests NFATc1 is the initiating factor for PD-1 expression. This further supports the idea that the -2.7 STAT site has no independent regulatory activity due to the lack of NFAT binding. It would be interesting to determine if addition of the -2.7 STAT site to the CR-C reporter construct, which lacks STAT binding, but binds NFATc1, allows for IL-6 augmentation as seen in the +17.1 reporter construct.

PD-1 expression is increased and prolonged in activated CD8 T cells treated with IL-6 and IL-12. Blimp-1 binds and evicts NFATc1 after 48 hours (Lu et al., In press), but IL-6 and IL-12 is able to alleviate this repression (Lu and Boss, unpublished data). The ability of cytokine driven STAT induction to block Blimp-1 repression of PD-1 could explain why exhausted T cells have high Blimp-1 expression but do not repress PD-1 (Shin et al., 2009). STAT3 mediated T cell exhaustion is supported by the overexpression of Blimp-1 as STAT3 positively regulates Blimp-1 expression (Diehl et al., 2008). Countering the STAT3 model is the finding that suppressor of cytokine signaling 3 (SOCS3) is overexpressed in chronic

LCMV infection (Pellegrini et al., 2011). SOCS3 negatively regulates STAT3 activation during IL-6 but not IL-10 signaling (Yasukawa et al., 2003). In preliminary experiments, IL-10 had no effect on PD-1 expression during *ex vivo* T cell activation (data not shown); however, IL-10's effects on IL-6 stimulated cells wasn't tested. A switch from IL-6 to IL-10 driven STAT3 activation would lead to SOCS3 being ineffective in repressing STAT driven gene expression, and explain how STAT3 could still be driving PD-1 expression.

In a study using IL-12 and type I IFNs to program cells it was discovered that IL-12 programmed CD8 T cells were able to respond well upon antigen restimulation as opposed to IFN-α programmed cells, which had high PD-1 expression and poor effector function following restimulation (Gerner et al., 2013). IL-12 has also been shown to rescue effector function of exhausted HBV-specific CD8 T cells (Schurich et al., 2013). This is counterintuitive to the studies presented here as IL-12 was shown to augment PD-1 expression. However, PD-1 expression was only monitored for 72 hours. It is still unknown if constant IL-12 stimulation would maintain PD-1 expression for longer periods or if levels would decrease over time. If STAT4 promotes proper programming of effector cells, then exhausted cells could potentially be reprogrammed by IL-12. Differential reexpression of PD-1 could be due to a difference in the chromatin architecture of the locus. Activating CD8 T cells in the presence of IL-12 or IFN-α and determining the chromatin state of the various regulatory regions at various timepoints could be informative and possibly explain why IFN-α cultured cells express more PD-1 following restimulation.

Proper programming and expression of PD-1 during an immune response has now been linked to the cytokine signals that CD8 T cells receive from the environment. As PD-1 is linked to effector function and memory formation, this discovery means that in addition to antigen induced TCR signaling, secondary signals from activated APCs in the form of

cytokines must be considered when devising vaccine strategies to produce effective immune responses and long-term protection. Moreover, the finding that STATs differentially augment PD-1 expression opens up new pathways to target in the treatment of chronic infections by reinvigoration of exhausted T cells.

Figure Legends

Figure 4-1. ChIP-seq identifies multiple STAT family member binding sites in PD-1. ChIP-seq data in reads per million (R.P.M.) of STAT3, STAT4, STAT5, STAT6, and CTCF across the PD-1 locus from Th17, Th1, Th17, Th2, and B cells, respectively. STAT3 and STAT5 data was made available as part of the ENCODE project (Consortium, 2011). STAT4 and STAT6 ChIP-seq was performed by Wei et al. (Wei et al., 2010). The data was accessed from the Gene Expression Omnibus (GEO) database under accession number GSE22105. CTCF ChIP-seq was performed by Majumder et al. (Majumder et al., In press). DNase I hypersensitive regions determined from Figure 2-1 are shown as black bars. The known regulatory regions of PD-1 are denoted as green bars. Amplicons used to validate STAT binding are presented as red bars.

Figure 4-2. IL-6 and IL-12 increase and prolong PD-1 expression of CD8 T cells by inducing STAT binding to multiple sites. (A) PD-1 mRNA expression from splenic CD8 T cells stimulated *ex vivo* with anti-CD3/CD8 beads, IL-2, IL-4, IL-6, IL-12, or IFN-γ as indicated was measured by real-time PCR. Three independent cell preparations were averaged. Error bars represent the standard deviation. **(B)** STAT3 and control IgG ChIP from freshly isolated splenic CD8 T cells or CD8 T cells stimulated by anti-CD3/CD28

beads in the presence or absence of IL-6 for 1 or 5 days. Data is presented as the average percent pulldown from three independent experiments with standard deviation.

Figure 4-3. IL-6 and IL-12 induce expression through the use of the same STAT binding sites. (A) Schematic showing the reagions cloned into the pGL3-Promoter firefly luciferase reporter vector. The -2.7 vector was made for this project. The CR-B/C, +17.1, and -3.7 vectors are the same as used in Figure 2-2. **(B)** EL4 cells transiently transfected with the construct indicated were cultured for 16 hours then subsequently stimulated with PMA/Io and/or IL-6. Lysates were collected 24 hours post stimulation and firefly luciferase activity was determined and normalized to the cotransfected *Renilla* luciferase plasmid. The data is presented as the average relative luciferase activity compared to the pGL3-promter vector from three independent experiments. Error bars represent the standard deviation.

Figure 4-1

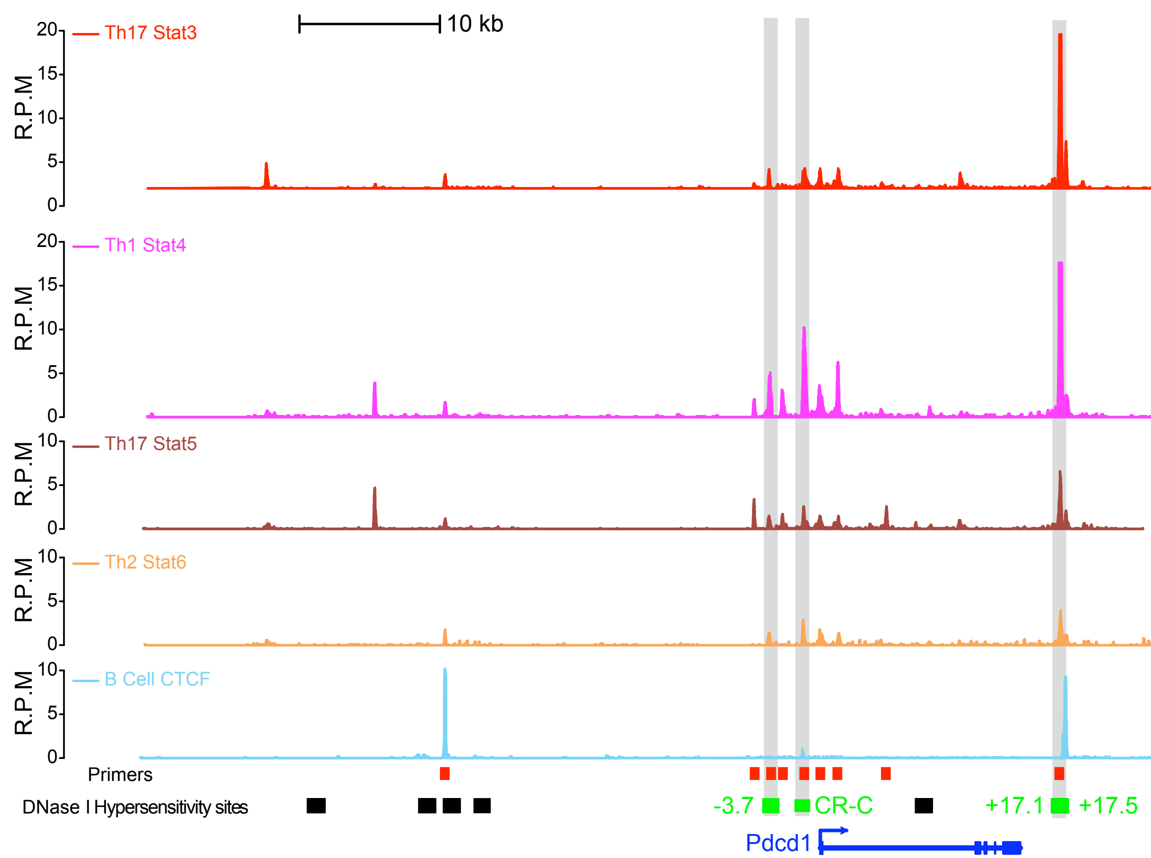


Figure 4-2

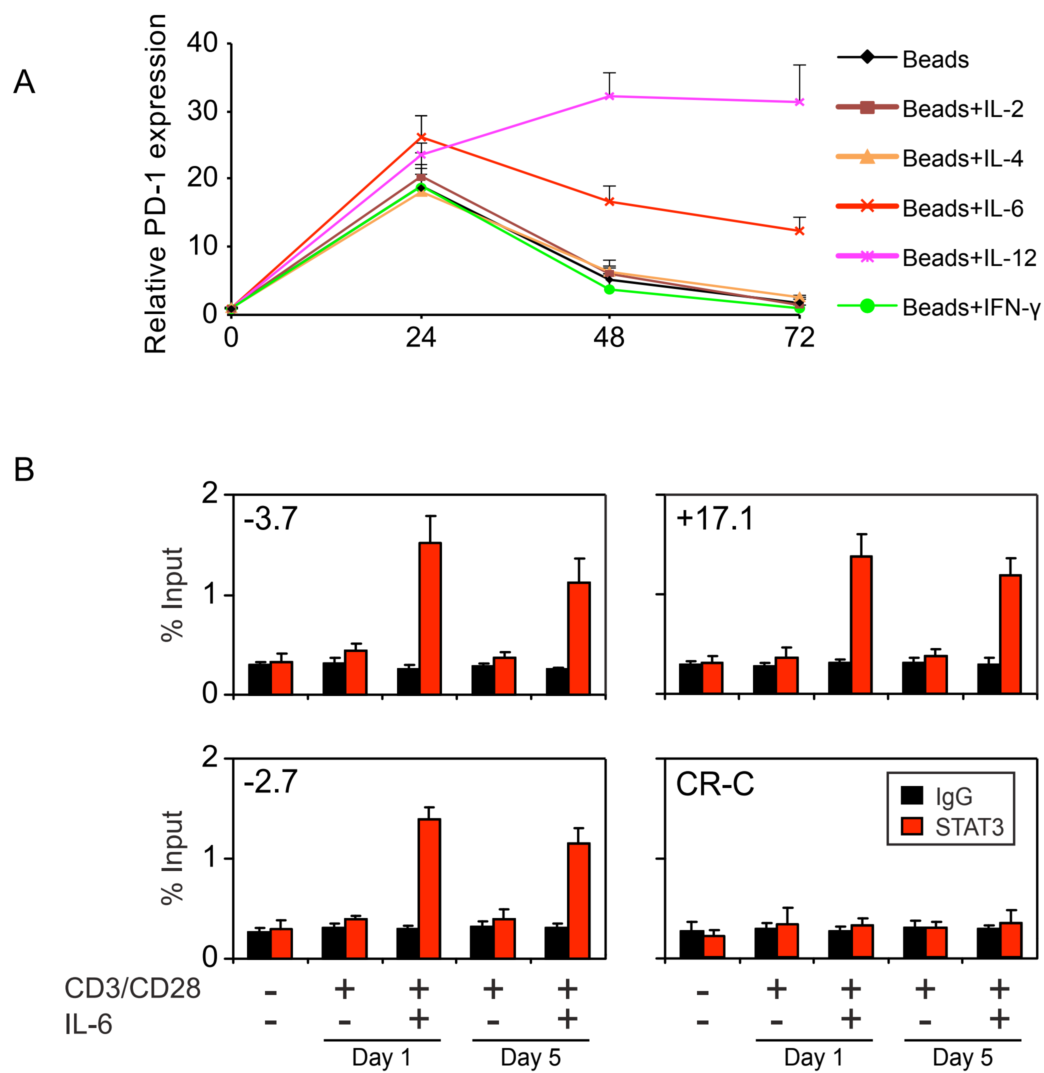


Figure 4-3

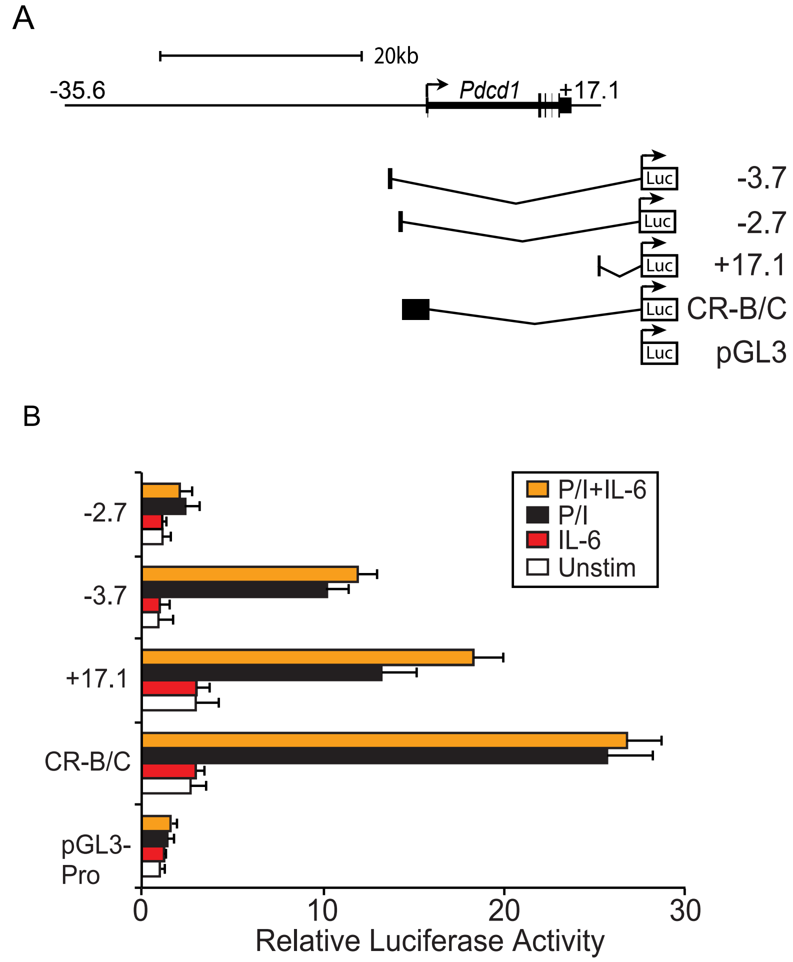


Table 4-1: Primers used in this study	
qRT-PCR Primers	
PD-1 Forward	CGTCCCTCAGTCAAGAGGAG
PD-1 Reverse	GTCCCTAGAAGTGCCCAACA
18S Forward	GTAACCCGTTGAACCCCATT
18S Reverse	CCATCCAATCGGTAGTAGCCG
ChIP Primers	
-3.7 Forward	AGGGTGAGCAGGCGAGAGCAA
-3.7 Reverse	AGCACAGGGAAGAAGCTGTTGGG
-2.7 Forward	GGCCCTGCAGCCCAGGTAAC
-2.7 Reverse	TCAAAGCAGGGCAGAACAGAGAGT
CR-C Forward	CCTCACCTCCTGCTTGTCTCTC
CR-C Reverse	GTGAGACCCACACATCTCATTGC
+17.1 Forward	GGAGGGGATAGGCGCTGGGT
+17.1 Reverse	TCTGGGCCAAGCCATCCGGT
Luciferase Cloning Primers	
-2.7 Forward	TAGTGCTAGCCAGCATAGTCGGAAAGTTGGG
-2.7 Reverse	TTGTGCTAGCCCTCTACAGCTCAGAGCCTATTG

Chapter 5. Discussion

Multiple *Cis* Elements Regulate PD-1 Expression

Using a non-biased DNase I hypersensitive screen, multiple hypersensitive sites were identified within the PD-1 locus. Two of these sites were found to have regulatory potential *in vivo*. Further investigation showed that NFATc1 and multiple STAT proteins including STAT1, STAT3, and STAT4 bound the regions when appropriate cytokine stimulation was added. CTCF was also shown to bind to two distal regions in the PD-1 locus. With the addition of these four novel elements, the number of known *cis* regulatory elements of PD-1 stands at 10 (relative position to the TSS in kb is listed): -26.7, -3.7, -1.9, -1.1 (CR-C), -0.8, -0.5, -0.1 (CR-B), +10.6, +17.1, and +17.5. The -2.7 region also binds STAT3 in response to IL-6 signaling, although as discussed in Chapter 4, it had no regulatory activity on its own. Thus, a potential 11th regulatory element exists, but may require additional elements to function. The -26.7 and +17.5 CTCF sites allow the locus to form a constitutive loop that is irrespective of PD-1 expression. The upstream CTCF site marks a boundary between an area of homology with human chromosome 5 and an area of homology with human chromosome 2, which contains the human *PDCD1* gene. The downstream CTCF site separates *Pdcd1* from the neighboring gene, *Neu4*, a neuraminidase highly expressed in the brain (Cohen and Eisenberg, 1991). This suggests that the CTCF sites create a chromatin loop to isolate *Pdcd1* from the neighboring genes, and insulating it from other regulatory regions and encroaching chromatin modification. Furthermore, this implies that all *cis* regulatory regions of PD-1 will be between these two CTCF sites.

The eight remaining *cis* elements all bind transcription factors that directly enhance or repress PD-1 expression. CR-C was the first element described to bind a factor,

NFATc1, which directly promotes PD-1 expression (Oestreich et al., 2008). Through the use of NFAT and calcineurin inhibitors it was shown that NFATc1 was necessary for PD-1 expression, and is likely the primary initiator of PD-1 expression following TCR engagement. As discussed in Chapter 2, two new NFATc1 binding sites were discovered at the -3.7 and +17.1 regions. Multiple STAT family members also bound these regions in response to their specific cytokine stimulation. Importantly, the activity of the two regulatory regions was dependent upon both TCR and cytokine stimulation. Although NFATc1 or STAT3/4 could bind in response to their respective stimuli, the chromatin state of the regions was different in singly-stimulated versus doubly-stimulated cells. TCR stimulation had no effect on the histone marks queried at the -3.7 and +17.1 regions by itself; however, cytokine stimulation led to the regions being marked by H3K4me1. Upon simultaneous TCR and cytokine stimulation the regions also become marked by H3K27ac. In combination, H3K4me1 and H3K27ac mark active enhancers, whereas H3K4me1 alone marks poised enhancers (Creyghton et al., 2010; Heintzman et al., 2007). Therefore, cytokine stimulation leading to STAT binding appears to lead to a poised state for the -3.7 and +17.1 regulatory regions. TCR stimulation in addition to cytokine stimulation induces both NFATc1 and STAT binding, respectively, and the regions become active and enhance PD-1 expression. Cytokine stimulation alone had no effect on PD-1 expression, further showing that the -3.7 and +17.1 regions depend on NFATc1 for functionality. Notably, the use of the two novel STAT-dependent regulatory regions allows for environmental cues to regulate PD-1 expression through cytokine stimulation.

CR-C is not bound by STAT3/4 in response to IL-6 or IL12, respectively. Although an ISRE resides in CR-C and is bound by the ISGF3 complex, which contains STAT1 and STAT2, in response to type I IFNs (Cho et al., 2008; Terawaki et al., 2011), CR-C is not

dependent on cytokine stimulation for activity. ChIP of H3K27ac supports this observation as H3K27ac is found at CR-B and CR-C following only TCR stimulation. The reason why -3.7 and +17.1 are dependent on both cytokine and TCR stimulation while CR-B and CR-C are only TCR-dependent is not clear. The use of multiple regulatory regions that respond to different signals could be used as a way of tuning the level of PD-1 expression in each cell type. For instance, in macrophages, the CR-B/C region has regulatory potential but the -3.7 and +17.1 regions do not (Bally and Boss, personal communication). In connection with this, activated macrophages express much less PD-1 than activated T cells. Alternatively, the answer could involve the looping of the enhancers into the promoter. As seen in the 3C assays from Figure 2-6, TCR stimulation induces interactions between the distal regulatory regions and the promoter. This implies that NFATc1 mediates these interactions. In addition, NFATc1 is critical for the initiation of PD-1 expression but not its maintenance. NFATc1 could be necessary for formation of the loops, but, once formed, STAT mediates the continued expression of PD-1. A similar system could exist for the CR-B/C regions where NFATc1 initiates PD-1 expression, but AP-1, which is also induced by TCR, is able to bind CR-B to maintain PD-1 expression.

Multiple Factors Effect the DNA Methylation of PD-1

Using model cell lines and the LCMV system, the DNA methylation of CR-B and CR-C was shown to inversely correlate with PD-1 gene expression. Naïve, non-PD-1 expressing T cells are marked by DNA methylation at both regions, which is lost upon CD8 T cell activation and PD-1 induction. The DNA methylation returns as viral loads are controlled, PD-1 expression wanes, and CD8 T cells transition into memory cells. In contrast, in chronic infections resulting in an overexpression of PD-1, DNA methylation is lost and does not

return. In studies of HIV infection, DNA methylation is lost and does not return despite controlled viral loads through natural immunity or the use of anti-retroviral drugs. This implies that exhausted T cells have been epigenetically programmed to become exhausted, and this program is not dependent on antigen for maintenance. It should be noted that while viral loads are controlled and PD-1 expression on HIV-specific cells decreases in elite controllers and patients undergoing successful HAART treatment, virus is not cleared nor is PD-1 expression completely lost. Earlier reports showed that when HIV antigenically shifts, the T cells that had been targeting the virus but subsequently lost their antigens, downregulate PD-1 expression (Streeck et al., 2008). A similar effect was seen in the chronic LCMV system (Blattman et al., 2009), thus it was proposed that continued antigen presentation was important in the development of T cell exhaustion. In both of these studies, viral antigens shifted early in infection, before the antigen-specific cells had become exhausted. This means that a cell does not immediately setup an exhaustion program upon chronic viral infection, but the program is put into place during the acute stage of infection. Antigen dose, irrespective of presentation route, is a driving force behind T cell exhaustion (Richter et al., 2012), yet once exhausted, a decrease in antigen is not enough to restore functionality of T cells. A change in the chromatin architecture of PD-1, including histone modifications and DNA methylation, provides an explanation for the stable, heritable exhaustive phenotype that is observed following loss of antigen signal. The small window between T cell activation and the setup of the exhaustion program may represent the optimal time to target for molecular therapies designed to prevent T cell exhaustion. Reinvigoration of exhausted T cells is only partially successful, likely due to the inability to fully reprogram the cells. Therapies designed to counter the exhaustion program while it is

still being setup could be more successful than the current therapies aimed at reversing already exhausted cells.

Many of the known transcription factors of PD-1 have been shown to recruit histone-modifying enzymes, including NFATc1, Blimp-1, and the STAT proteins, suggesting they also regulated the DNA methylation of the PD-1 locus (Garcia-Rodriguez and Rao, 1998; Gyory et al., 2004; Paulson et al., 1999). A possible pathway to explain the remethylation of PD-1 during acute viral clearance begins with the repressor Blimp-1. Blimp-1 interacts with LSD1, an H3K4 demethylase. H3K4me3 is observed at the PD-1 promoter during an active gene state but not prior to T cell induction or after PD-1 is silenced. LSD1 demethylation of H3K4 leads to an H3K4me0 state, which is a target for the DNMT3 complex. Testing of this possible pathway through the use of conditional knockouts of Blimp-1 or LSD1 suggest that these two proteins play a role in the remethylation of PD-1 as DNA methylation is reduced or delayed, respectively, in conditional knockout animals following LCMV infection. Though significant, there is not a complete inability to remethylate, suggesting other mechanisms are also involved. T-bet has not been shown to interact with any chromatin remodeling complexes that inhibit gene expression, therefore at this time there is no evidence to suggest that T-bet is critical for the epigenetic silencing of PD-1. Transcription is an active process; therefore, loss of activators, such as NFATc1, binding to the PD-1 locus would halt transcription of PD-1. Furthermore, loss of the activators would result in a loss of targeting of the histone modifying enzymes that maintain the active gene state. As cells divide, the active histone marks would be lost as histones are replaced following DNA replication. Newly placed histones that are in the H3K4me0 state would become targets for DNMT3, thereby allowing for remethylation of PD-1. This could explain why there is a delay, but not a complete loss of remethylation.

Alternatively, another unknown repressor could still recruit the chromatin modifying enzymes, but do so at a later time. Likewise, another H3K4 demethylase such as RBP2 (Klose et al., 2007) could be used instead of LSD1, but recruitment of that enzyme could be delayed compared to LSD1. A double conditional knockout mouse of LSD1 and Blimp-1 could result in a more robust phenotype, as knocking out two of the putative pathway members would be harder to overcome than a single knockout.

Prolonged PD-1 Expression in Response to Cytokine Signaling

As shown in Chapter 4, both IL-6 and IL-12 prolong PD-1 expression of activated CD8 T cells stimulated *ex vivo* with CD3/CD28 beads. STAT3 is bound to the -3.7 and +17.1 regions five days post stimulation, suggesting the maintenance of PD-1 expression is due to the continued activation by STAT3. Alternatively, STAT3 may be inhibiting the repression of PD-1 by Blimp-1. It should be noted that NFATc1 binding was not observed at day 5 post stimulation and is not responsible for the continued expression of PD-1 (data not shown). While not yet tested, STAT4 is hypothesized to be bound to the same regions at day 5 post-stimulation in CD3/CD28 and IL-12 costimulated cultures. The data also suggest that IL-12 is better at maintaining expression of PD-1 than IL-6. This would be consistent with the data from Chapter 2 showing that IL-12 induces a higher expression of PD-1 than IL-6 (Figure 2-3). Previous work showed that restimulation of exhausted HBV-specific cells with peptide and IL-12 had less PD-1 on their surface than peptide only cultured cells (Schurich et al., 2013). The authors found that the expression of the PD-1 repressor T-bet was increased, which they suggest as the mechanism for decreased PD-1 expression. It is unclear why IL-12 had opposite effects between their study and ours. There is a difference in both the chromatin architecture and the transcription factors bound between naïve and

exhausted cells. This could inhibit/promote the binding of other factors that regulate PD-1, thereby altering the effects of IL-12. The different IL-12 effects could also be explained by the different culture systems. In our system CD8 T cells from uninfected mice were isolated and cultured *ex vivo* with simultaneous TCR and cytokine stimulation. In the other study total human PBMCs were isolated and stimulated with peptide and cytokine. IL-12 stimulation of macrophages and DCs induces production of multiple cytokines that could alter PD-1 expression (Chan et al., 2012; Grohmann et al., 2001; Xing et al., 2000); therefore, the dampening of PD-1 expression may due to a secondary effect of IL-12 stimulation or species-specific.

It has been shown that IFN-α and IL-12 differentially program PD-1 in restimulated T cells (Gerner et al., 2013). Intriguingly, PD-1 expression is enhanced through IL-12 or IFN-α stimulation of activated T cells, with IL-12 having a more pronounced effect, suggesting that the T cell programming is not linked to the magnitude of PD-1 expression. In addition to the magnitude of PD-1 expression, both IFN-α (Terawaki et al., 2011) and IL-12 prolong PD-1 expression in activated T cells, although it is still not known how long PD-1 expression is prolonged following stimulation with either cytokine. The possibility exists that IFN-α will prolong PD-1 expression to a greater extent than IL-12. If true, this implies that duration of PD-1 is a better indicator of T cell functionality upon restimulation than the maximal magnitude of expression. Alternatively, the cytokines could set up different chromatin states that allows for a more robust response upon restimulation of the IFN-α stimulated cells. Determining the histone modifications and DNA methylation status following cytokine programming could be helpful in determining how the epigenetic program of the PD-1 locus is established.

Altered chromatin states in IFN- α compared to IL-12 induced cells could be due to differences in STAT1 compared to STAT4. STAT1 plays a critical role in type I IFN-mediated inhibition of proliferation. Non-antigen-specific CD8 T cells proliferate during the early immune response (prior to day 4) in response to LCMV infection of STAT1-deficient mice, demonstrating the importance of STAT1 in controlling antigen specificity (Gil et al., 2006). Following LCMV infection, STAT1 is upregulated in CD8 T cells; however, antigen-specific CD8 T cells that undergo expansion also upregulate expression of STAT4 in response to TCR signals and have lower STAT1 levels compared to non-proliferating cells (Gil et al., 2012; Gil et al., 2006) (Figure 5-1). Once activated, CD8 T cells preferentially activate STAT4-dependent genes following type I IFN stimulation, showing that type I IFNs are capable of activating multiple STAT family members. The ratio of STAT4 to STAT1 in the cell determines which of the pathways are used during type I IFN signaling. In addition to regulating proliferation, the usage of STAT4 or STAT1 during type I IFN signaling determines if IFN-γ is positively or negatively regulated, respectively (Nguyen et al., 2002). During type I IFN signaling, STAT1 acts a repressor of immune function while STAT4 acts as a proimmune factor. Envisioning a similar system regulating PD-1 fits with the previous experiments showing the differential programming of PD-1 during IFN-α or IL-12 signaling (Gerner et al., 2013). Preferential activation of STAT1 predisposes the cell to reexpress PD-1 to higher levels following restimulation, keeping with the idea of STAT1 as a repressor of immune function. In contrast IL-12 programmed cells are functional and better at protection following restimulation, suggesting they are better memory cells. The STAT4:STAT1 ratio of LCMV-specific CD8 T cells increases throughout the course of acute infection leading from a ratio of 1:1 in naïve cells to a ratio of 4:1 in LCMV-specific T cells at day 30 (Figure 5-1) (Wherry et al., 2007). In contrast, during chronic infection, the

STAT4:STAT1 ratio peaks around 3:2 at day 6, decreases slightly at day 8, then is constant through day 30. The potential exists that PD-1 expression in exhausted T cells is being driven by STAT1. Increased STAT1 is also consistent with the loss of IFN-γ production during exhaustion.

Additional Potential Regulatory Factors of PD-1

With the addition of CTCF and multiple members of the STAT family regulating PD-1 as described above, there are 9 known transcription factors/complexes that directly regulate PD-1 expression: NFATc1, AP-1, T-bet, Blimp-1, Notch, CTCF, STAT3, STAT4, and the ISGF3 complex (STAT1, STAT2, and IRF9) (Cho et al., 2008; Kao et al., 2011; Lu et al., In press; Mathieu et al., 2013; Oestreich et al., 2008; Terawaki et al., 2011; Xiao et al., 2012). Although multiple transcription factors of PD-1 are now known, multiple lines of evidence suggest there are even more transcription factors that regulate PD-1.

ChIP-seq experiments of IRF4 in IL-21 stimulated CD4 T cells identified 2 peaks, located at -26.7 and +17.1 (Kwon et al., 2009). A strong peak located at +17.1 contains enough normalized reads to suggest the peak is real; however, the peak at -26.7 has a drastically reduced read count. The small peak at -26.7 is common in many other data sets, (Consortium, 2011; Durant et al., 2010; Wei et al., 2010), suggesting that this region produces a false positive. Indeed, we have confirmed that although multiple factors are suggested by ChIP-seq data to bind the -26.7 region, none have been validated except CTCF. The 3C data shows that the -26.7 region interacts with both the +17.5 and -3.7 regions. This interaction is likely maintained upon crosslinking during the ChIP-seq procedure. Pulldown of factors bound to interacting regions can allow for small amounts of the -26.7 region to be pulled down, leading to the false positive peaks. IRF4-STAT3

interactions are seen at the Blimp-1 locus during IL-21 stimulation (Kwon et al., 2009). Furthermore, IRF4 was important for STAT3 binding to target sites as *Irf4* knockout mice have reduced STAT3 binding at co-occupied sites. If IRF4 is also found to be bound at PD-1 like suggested, STAT3-IRF4 cooperative gene expression could explain the high level of both Blimp-1 and PD-1 in exhausted cells.

Using a genomics approach to identify genes that are induced following PD-1 ligation, the transcription factors basic leucine zipper transcription factor, ATF-like (BATF), IRF9, and STAT1 were identified as being upregulated in exhausted T cell (Quigley et al., 2010). STAT1 and IRF9 have already been shown to bind to the ISRE located within CR-C to positively regulate PD-1 (Cho et al., 2008; Terawaki et al., 2011). BATF has been shown to interact with IRF4 and IRF8 at AP-1–IRF composite elements (AICEs) (Ciofani et al., 2012; Glasmacher et al., 2012; Li et al., 2012b; Tussiwand et al., 2012). In addition, BATF has been shown to bind to the same sites as STAT3 and IRF4 (Li et al., 2012b), suggesting that BATF may bind the +17.1 region. Indeed, ChIP-seq from CD4 T cells indicates that BATF binds to the +17.1 region (Li et al., 2012b). Infection of CD4 or CD8 T cells with a retrovirus expressing BATF inhibited effector functions (Quigley et al., 2010). Likewise, knockdown of BATF increased IL-2 and IFN-γ production, lending more support to the idea that BATF could regulate PD-1 expression. Together, available ChIP-seq data and molecular interactions indicate that IRF4 and BATF potentially bind the +17.1 to regulate PD-1 expression.

Many of the known regulators of PD-1 such as T-bet and Blimp-1 have also been shown to regulate memory formation of CD8 T cells, suggesting that other regulators of memory formation may regulate PD-1 (Rao et al., 2012; Rutishauser et al., 2009). Eomesodermin (Eomes) is a T-box transcription factor that shares homology with T-bet (Yi

et al., 1999). Eomes and T-bet share overlapping functions in a number of cell types including regulating memory formation in CD8 T cells (Intlekofer et al., 2005). A key difference between T-bet and Eomes is their expression during CD8 T cell differentiation. T-bet is highly expressed in early effectors and decreases as cells transition into a memory phenotype (Joshi et al., 2011). In contrast, Eomes expression is induced in the early effectors, but levels continue to rise through the late effectors and into memory cells, which maintain high expression of Eomes (Banerjee et al., 2010; Joshi et al., 2011; Pipkin et al., 2010). A key factor in controlling the expression of T-bet and Eomes is IL-12. IL-12 induces T-bet expression while simultaneously inhibiting Eomes expression (Takemoto et al., 2006). IL-12 levels decrease as antigen in cleared, suggesting that T-bet mediated repression of PD-1 would be lost during the late effector stage. Eomes could solve this conundrum as Eomes expression increases when IL-12 decreases. As mentioned above, T-bet and Eomes have several overlapping functions, setting a precedent for this possibility.

PD-1 Regulation In Exhausted T Cells

As discussed above, PD-1 is regulated by a variety of mechanisms including DNA methylation, histone modifications, and multiple transcription factors binding to 10 *cis*-regulatory regions. In addition, there are other factors that have the potential to regulate PD-1 as well. TCR signaling is clearly necessary for PD-1 induction in CD8 T cells as in no setting has PD-1 expression been observed in the absence of T cell activation. Augmenting or prolonging PD-1 expression can be accomplished through a variety of mechanisms including stimulation with multiple cytokines. Despite the progress made, the regulation of PD-1 in exhausted T cells is still not well understood.

There are clear differences between naïve, early effector, late effector, and exhausted CD8 T cells with regard to the transcription factors bound and the epigenetic marks (DNA methylation and histone modifications) associated with the regulatory regions (Figure 5-2). Naïve CD8 T cells lack repressive and active histone marks, but CR-B and CR-C are marked by DNA methylation. Naïve cells also are not bound by any currently known transcription factor of PD-1, although CTCF is present as the boundaries of the locus. Once activated, the DNA methylation is rapidly lost, the activating transcription factors (NFATc1, AP-1, STATs, ISGF3, Notch) bind, and the locus is marked with active histone marks. Late effector stage cells lose the binding of the activating transcription factors and are replaced with the repressors Blimp-1 and T-bet. In conjunction with the change in transcription factors, the histone modifications associated with the locus change from the active to repressive marks, and there is *de novo* DNA methylation of the locus (Figure 5-2). In exhausted CD8 T cells, the factor(s) driving increased PD-1 expression is not well defined. Active histone modifications and an unmethylated DNA state are associated with exhausted cells. It is also clear that NFATc1 is not a driving force for the upregulation of PD-1 as NFATc1 translocation into the nucleus is impaired in chronic LCMV infection (Agnellini et al., 2007). Counterintuitively, Blimp-1 is bound to its site between CR-B and CR-C in exhausted cells (Lu and Boss, personal communication). The binding status of the rest of the transcription factors is unknown in exhausted T cells. T-bet is unlikely to be bound as T-bet expression is inversely correlated with T cell exhaustion, with the most exhausted cells having the lowest T-bet expression (Kao et al., 2011). The most likely known factors to be the cause of the PD-1 upregulation are the STATs (Figure 5-2). The STAT factors have the strongest effect *ex vivo* in upregulating PD-1 expression, and are still expressed in exhausted cells (Wherry et al., 2007), and as shown in Figure 4-2, can prolong PD-1 expression *ex vivo*.

It is still unknown which, if any, STATs are bound in exhausted CD8 T cells. The case for STAT1 being the proposed "exhaustive" STAT was discussed above, as was the case against STAT4. Like STAT1, STAT2 also binds to the ISRE in CR-C as part of the ISGF3 complex. There is no evidence of STAT2 homodimers binding CR-C or any other regulatory region of PD-1. Therefore, if STAT2 is driving the upregulation of PD-1 it is likely as part of a complex with STAT1. STAT5 and STAT6 have not been shown to bind to the PD-1 locus in CD8 T cells. Chip-seq suggests that each can bind in differentiated CD4 T cells; however, IL-2 and IL-4, cytokines that lead to STAT5 and STAT6-mediated signaling, had no effect on PD-1 expression from CD8 T cells, suggesting they do not induce STAT binding. STAT3 binds to the -3.7, -2.7, and +17.1 regions in activated CD8 T cells stimulated with IL-6. Serum IL-6 levels of people coinfected with HIV and HCV are predictive of individuals who do not respond to antiviral treatment, in which high IL-6 levels correlates with a worse antiviral response. Blockade of IL-10, another activator of STAT3, leads to clearance of a chronic LCMV infection (Ejrnaes et al., 2006). Together, this implies STAT3 is a mediator of chronic infection. It is unlikely that IL-6 is the continued source of activated STAT3 in chronic infection as exhausted CD8 T cells upregulate SOCS3, an inhibitor of JAK2, and a critical kinase in the activation of STAT3 by the IL-6R. As mentioned above, STAT3 is also activated by IL-10. IL-10 signaling is not dependent on JAK2 for STAT3 activation, thus SOCS3 does not repress STAT3 activation by the IL-10R (Niemand et al., 2003). However, when activated CD8 T cells were cultured *ex vivo* with IL-10 no effect on PD-1 expression was observed. Pleiotropic effects of IL-10 on naïve and activated T cells have been observed *ex vivo* (de Waal Malefyt et al., 1993; MacNeil et al., 1990); therefore, it is possible that the timing of IL-10 stimulation is critical to observe an effect. In the studies presented here, IL-10 was used concomitantly with CD3/CD28 bead

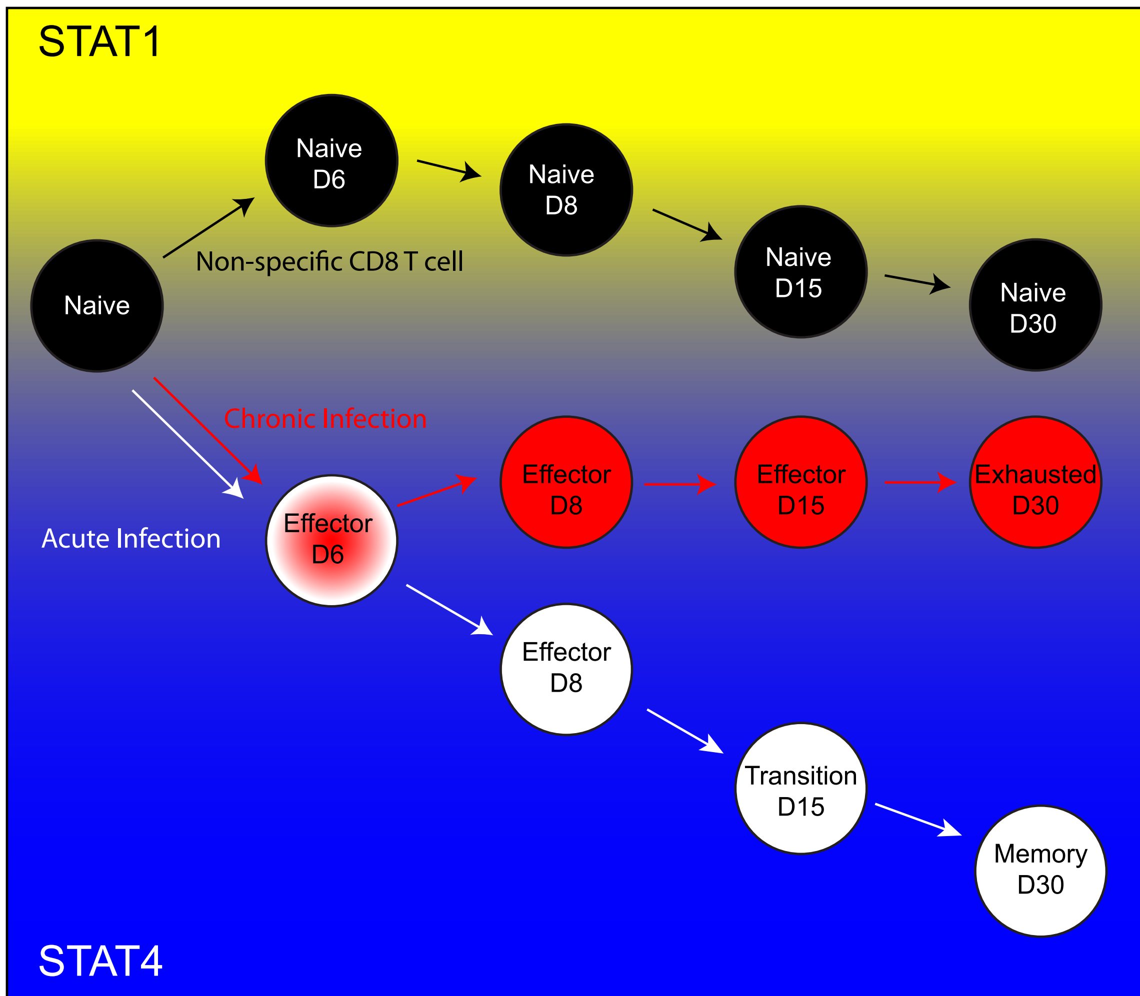
stimulation instead of being added following T cell activation. It is also possible that IL-10 was not able to establish STAT3 mediated PD-1 expression, but can maintain it. Studies to determine which factor(s) regulate PD-1 in exhausted cells will provide specific targets for molecular therapies. Cytokine therapies have already been shown to synergize with anti-PD-1/L1 therapy to reinvigorate T cells (Pellegrini et al., 2009; West et al., 2013). It is envisioned that with a greater understanding of PD-1 regulation more targeted combination therapies will be developed to reinvigorate exhausted T cells.

Figure Legends

Figure 5-1. STAT1 and STAT4 are differentially expressed in CD8 T cells from acute versus chronic infection. The ratio of STAT1 (yellow) compared to STAT4 (blue) expression in each cell is represented by the vertical placement of the cell. Naïve, unstimulated T cells (black) express roughly equal amounts of STAT1 and STAT4. Non-specific cells slightly upregulate STAT1 expression early during infection. STAT1 expression decreases following the initial effector response, eventually returning to baseline. LCMV-specific cells that are stimulated during an infection upregulate STAT4 expression so that by day 6 after LCMV infection more STAT4 is expressed compared to STAT1. LCMV-specific CD8 T cells from acute infection (white cells and lines) continue to increase the STAT4:STAT1 ratio through the course of infection, through the transitional memory stage, culminating with memory cells having a 4:1 ratio of STAT4:STAT1. In contrast, LCMV-specific cells from a chronic LCMV infection upregulate STAT4 expression early (Day 6), but there is a slight loss of STAT4 expression by day 8. The ratio of STAT4:STAT1 in LCMV-specific cells from a chronic infection stays at 3:2 from day 8 through day 30.

Figure 5-2. An exchange of transcription factors alters PD-1 expression in CD8 T cells during an immune response. The binding of transcription factors to the PD-1 locus in naïve, early effector, late effector, and exhausted CD8 T cells is shown. The DNA methylation status is also indicated as either black (methylated) or white (unmethylated) lollipops. Histone modifications at the regulatory regions are indicated as white circles below the regulatory regions. Regions with unknown histone modifications are indicated as a circle with a "?". The status of PD-1 transcription is indicated by either a black arrow (active) or red line (repressed). Proposed binding of factors in exhausted cells is indicated as a white "?" within the factor. The background color of the box represents the epigenetic state of the locus, with yellow representing neutral, green representing active, and red representing repressed states.

Figure 5-1



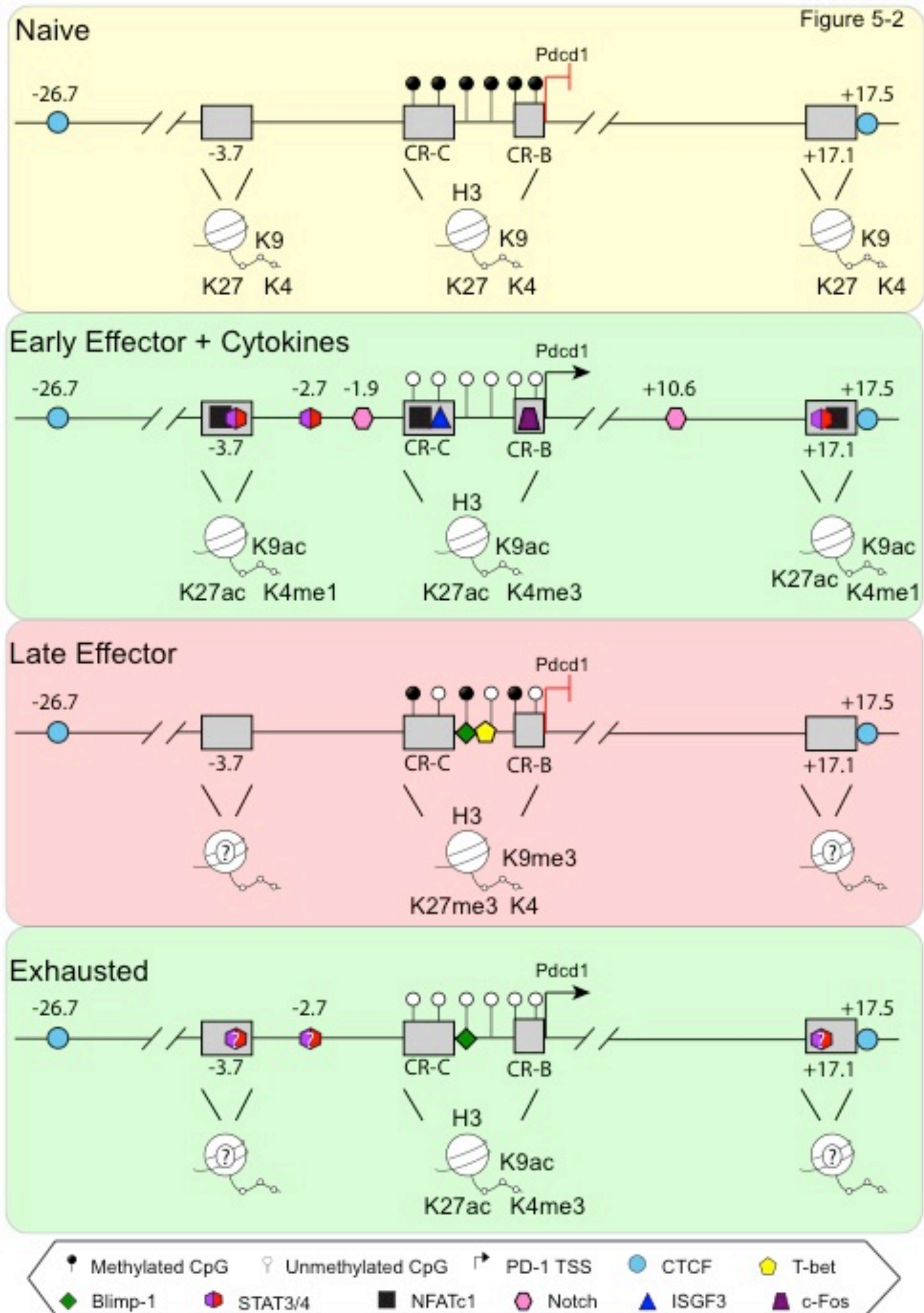


Figure 5-2
Naive
Pdcd1
-26.7
+17.5
-3.7
CR-C
CR-B
+17.1
H3
K9
K27
K4
Early Effector + Cytokines
-2.7
-1.9
+10.6
K9ac
K27ac
K4me1
K4me3
Late Effector
K9me3
K27me3
Exhausted
Methylated CpG
Unmethylated CpG
PD-1 TSS
CTCF
T-bet
Blimp-1
STAT3/4
NFATc1
Notch
ISGF3
c-Fos

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