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CIITA promoter I and isoform I expression and function in cells of the myeloid lineage

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CIITA promoter I and isoform I expression and function in cells of the myeloid lineage

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B.S University of North Carolina at Chapel Hill, 2000

Advisor: Jeremy Boss, Ph.D.

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Abstract

CIITA promoter I and isoform I expression and function in cells of the myeloid lineage

By Wendy Zinzow-Kramer

The major histocompatibility class II transactivator (CIITA) is controlled by three conserved promoters (pI, pIII, and pIV). Each promoter encodes a unique first exon, which is spliced into a common second exon, resulting in three isoforms that differ only at the N-terminus of the protein. The first exon of isoform I, which is expressed primarily by myeloid cells (dendritic cells and macrophages), encodes a region with homology to a caspase recruitment domain (CARD). CIITA isoform I is a member of the nucleotide-binding and leucine-rich repeat containing family (NLR). The CARD domain is protein-protein interaction domain, and is commonly associated with proteins involved in inflammation and apoptosis. Studies of CIITA pIV and CIITA pIII/pIV double knockout mouse models have demonstrated the requirement for CIITA pIII in B cells and CIITA pIV in IFN-γ induced non-hematopoietic cells. The activity and function of isoform I has not been well studied, and it has been suggested that the CARD domain enhances CIITA activity. To address whether CIITA isoform I has a unique function, attributable to the presence of the CARD domain, a mouse model in which isoform I was replaced with isoform III was generated. In addition, to study expression of *Ciita* pI, a mouse line in which the promoter and first exon of *Ciita* pI were deleted was also created. No defects the formation of CD4 T cells or in responses to viral or bacterial challenge were seen in mice lacking isoform I. In addition, in the pI knockout model, only a slight decrease in *Ciita* and MHC-II expression was observed in dendritic cells, and no change in expression was observed in macrophages. These results suggest that control of pI is mediated by an unknown distal enhancer element that is also capable of promoting transcription from pIII, the B cell specific promoter. Recent work in our lab and others has identified a regulatory region, HSS1 (DNase hypersensitivity site 1), which is required for transcription of pIII in B cells and pIV in IFN-γ induced HeLa cells. Analysis of chromatin structure at the CIITA locus suggests that HSS1 may also interact with CIITA pI. In B cells, CIITA pI silencing is likely due to heavy methylation at this promoter. In non-hematopoietic cells, pIV is inactive unless IFN-γ induced transcription factors are present. Thus, HSS1 may be a master enhancer for CIITA, the activity of which is mediated by DNA methylation and availability of transcription factors.

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Chapter 1: General Introduction

I. Adaptive Immunity: Major Histocompatibility Class II

Major histocompatibility class I and II (MHC-I and MHC-II) molecules play an important role in the adaptive immune response (reviewed in (Benacerraf 1981)). The major function of these molecules is to present peptides to CD8 and CD4 T lymphocytes, respectively. All nucleated cells express MHC-I, whereas constitutive expression of MHC-II molecules is limited to professional antigen presenting cells (APCs) (dendritic cells, macrophages, B lymphocytes, and thymic epithelial cells), though expression can be induced in other cells types by inflammatory cytokines (particularly interferon gamma (IFN-γ)). Interaction of the T cell receptor (TCR) with peptide-MHC complexes is required for development, activation, and regulation of T lymphocytes. Expression of MHC-II is required for CD4 T cell survival and is critical to the formation of an adaptive immune response. However, inappropriate expression of MHC-II molecules can be just as detrimental, as this can lead to CD4 T cell-mediated autoimmune disorders. The expression of MHC-II molecules and associated proteins is coordinately regulated by a set of transcription factors that recognize a shared regulatory motif.

MHC-II and Disease

The appropriate expression of MHC-II molecules is tightly linked with a healthy immune response. Epidemiological studies consistently show strong linkage between the MHC-II locus and multiple autoimmune diseases (Fernando, Stevens et al. 2008;

Handunnetthi, Ramagopalan et al. 2010). Among those diseases associated with the MHC-II locus are: rheumatoid arthritis, multiple sclerosis, type I diabetes, systemic lupus erythematosus, inflammatory bowel disease, celiac disease, Crohn's disease, and ulcerative colitis (Haines, Ter-Minassian et al. 1996; Vyse and Todd 1996; Friese, Jones et al. 2005; Swanberg, Lidman et al. 2005; Barcellos, Sawcer et al. 2006; Kanazawa, Ota et al. 2006; Hill, King et al. 2008; Forabosco, Bouzigon et al. 2009). Expression of MHC-II molecules also plays a role in cancers and immune system surveillance. Many cancers display altered levels of MHC-II molecules, which is often correlated with disease prognosis. For example, melanoma cells often constitutively express MHC-II molecules, a characteristic not seen in normal melanocytes, and melanoma expression of MHC-II molecules is correlated with poor prognosis (Goodwin, Xi et al. 2001). Some viruses and bacteria are also known to reduce MHC-II levels on infected cells by targeting the master regulator of MHC-II expression (Pennini, Pai et al. 2006; Lee, Wang et al. 2011). Abnormal MHC-II expression is most often the result of altered expression from the master regulator of MHC-II genes (CIITA, discussed below) (Holling, Schooten et al. 2004; Radosevich, Jager et al. 2007; van der Stoep, Quinten et al. 2007). Reduction of MHC-II expression reduces the affected cell's potential to present antigenic peptides to T cells, while increased MHC-II expression (without a corresponding increase in costimulatory molecules) may promote T cell anergy. Therefore, a characteristic of some cancers, bacteria and virus-infected cells is aberrant expression of MHC-II genes, which aids in evasion of immune system surveillance.

Bare Lymphocyte Syndrome: Bare lymphocyte syndrome (BLS) is a hereditary genetic disorder characterized by the lack of both constitutive and inducible expression of MHC-II molecules (Clement 1990). BLS is a deadly autosomal recessive immunodeficiency disorder that results in severe defects in cellular and humoral immunity. BLS patients suffer from recurrent infections, failure to thrive, and often death at an early age (Griscelli,

Lisowska-Grospierre et al. 1989; Mach, Steimle et al. 1994). Though the disease is genetically heterogeneous, the clinical outcome is the same, regardless of the genetic lesion. Because patients with BLS lack both constitutive and inducible expression of all MHC-II molecules, cell lines derived from these patients became a valuable tool for identifying transcription factors required for the expression of MHC-II genes.

MHC-II genes and accessory molecules

MHC-II genes encode cell surface glycoproteins that present exogenous antigen to CD4 T cells. There are three isotypes: human leukocyte antigen (HLA) –DR, -DQ, and -DP, which are expressed as αβ heterodimers. These genes are located in a gene dense region on the short arm of chromosome 6 (chr 17 in mice) (mapped in reference (1999)) (Figure 1). In mice, these genes are known as *I-A* and *I-E*, and both are also heterodimers of alpha and beta chains. In addition, there are several accessory genes (*HLA-DM, HLA-DO*, and *Ii*), which are coordinately regulated and involved in various aspects of peptide processing, selection, and loading onto the MHC molecules. In the endoplasmic reticulum (ER), MHC-II alpha and beta molecules form a heterodimer that associates with the invariant chain (Ii). Ii binds to the peptide groove of the MHC-II heterodimer. The Ii serves multiple functions: Ii stabilizes the MHC-II complex and prevents MHC-II molecules from binding endogenous peptides present in the ER, Ii also directs the complex to specialized endosomal compartments (Bakke and Dobberstein 1990; Bangia and Watts 1995; Cresswell 1996). The low pH in the endosomal compartment promotes cleavage of Ii, leaving a short fragment bound to the peptide-binding groove of MHC-II, called the MHC-II associated peptide (CLIP). The heterodimer HLA-DM/DO is also transported from the ER to the lysosomal compartment, where HLA-DM associates with the CLIP bound MHC-II heterodimer. HLA-DM acts as a chaperone molecule, promoting exchange of CLIP for another peptide (presumably non-

self) (Denzin and Cresswell 1995; Roche 1995). HLA-DM mediates peptide exchange with the MHC-II heterodimer, promoting interaction between the MHC-II heterodimer and a peptide with high affinity for the peptide-binding groove. HLA-DO molecules have been detected in B cells, thymic medullary epithelial cell, and dendritic cells (Karlsson, Surh et al. 1991; Chen, Reed-Loisel et al. 2006; Hornell, Burster et al. 2006). HLA-DO functions as an inhibitor of HLA-DM (Denzin, Sant'Angelo et al. 1997), though the actual *in vivo* function is more complex. HLA-DO in B cells has been proposed to modulate the antigenic repertoire presented by MHC-II molecules (Perraudeau, Taylor et al. 2000; van Ham, van Lith et al. 2000). Once formed, the peptide-bound MHC-II heterodimer is transported to the cell surface membrane, where it is able to interact with cognate CD4 T cells.

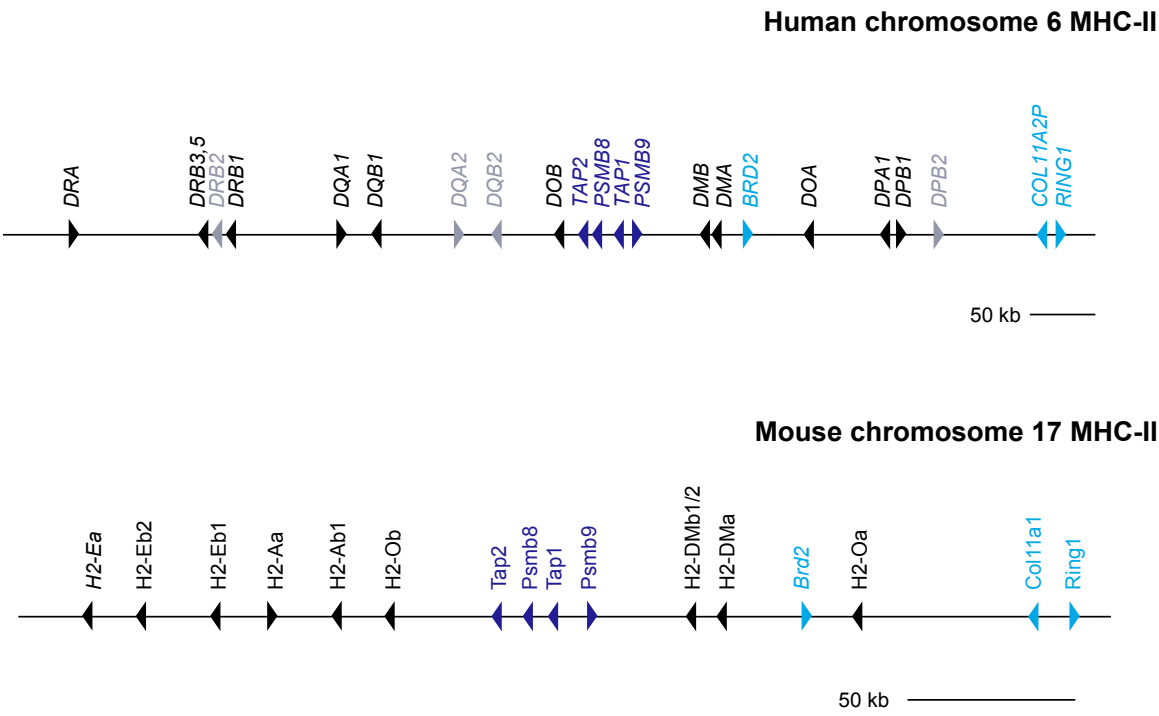


Figure 1: MHC-II locus. Schematic of classical and non-classical MHC-II genes (black), pseudogenes (grey), antigen processing and proteosome genes (purple), and non-MHC genes (blue). Figure modified from (Choi, Majumder et al. 2011).

MHC-II expression in antigen presenting and non-hematopoietic cells

Expression of MHC-II molecules is tightly regulated, both temporally and spatially. Constitutive expression is limited to particular cell types, and expression can be induced or repressed depending on the stimuli present. Professional antigen presenting cells (APCs: B cells, dendritic cells, and macrophages) are specialized for the presentation of foreign antigens to CD4 T cells, thus initiating an adaptive immune response. APCs express high levels of MHC-II molecules, though almost all cells can be stimulated to express MHC-II. Although APCs highly express MHC-II genes, MHC-II levels continuously change as these cells undergo developmental changes.

Antigen Presenting Cells: B cells constitutively express MHC-II molecules, and expression is modulated throughout development of the B-lymphocyte lineage (Kara and Glimcher 1993). Early pro-B cells are MHC-II negative, low levels are detected in pre-B cells, and expression is increased by exposure to interleukin-4 (IL-4). Mature B cells constitutively express high levels of MHC-II molecules, and expression can be further increased by exposure to IL-4. As activated B cells transition to antibody secreting plasma cell, expression of MHC-II is turned off (Silacci, Mottet et al. 1994). B cells are specialized for the presentation of immunoglobulin captured antigen.

Dendritic cells (DC) are highly efficient at antigen capture and presentation and DC function is dependent on expression of MHC-II molecules. Circulating DC and tissue residing DC (Langerhans) can be recruited to the sight of pathogen invasion by cytokine signals. Immature dendritic cells collect antigen at the site of infection. As the dendritic cells process the antigen, they migrate to lymph nodes and mature into potent immunostimulatory DC. In immature DC, most MHC-II molecules reside in internal compartments, and turnover of surface MHC-II molecules is rapid. Upon exposure to pathogenic molecules, DC turn off transcription of *MHC-II* genes while at the same time

increase surface expression of MHC-II molecules (Landmann, Muhlethaler-Mottet et al. 2001). This is accomplished by increasing the protein stability of existing MHC-II molecules and transporting MHC-II molecules bound to antigenic peptides to the surface (Askew, Chu et al. 2000). Shutting down production of new MHC-II molecules in effect "freezes" the peptide repertoire expressed by DC to antigens present at the time of stimulation. Upon arrival at the lymph node, the now mature DC is capable of stimulating a potent T cell mediated immune response.

Dendritic cells can be derived from multiple lineages and can vary in antigen processing capacity and effecter functions (Vremec and Shortman 1997). For example, thymic dendritic cells, in contrast to Langerhans or circulating DC, present self-antigen to developing T cells, and play an important role in T cell negative selection. Dendritic cells can be divided into a number of subtypes based on cell surface markers. There are two major groups: conventional, characterized by high CD11c expression, and plasmacytoid, characterized by intermediate CD11c expression and CD45RA (Vremec and Shortman 1997). Dendritic cells can also activate NK cells, a component of innate immunity. Therefore, DCs play an important role in both innate and adaptive immune responses.

Macrophages, like DC, also constitutively express low levels of MHC-II molecules, and can be recruited to the site of pathogen infection by cytokine signals. Activation, particularly by stimulation with the cytokine IFN-γ, increases MHC-II expression. Macrophages are phagocytic cells, and often uptake antigen though the phagocytosis of damaged cells and pathogens. Both macrophages and dendritic cells are specialized to present pathogenic molecules to cognate T cells. Also, macrophages and dendritic cells are unique from B cells in that they function in both innate and adaptive immunity.

Non-bone marrow derived cells: Non-professional antigen presenting cells typically do not constitutively express MHC-II molecules. However, most cells can be induced to express MHC-II molecules by a variety of stimuli, IFN-γ being the most potent. An exception to this is fetal throphoblast cells. These cells are refractory to IFN-γ induction, a characteristic important for preventing maternal immune responses against the fetus. T cell development in the thymus is also dependent on the expression of MHC-II molecules. MHC-II molecules are present on thymic epithelial cells, in both the cortex and medulla, and on interdigitating reticular cells (Chang, Guerder et al. 1996).

Thymocyte Education

T lymphocytes undergo a process known as "thymic education" during development in the thymus. This a sequential process in which T cells must first receive survival signals in the cortex of the thymus (positive selection), and then survive negative selection in the medulla of the thymus. The goal of this process is to end up with T cells that recognize MHC-II molecules bound to foreign peptides, but that do not respond to MHC-II molecules bound to self-peptides.

Positive Selection. Double-negative CD4-CD8- T lymphocytes enter the cortex of the thymus through blood vessels. These cells develop into double-positive CD4+CD8+ T cells, which encounter peptide bound to MHC-II molecules presented by cortical thymic epithelial cells (Benoist and Mathis 1989). This interaction is required for survival, and cells that fail to interact with cognate cortical thymic epithelial cells die by neglect. The majority of double positive T cells die in the thymic cortex (Egerton and Scollay 1990). During this process, only T cells that recognize peptide bound to MHC molecules receive survival signals. These cells also undergo lineage commitment, becoming single-positive (CD4+ or CD8+) (Benoist and Mathis 1989). Antigen presenting cells in the thymus are limited to

presenting self-peptide bound to self-MHC. Hence, all T cells must be selected to recognize self peptide-MHC complexes (Benacerraf 1985). This poses a problem, as an interaction between a T lymphocyte and self-peptide bound to MHC in the periphery would result in an autoimmune reaction. After surviving positive selection, T lymphocytes proceed to the medulla of the thymus, where they undergo negative selection.

Negative Selection. Single positive thymocytes proceed from the thymic cortex to the thymic medulla, where they encounter peptide bound to MHC molecules presented by dendritic cells and medullary thymic epithelial cells (mTEC). In this setting, interaction with cognate peptide-MHC leads to death by negative selection. This process is crucial for central tolerance by eliminating cells that would be self-reactive (Klein, Hinterberger et al. 2009). While thymic dendritic cells play a crucial role in central tolerance, this is not the only cell type capable of mediating T cell negative selection. When CD11c high dendritic cells were selectively eliminated, neither thymic negative selection nor regulatory T cell generation was markedly affected (Birnberg, Bar-On et al. 2008). Experiments utilizing bone marrow chimeras indicated that half to two-thirds of CD4 single positive cells (CD4SP) (that survived positive selection) were eliminated by negative selection (van Meerwijk, Marguerat et al. 1997). In this model, MHC-II molecules were expressed on radiation resistant thymic epithelial cells (allowing positive selection), but not hematopoietic cells (dendritic cells), which were transferred from a mouse lacking MHC-II gene expression. Reduction of MHC-II molecules on mTECs also changes the T cell repertoire. Hinterberger et al. (Hinterberger, Aichinger et al. 2010) demonstrated the importance of MHC-II expression in mTEC by selectively knocking down expression using a designer micro-RNA (miRNA). The miRNA was driven by an mTEC specific promoter and targeted the gene encoding the master regulator of MHC-II (CIITA, discussed below). This resulted in reduced mRNA expression of all MHC-II genes and several accessory molecules (*H2-Oa, -Ob, -DMB2, -*

Aa, -Ab1, -Ea, -Eb1, and CD74), and correspondingly decreased surface expression of MHC-II molecules specifically on mTECs (Hinterberger, Aichinger et al. 2010). Importantly, there was no reduction in MHC-II expression on DC. Reduction of MHC-II molecules only on mTECs resulted in an increased number of CD4 T cells, and an increase in the ratio of $Foxp3^+$ T_{reg} cells present in the CD4 single positive T cell compartment. Additionally, if radiation treated mice were reconstituted with bone marrow cells deficient in MHC-II gene expression (resulting in MHC-II deficient mTEC and DC), the number of CD4 T cells was further increased. These experiments demonstrated an additive, non-redundant role for MHC-II molecule expression on both mTEC and DC during CD4 single positive T cell negative selection in the thymic medulla, leading to central tolerance.

Therefore, during development, interaction with peptide-MHC is both required for survival and lineage commitment (positive selection), but may also induce cell death (negative selection) (Benoist and Mathis 1989; Klein, Hinterberger et al. 2009). Low or no interaction leads to death by neglect in the thymic cortex, whereas strong interaction leads to death by negative selection in the thymic medulla. These fate-determining interactions occur in the thymus, where T cells interact with peptide-MHC presented by thymic epithelial cells and dendritic cells.

II. Regulation of MHC-II expression: The WXY box

Expression of MHC-II molecules is controlled primarily at the level of transcription (Benoist and Mathis 1990; Boss 1997). Therefore, in order to understand MHC-II expression, the promoter region of MHC-II genes and required transcription factors have been extensively studied. The study of BLS patients, which are characterized by lack of MHC-II molecule expression, as well as reduction in the accessory molecules HLA-DM, HLA–

DO, and the Ii chain, has played a central role in understanding the regulation of MHC-II genes (Griscelli, Lisowska-Grospierre et al. 1989; Reith and Mach 2001). The generation of cell lines from BLS patients, as well as *in vitro* derived MHC-II deficient cells lines, allowed for the identification of four complementation groups (A, B, C, and D) (Calman and Peterlin 1987; Hume and Lee 1989; Benichou and Strominger 1991; Stimac, Urieli-Shoval et al. 1991; Seidl, Saraiya et al. 1992). Each of the genetic defects identified in these four complementation groups are located in transcription factors required for expression of MHC-II molecules, not in the MHC-II genes themselves (Seidl, Saraiya et al. 1992; Steimle, Otten et al. 1993; Steimle, Durand et al. 1995; Durand, Sperisen et al. 1997; Moreno, Rogers et al. 1997; Nagarajan, Louis-Plence et al. 1999). Knocking out the regulatory mechanism responsible for MHC-II gene expression results in a more severe phenotype than merely losing expression of one of the MHC-II molecules. These transcription factors are common to all cell types, and interact with a regulatory motif shared by all MHC-II genes and associated molecules.

MHC-II transcription factors and the WXY box

Promoters of MHC-II genes and accessory genes (*Ii, HLA-DM*, and *HLA-DO*) are characterized by an upstream enhancer element known as the WXY (or SXY) module, which is composed of the W/S/Z, X1, X2, and Y box elements (Benoist and Mathis 1990). Most of the factors that bind the WXY box have been identified. Nuclear factor Y (NF-Y, consisting of NF-Ya, NF-Yb, and NF-Yc) complex binds to the Y box (Hooft van Huijsduijnen, Li et al. 1990; Mantovani, Pessara et al. 1992; van den Elsen, Holling et al. 2004). The W/S box is important for recruitment of the MHC-II transactivator (CIITA), though the mechanism is not known. The X box interacts with multiple transcription factors. These elements allow

for coordinated regulation of the MHC-II genes and MHC-II associated genes necessary for peptide-MHC-II expression.

Multiple proteins bind the X box, which is subdivided into X1 and X2. The cAMP response element binding protein (CREB)/ activating transcription factor (ATF) binds at the X2 box (Moreno, Beresford et al. 1999). Studies of cell lines comprising the four BLS complementation groups A, B, C, and D, identified factors interact with the X box. BLS patient derived cells lines Ramia and SJO were classified into groups B and C, respectively (Benichou and Strominger 1991; Lisowska-Grospierre, Fondaneche et al. 1994). Additionally, the experimentally derived cell lines RJ2.2.5 (derived from the human B cell line Raji) and 6.1.6 was classified into groups A and D, respectively (Gladstone and Pious 1978; Accolla 1983; Calman and Peterlin 1987). Complementation cloning of the genes defective in groups B, C, and D, identified factors that form the regulatory factor X (RFX) complex and bound the X1 box (Reith, Steimle et al. 1995; Steimle, Durand et al. 1995; Durand, Sperisen et al. 1997; Nagarajan, Louis-Plence et al. 1999). The RFX complex consists of RFX5, RFX-B (RFXANK), and RFXAP (Moreno, Rogers et al. 1997; Masternak, Barras et al. 1998). Complementation cloning in the cell line RJ2.2.5, which was classified into complementation group A, identified a factor that is now known as the MHC-II transactivating factor, or CIITA (Steimle, Otten et al. 1993).

In Groups B, C, and D, the promoter region of MHC-II genes is not occupied, this contrasts to Group A in which the WXY region is occupied (Kara and Glimcher 1991). This is because the factors that bind the WXY motif cooperatively bind the promoter region, and if one factor is missing, a stable RFX complex is not able to form (Moreno, Emery et al. 1995; DeSandro, Nagarajan et al. 2000). The factors that bind the WXY region are ubiquitously expressed; however, transcription does not occur unless the MHC class II transactivator (CIITA) is also present (Chang, Fontes et al. 1994; Riley, Westerheide et al. 1995; Chang,

Guerder et al. 1996; Morris, Riley et al. 1998). CIITA does not bind DNA, but has been shown to interact with the complex that binds the WXY box, including: RFX5, RFXANK, CREB, NF-Yb and NF-Yc (Masternak, Muhlethaler-Mottet et al. 2000; Beresford and Boss 2001) (Riley, Westerheide et al. 1995; DeSandro, Nagarajan et al. 2000; Zhu, Linhoff et al. 2000). CIITA is the limiting factor in MHC-II gene expression, and is quantitatively correlated with MHC-II expression (Otten, Steimle et al. 1998; Swanberg, Lidman et al. 2005). Mice lacking CIITA do not express MHC-II molecules in almost all cell types (discussed below). Therefore, CIITA has become known as the master regulator of MHC-II expression, as expression of MHC-II is primarily controlled at the level of transcription, which in turn is controlled by expression of CIITA.

III. Regulation of MHC-II expression: CIITA

Successful antigen presentation is dependent not only on the proper expression of MHC-II molecules, but also on the production of accessory molecules, such as HLA-DM and Ii, which are required for the processing of antigen and loading of antigen onto MHC-II molecules (Roche 1995). The master regulator of MHC-II genes, major histocompatibility class II transactivator (CIITA), coordinately regulates these genes (Chang and Flavell 1995). CIITA is a non-DNA binding protein that interacts with the enhanceosome complex present at the WXY box upstream of MHC-II promoters (Beresford and Boss 2001). CIITA promotes transcriptional activity by recruiting histone modifying enzymes, chromatin modifiers, and general transcription factors.

The gene encoding CIITA (the aIr-1 locus (Accolla, Jotterand-Bellomo et al. 1986)) resides on chromosome 16 in both mice and humans and spans about 50 kb (40 kb in mice). It contains four promoters, though promoter II is not conserved in mice, and little is known

about the function of this promoter (Muhlethaler-Mottet, Otten et al. 1997). Each promoter encodes a unique first exon, which is spliced into a common second exon (Figure 2). Downstream exons 2-20 (2-19 in mice) are transcribed from all three promoters for a total mRNA length of about 3 kb (5 kb in mice) that differs only at the 5' end. The first exon of promoters I and III contains a translation start site, while translation from promoter IV starts in the second exon, resulting in three isoforms differing at the N-terminus (Muhlethaler-Mottet, Otten et al. 1997). Translation of the transcript produced by promoter I results in an additional 90 amino acids, and promoter III adds 17 amino acids, compared to the protein produced by promoter IV (Figure 2).

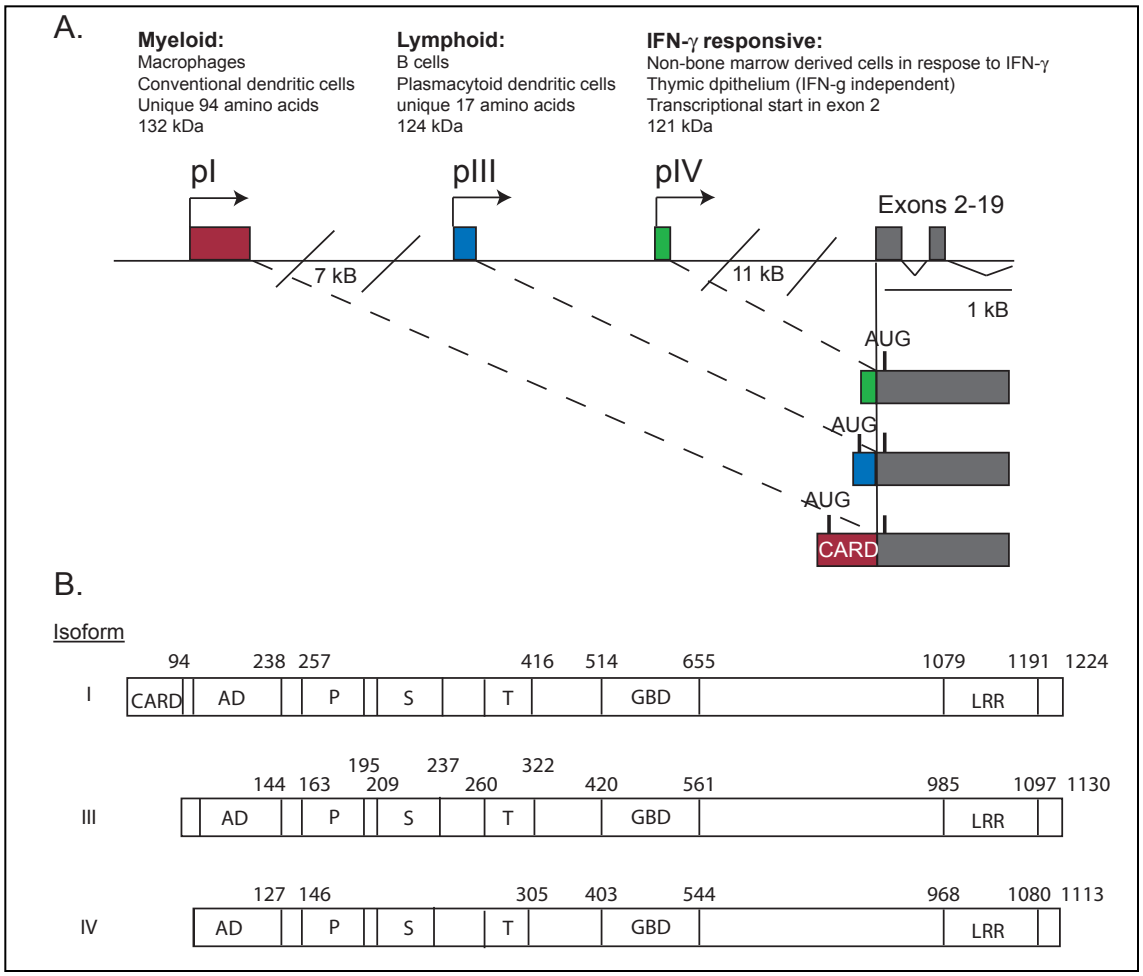


Figure 2: CIITA promoter region. A. Schematic representation of the three promoters of CIITA conserved between human and mouse. Each promoter encodes a unique first exon, which is spliced into a common exon 2. B. Diagram of CIITA domains. Numbers represent the amino acid at which each domain begins or ends. The PST region can be divided into separate proline, serine, and threonine rich regions. For clarity, the amino acids delineating these regions are indicated for isoform III only. CARD: caspase recruitment domain; AD: activation domain; P/S/T: proline, serine, and threonine-rich region; GBD: GTP-binding domain; LRR: leucine-rich repeat containing domain.

CIITA is required for the expression of MHC-II, and enhances expression of MHC-II associated genes

Expression of CIITA is required for transcription from MHC-II genes, as evidenced by a subset of BLS patients in which CIITA expression is lacking. In cell lines, CIITA has been shown to regulate expression of HLA-DR, HLA-DM, Ii, and IFN-γ induced MHC-I genes (Chang and Flavell 1995; Kern, Steimle et al. 1995; Martin, Chin et al. 1997). Extopic expression of CIITA in multiple MHC-II null cell lines has been shown to induce MHC-II (Chang, Fodor et al. 1992; Chang, Fontes et al. 1994; Chang and Flavell 1995; Chang, Hong et al. 1995; Lennon, Ottone et al. 1996; Bradley, Fernandez et al. 1997). Several mouse models have been developed to aid in the study of CIITA function *in vivo*.

Two CIITA null models have been developed, (Chang, Guerder et al. 1996; Williams, Malin et al. 1998), and a third model in which the GTP region of CIITA was deleted resulted in a mouse that was essentially null for CIITA expression (Itoh-Lindstrom, Piskurich et al. 1999). The mice recapitulate the T cell defects seen in MHC-II null mice (H2-Aβ $^{-/-}$ mice) (Cosgrove, Gray et al. 1991; Grusby and Glimcher 1995) and most of the phenotypes observed in BLS patients.

In all three CIITA$^{-/-}$ mouse models, MHC-II expression was significantly reduced on B cells, macrophages, and splenic dendritic cells (Chang, Guerder et al. 1996; Williams, Malin et al. 1998; Itoh-Lindstrom, Piskurich et al. 1999). In one CIITA$^{-/-}$ mouse model H-2M-β (an MHC-I molecule) expression was also significantly reduced in B cells and splenic dendritic cells, while H2-O-α and H2-O-β appeared to be unaltered (Chang, Guerder et al. 1996). Ii was also reduced, but to a lesser extent than MHC-II molecules (Chang, Guerder et al. 1996; Itoh-Lindstrom, Piskurich et al. 1999). Cytokine induction (IFN-γ, LPS, or IL-4) of MHC-II expression was also abrogated in peritoneal macrophages, as well as cells from the spleen, thymus, liver, and lung (Chang, Guerder et al. 1996; Itoh-Lindstrom, Piskurich et al. 1999).

There appeared to be some non-CIITA directed MHC-II molecule expression, as MHC-II molecules were detected on subsets of cells in the thymus, spleen, and lymph nodes (Chang, Guerder et al. 1996; Williams, Malin et al. 1998; Itoh-Lindstrom, Piskurich et al. 1999). The cells expressing MHC-II molecules in the spleen and lymph nodes are most likely B cells and dendritic cells, respectively (Williams, Malin et al. 1998). One CIITA knockout also expressed MHC-II in the germinal center (Williams, Malin et al. 1998). The possibility exists that there may be a truncated form of CIITA, as the entire CIITA gene was not deleted in any of these CIITA knockout models. This is not likely the case because the three targeting constructs deleted different regions of the CIITA gene, and MHC-II expression was very similar in all of the CIITA$^{-/-}$ mouse lines generated. The targeting construct generated by Chang et al. deleted exons 13 and 14 (Xu, McDonald et al. 2007), the construct generated by Williams et al. deleted exons 2 and 3, and the construct designed by Itoh-Lindstrom deleted the GTP binding domain. More likely, there is some MHC-II gene transcription that is independent of CIITA, or even independent of transcription factors binding to the WXY box. This is consistent with the observation that MHC-II surface expression is not completely abrogated in most BLS patients, nor is it completely abrogated on dendritic cells and B cells from RFX5-deficient mice (Clausen, Waldburger et al. 1998), or CIITA/RFX5 double-deficient mice (Buch, Polic et al. 2006).

Observations of T cells in the CIITA$^{-/-}$ models demonstrate the importance of MHC-II expression for CD4 T cell positive selection. In all of the CIITA$^{-/-}$ models, the small amount of MHC-II observed in the thymus of the CIITA$^{-/-}$ mice was insufficient to promote efficient positive T cell selection, as these mice had severely reduced numbers of CD4 T cells in the periphery, and single positive CD4 T cells in the thymus were not detected, or were present at very low levels (Chang, Guerder et al. 1996; Itoh-Lindstrom, Piskurich et al. 1999). Even though CD4 T cells were severely reduced, there appeared to be a low level of positive

selection, probably mediated by the residual levels of MHC-II detected in the thymus. This conclusion is based on the observation that the low numbers of CD4 T cells observed were higher than that seen in H2-Aβ$^{-/-}$, which were completely MHC-II null (Williams, Malin et al. 1998). C57BL/6 H2-Aβ$^{-/-}$ mice do not express surface MHC-II molecules due to the introduced deletion of the H2-Aβ chain and an existing deletion in the H2-Eα gene. Again, this recapitulates the phenotype seen in human BLS patients, as CD4 T cells are present, though at low levels (Griscelli, Lisowska-Grospierre et al. 1989). Cell transfer experiments into irradiated mice demonstrated that there was no intrinsic defect in T cells lacking CIITA, rather the deficiency in CD4 T cells observed in the CIITA$^{-/-}$ mice is most likely due to lack of MHC-II expression on cells of the thymus that interact with T cells during T cell development (Chang, Guerder et al. 1996).

The residual amount of MHC-II detected in all of the CIITA$^{-/-}$ mouse models suggests that there is some MHC-II gene transcription that is CIITA independent. The amount of CIITA-independent MHC-II expression produced is low, and the numbers and type of cells displaying this phenotype appears to be limited. Collectively, these models demonstrate that the loss of CIITA results in the (almost complete) loss of constitutive and inducible MHC-II protein expression and CD4+ T cell positive selection, as well as a reduction in the expression the invariant chain (Ii), a MHC-II accessory molecule.

IV. CIITA and expression of non-MHC-II genes

In addition to mouse knockouts, several approaches have been utilized to hunt for additional targets of CIITA. A microarray analysis was conducted to compare gene expression in the Raji B cell line compared to the CIITA deficient RJ2.2.5 B cell line (Nagarajan, Bushey et al. 2002). Representational difference analysis (RDA) was used to

compare cDNA from G3A fibrosarcoma cells stably transfected with CIITA compared to vector control (Taxman, Cressman et al. 2000). RDA analysis is a method by which cDNA sequences that are enriched in CIITA transfected vs. vector control samples are enriched for by subtractive hybridization, amplified by PCR, and identified by cloning and sequencing. These cDNA analyses identified many genes that were either up-regulated or down-regulated in the CIITA containing cell line vs. the CIITA null line. Analysis of mRNA expression does not demonstrate a direct relationship between CIITA and gene expression. Therefore, chromatin immunoprecipitation (ChIP) coupled with microarray analysis (ChIP-chip using a promoter microarray) was used to search genome-wide for targets that are bound by CIITA (Krawczyk, Seguin-Estevez et al. 2008). These analyses confirmed CIITA binds to MHC-II genes and MHC-II associated genes including: *HLA-DRA, -DRB1, -DRB5, -DQA, -DQB, -DOA, -DOB, -DMA, -DMB, -DPA, -DPB*, and *Ii*. In addition, several new potential target genes were identified.

The ChIP-chip analysis identified nine new potential target genes for CIITA, for a total of 21 genes bound by CIITA in B cells and dendritic cells. Quantitative ChIP analysis confirmed CIITA was present at all of these genes in Raji cells, and at eight of these genes in dendritic cells (Krawczyk, Seguin-Estevez et al. 2008). These genes are: *RAB4B, TRIM26, FLJ45422*, *KIAA0841, RFX5, ZNF672*, *MYBPC2, TPP1*, and *PSMD3*. Compared to other known transcription factors, CIITA binds relatively few genes, and binding of CIITA is dependent on RFX5. The selectivity seen in CIITA binding is likely a result of the fact that CIITA is dependent on the formation of an enhanceosome complex at the WXY box of these promoters.

Even though CIITA has been confirmed to bind at multiple genes, the role of CIITA in expression of several of these genes has been controversial. For example, CIITA binds *HLA-DO*, but may not be required for expression of this gene. No change in expression of *HLA-*

DOB was observed in the RDA analysis (Taxman, Cressman et al. 2000). Furthermore, no change in expression in *H2-Ob* was observed in spleens from CIITA$^{-/-}$ mice (Chang, Guerder et al. 1996). In contrast, CIITA does appear to be required for maximal expression of *HLA-DOB* in Raji cells (Nagarajan, Bushey et al. 2002; Nagarajan, Lochamy et al. 2002). The role of CIITA in expression of *COL1A2* is also questionable. CIITA was found to represses collagen expression in IFN-γ induced cells, however, no effect on collagen expression was observed in cells generated from CIITA$^{-/-}$ mice (Xu, Wang et al. 2004; Xu, McDonald et al. 2007). The finding that CIITA binds the promoter of a gene does not *a priori* indicate a requirement for CIITA in expression.

MHC-I genes are also upregulated by CIITA binding under specific circumstances (Martin, Chin et al. 1997). CIITA increased expression of MHC-I genes in cell lines in response to IFN-γ (Gobin, Peijnenburg et al. 1997; Martin, Chin et al. 1997). In contrast, MHC-I gene expression was not affected in APCs, even after stimulation with IFN-γ from CIITA null mice (Williams, Malin et al. 1998; Itoh-Lindstrom, Piskurich et al. 1999). All of the cell types examined in the mouse knockout models expressed high constitutive levels of MHC-I genes. CIITA appears to enhance cytokine induced MHC-I expression only in cells that express low levels of MHC-I, while cells that typically express high levels of MHC-I are not dependent on CIITA for efficient expression of MHC-I genes (Martin, Chin et al. 1997).

CIITA has only been shown to directly regulate the expression of about 21 genes. CIITA expression is essential for transcription MHC-II genes, and enhances expression of MHC-II associated genes such as *Ii* and *HLA-DM*. The transactivator function of CIITA is dependent on the formation of an enhanceosome complex at the WXY box found at the promoters of all MHC-II genes. The role of CIITA in expression of genes such as non-classical MHC-II genes *HLA-DO* and *HLA-DM*, *COL1A1*, *PLXNA1*, *IL-4* and *MHC-I* may be to modulate expression in a cell-type and context dependent manner.

V. CIITA Gene Regulation

The region containing *CIITA* promoters spans 13 kb (10kb in mice) (Muhlethaler-Mottet, Otten et al. 1997). Sequence comparison of *CIITA* mRNA revealed four cDNA sequences, which differed only at the 5' end (Muhlethaler-Mottet, Otten et al. 1997). Mapping these sequences revealed that *CIITA* is expressed from four independent promoters, termed pI, pII, pIII, and pIV; each of which encodes a unique first exon (Muhlethaler-Mottet, Otten et al. 1997). Promoter II is not conserved in mouse, and expression in human samples was weak. Promoter II expression has been reported in melanoma cell lines (van der Stoep, Quinten et al. 2007), though expression from pII is weak and the role of pII is not understood. Promoters I, III, and IV are expressed in a tissue specific manner.

Promoter I: CIITA promoter I expression is expressed primarily by cells of the myeloid lineage. Dendritic cells express CIITA primarily from pI (Muhlethaler-Mottet, Otten et al. 1997; Pai, Askew et al. 2002) until maturation, at which point CIITA expression is turned off. Unstimulated macrophages express very low levels of CIITA and MHC-II, but exposure to IFN-γ induces CIITA expression from both promoters I and IV (Muhlethaler-Mottet, Otten et al. 1997; Waldburger, Suter et al. 2001; Pai, Askew et al. 2002; LeibundGut-Landmann, Waldburger et al. 2004). Although dendritic cells and macrophages express CIITA primarily from pI, promoters III and IV are also expressed. Deletion of pIII and pIV does not alter expression of MHC-II in these cell types (LeibundGut-Landmann, Waldburger et al. 2004). Therefore, not only is pI the dominant promoter expressed in myeloid cells, but pI is also sufficient for proper MHC-II expression in myeloid cells.

Promoter I was also determined to be sufficient for negative selection. In both pIV and pIII/pIV double knockout mouse models, it was determined that while positive selection was severely impaired, negative selection remained intact (Waldburger, Rossi et al. 2003; LeibundGut-Landmann, Waldburger et al. 2004). Thus it was concluded that the cell type required for negative selection must express CIITA via promoter I. The cell types most likely mediating positive selection are medullary thymic dendritic cells, as these cells maintained MHC-II expression in the pIII/pIV knockout model.

Promoter III: MHC-II is constitutively present on B cells, and disappears as B cells transition to plasma cells (Silacci, Mottet et al. 1994; Lennon, Ottone et al. 1997; Muhlethaler-Mottet, Otten et al. 1997; Piskurich, Wang et al. 1998). CIITA isoform III is the primary transcript detected in B cells, though some isoform IV can also be detected (Muhlethaler-Mottet, Otten et al. 1997; Nickerson, Sisk et al. 2001; Pai, Askew et al. 2002). Dendritic cells and human T cells also express low levels of CIITA isoform III (Muhlethaler-Mottet, Otten et al. 1997; Nickerson, Sisk et al. 2001; Holling, van der Stoep et al. 2002). Promoter III is also induced by IFN-γ in some human primary cells and tumor cell lines (Piskurich, Wang et al. 1998; Piskurich, Linhoff et al. 1999). The importance of CIITA pIII in B cells was demonstrated in CIITA promoter specific knockouts. B cells and dendritic cells isolated from pIV knockout mice continued to express MHC-II molecules at levels comparable to wild-type (Waldburger, Suter et al. 2001). The additional deletion of pIII (pIII/IV$^{-/-}$ double knockouts) resulted in loss of MHC-II gene expression in B cells, but not conventional dendritic cells, IFN-γ induced macrophages, or IFN-γ induced microglia (LeibundGut-Landmann, Waldburger et al. 2004). Plasmacytoid dendritic cells isolated from pIII/IV$^{-/-}$ knockout mice also lost MHC-II gene expression (LeibundGut-Landmann, Waldburger et al. 2004). CIITA pIII is required for expression of MHC-II molecules on both B cells and plasmacytoid dendritic cells.

CIITA promoter IV: Promoter IV is inducible by IFN-γ in cells of non-hematopoietic origin such as endothelia, epithelia, astrocytes, and fibroblasts (Muhlethaler-Mottet, Otten et al. 1997; Waldburger, Suter et al. 2001), as well as APCs such as monocyte/ macrophages (Muhlethaler-Mottet, Otten et al. 1997; Muhlethaler-Mottet, Di Berardino et al. 1998; Dong, Rohn et al. 1999; Piskurich, Linhoff et al. 1999; Rohn, Tang et al. 1999; Pai, Askew et al. 2002). In a CIITA $pIV^{-/-}$ mouse model, deletion of pIV abrogated IFN-γ induced expression of MHC-II genes on fibroblasts and astrocytes (non-bone marrow derived glial cells) (Waldburger, Suter et al. 2001). This effect was quantitative, as expression of MHC-II molecules on $pIV^{+/-}$ fibroblasts was intermediate between wild-type and knockout. No effect was seen on IFN-γ induced expression of peritoneal macrophages, dendritic cells, or microglia (central nervous system macrophages) (Waldburger, Suter et al. 2001). Furthermore, no change in constitutive MHC-II expression was observed any bone marrow derived cells examined, including: B cells, dendritic cells, and macrophages. MHC-II expression was also lost on cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) in the $pIV^{-/-}$ model (Waldburger, Suter et al. 2001; Waldburger, Rossi et al. 2003). Loss of MHC-II on cTEC resulted in severely reduced numbers of CD4+ T cells (Waldburger, Suter et al. 2001). This was determined to be due to the loss of positive selection, and negative selection was determined to remain intact (Waldburger, Rossi et al. 2003). Unlike expression of *CIITA* pIV in other non-bone marrow derived cell types, pIV expression in thymic epithelial cells is independent of IFN-γ stimulation (Waldburger, Suter et al. 2001). Expression of *CIITA* pIV is required for MHC-II gene expression in IFN-γ induced non-bone marrow derived cells and thymic epithelial cells.

VI. Protein structure and function of CIITA

Domains of CIITA

The human genome encodes nearly 20-25,000 protein coding genes (2004), though only a select few are active at any given time. DNA organization in the nucleus is crucial for control of gene expression. Central to this organization are histones: proteins around which the DNA is wrapped. Histones play a central role in DNA accessibility and transcriptional activity: transcriptionally active genes must be able to interact with components of the transcriptional machinery. Histones may be modified, which alters accessibility, thereby altering the transcriptional potential of a given region of DNA. CIITA acts as the master regulator of MHC-II genes primarily by promoting transcription of the genes to which it binds. The major functions CIITA appear to be to recruit a host of factors. These factors include histone modifying enzymes, nucleosome remodeling enzymes, and components of the basal transcriptional machinery.

Several structural motifs have been identified in the CIITA protein (Figure 2) (Steimle, Otten et al. 1993). Four of these domains are shared by all isoforms: an acidic activation domain (AD) (Riley, Westerheide et al. 1995; Zhou and Glimcher 1995), three proline-, serine-, and threonine-rich (PST) regions, a GTP-binding domain (GBD) (Chin, Li et al. 1997; Harton, Cressman et al. 1999), and four leucine rich repeats (LRR) (Hake, Masternak et al. 2000; Harton and Ting 2000). Additionally, the protein contains several nuclear localization sequences (NLS). A fifth domain, the N-terminal caspase recruitment domain (CARD), is unique to CIITA isoform I. Each of the shared domains has been identified as being required for proper function of the CIITA protein.

CIITA recruits transcription factors and mediate chromatin modifications

Many CIITA constructs containing mutations in various regions have been made to elucidate the functions of these domains (Zhou and Glimcher 1995; Chin, Li et al. 1997; Yun, Gustafsson et al. 1997; Zhou, Su et al. 1997; Brown, Rogers et al. 1998; Linhoff, Harton et al. 2001; Sisk, Nickerson et al. 2003). The majority of these constructs were based on the original form of CIITA discovered by Stiemle, et al., (Steimle, Otten et al. 1993), which was later identified as isoform III, and does not contain the CARD domain. The various functions of these domains as they relate to CIITA function are discussed below.

CIITA recruits components of the basal transcription machinery. The N-terminal domain contains an acidic activation domain (AD) (Riley, Westerheide et al. 1995; Zhou and Glimcher 1995), and is required for efficient recruitment of transcription factors and transcriptional activation of *MHC-II* promoters (Figure 3). CIITA has been shown to interact with the transcriptional activators TATA-binding protein (TBP) (Mahanta, Scholl et al. 1997), TBP-associated factor$_{II}$B ($TF_{II}B$), TBP-associated factor$_{II}$32 ($TAF_{II}32$) (Fontes, Jabrane-Ferrat et al. 1997), and positive transcription elongation factor b (P-TEFb) (Kanazawa, Okamoto et al. 2000; Drozina, Kohoutek et al. 2006).

CIITA coordinates histone acetylation at MHC-II promoters. In wild-type cells, DNA binding proteins constitutively bind the WXY box in front of MHC-II promoters. This binding is independent of CIITA, as proteins remain bound to the WXY box even in the absence of CIITA (Kara and Glimcher 1991). This was observed in the B cell line RJ2.2.5, which expresses wild-type RFX, but does not express functional CIITA. Even though the WXY box in RJ2.2.5 cells remained bound by multiple DNA binding proteins, the lack of CIITA resulted in altered chromatin status at *MHC-II* promoters. In RJ2.2.5 cells, decreased acetylation of H3 and H4 at the HLA-DRA promoter was observed; transfection with a constitutively expressing CIITA vector rescued this acetylation phenotype (Beresford and

Boss 2001). Transfection with a mutant CIITA protein lacking the N-terminal acidic activation resulted in acetlyation of H4, but not H3, at the HLA-DRA promoter (Beresford and Boss 2001). The GTP binding domain is also required for recruitment of histone acetyltransferases (Bewry, Bolick et al. 2007). CIITA has weak intrinsic histone acetyl transferase (HAT) activity located at the N-terminus (Raval, Howcroft et al. 2001), and the GTP-binding domain regulates its activity (Chin, Li et al. 1997). Additionally, CIITA recruits the HATs GCN5, p300/CBP and p300/CBP associated factor (PCAF) (Figure 3) (Fontes, Jabrane-Ferrat et al. 1997; Mahanta, Scholl et al. 1997; Kretsovali, Agalioti et al. 1998; Fontes, Kanazawa et al. 1999; Sisk, Gourley et al. 2000; Spilianakis, Papamatheakis et al. 2000; Ting and Trowsdale 2002; van den Elsen, Holling et al. 2004; Choi, Majumder et al. 2011). Removing the acidic activation region creates a dominant negative protein (Yun, Gustafsson et al. 1997; Brown, Rogers et al. 1998). Thus, CIITA mediates multiple HAT activities, and the recruitment of histone modifying enzymes is dependent on the presence of the AD and the GBD.

CIITA recruits chromatin remodeling enzymes. CIITA recruits the ATP-dependent chromatin remodeling factor Brahma-related gene 1 (BRG-1). BRG-1 is required for proper *MHC-II* gene induction (Mudhasani and Fontes 2002) and interacts with the GTP binding domain of CIITA (Mudhasani and Fontes 2005). BRG-1 is a component of the SWI/SNF (mating type switching/ sucrose non-fermenting) complex, which facilitates chromatin remodeling and nucleosome eviction at the WXY box (Choi, Majumder et al. 2011).

CIITA function is dependent on recruitment to the WXY box at MHC-II gene promoters. Domains downstream of the AD are required for interaction with proteins composing the RFX complex, and subsequently are responsible for targeting of CIITA to the WXY box (Zhou and Glimcher 1995) (Figure 3).

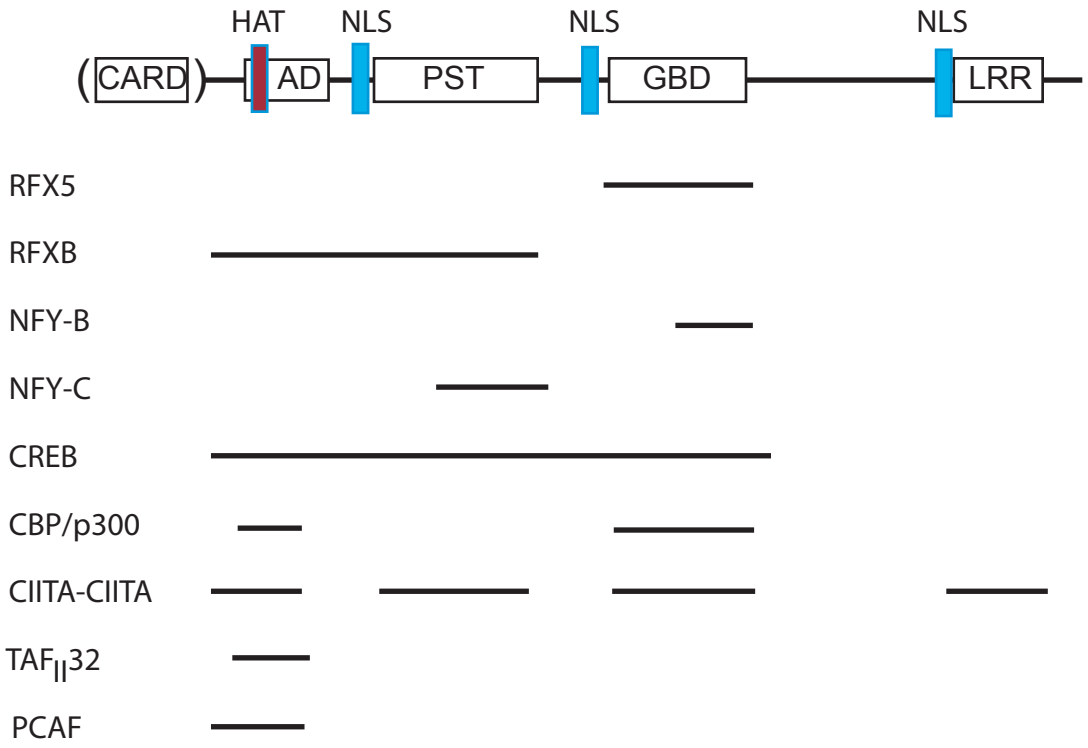


Figure 3: CIITA-protein interactions. Schematic representation of CIITA domains and the proteins they interact with. Black bars indicate the region of CIITA shown to be required for interaction with the indicated protein.

CIITA protein modification modulates activity

The transcriptional activity of CIITA can be modulated by phosphorylation and subsequent ubiquitylation. For example, mono-ubiquitylation of CIITA increased binding of P-TEFb to *MHC-II* promoters, which resulted in increased transcription of *MHC-II* genes (Tosi, Jabrane-Ferrat et al. 2002; Greer, Zika et al. 2003; Drozina, Kohoutek et al. 2006). Phosphorylation also increased CIITA activity (Tosi, Jabrane-Ferrat et al. 2002). Phosphorylation likely increases activity by altering CIITA protein structure, increasing oligomerization (Tosi, Jabrane-Ferrat et al. 2002) and nuclear localization, or by promoting mono-ubiquitylation.

Protein Stability: The N-terminal domain mediates the half-life of the protein. Endogenous CIITA has a half-life of about 30 minutes (Schnappauf, Hake et al. 2003). In contrast, N-terminally truncated CIITA proteins had greatly increased half-lives (over four hours) (Schnappauf, Hake et al. 2003). Addition of amino acids to the N-terminus had the same effect. Labeling the N-terminus of CIITA with GFP, Flag, Myc, or HA tags also increased the half-life to over 4 hours (Greer, Zika et al. 2003; Schnappauf, Hake et al. 2003). Two regions that promote proteolytic cleavage (termed degrons) have been identified in the N-terminus of CIITA (Schnappauf, Hake et al. 2003). One of these degrons is located in acidic activation domain, the other is just upstream of the PST region. Phosphorylation of a serine in the degron adjacent to the PST domain promotes ubiquitylation. Subsequent polyubiquitylation leads to rapid degradation (Drozina, Kohoutek et al. 2006). These experiments suggest that N-terminal amino acid additions (such as HA tags and GFP) interfere with the N-terminal degradation signals, increasing protein stability.

Nuclear localization: CIITA shuttles between the nucleus and cytosol. This activity is regulated in part by multiple nuclear localization signals (NLS), GTP-binding, and the LLR region (Chin, Li et al. 1997; Cressman, Chin et al. 1999; Harton, Cressman et al. 1999; Hake, Masternak et al. 2000; Sisk, Roys et al. 2001). CIITA contains multiple nuclear localization signals (NLS): the first NLS is located between the AD and PST domains, the second NLS is found just upstream of the GTP domain, and a third NLS is located upstream of the LRR (Figure 3) (Cressman, O'Connor et al. 2001). CBP and pCAF acetylation of CIITA, and phosphorylation of the PST region, promotes nuclear accumulation (Spilianakis, Papamatheakis et al. 2000; Tang, Trzaska et al. 2008). Mesenchymal stem cells are able to inhibit nuclear localization of CIITA, thereby decreasing *MHC-II* transcription without altering total levels of CIITA protein (Tang, Trzaska et al. 2008). Isoforms III and IV translocate to the nucleus with similar efficiency (Barbieri, Deffrennes et al. 2002). Altering

nuclear localization of CIITA, and therefore controlling access of CIITA to *MHC-II* promoters, modulates MHC-II transcription without changing CIITA protein expression.

Self association: Two molecules that share structural homology with CIITA are NOD1 and APAF-1. All three of these molecules contain an N-terminal CARD, a nucleotide binding domain (NBD), and a C-terminal domain (LLR in CIITA and NOD1, WD-40 repeats in APAF-1) (Adrain, Slee et al. 1999; Inohara, Koseki et al. 1999). NOD1 and APAF-1 are involved in inflammation and apoptosis and are known to self-interact. This interaction has been shown to be important for regulating activity (Hu, Ding et al. 1998; Bertin, Nir et al. 1999; Inohara, Koseki et al. 1999). CIITA has been shown to interact with itself in a similar manner. Both the GDB and LRR domains mediate CIITA self-associations, allowing for multiple potential inter- and intra-molecular interactions (Figure 3) (Linhoff, Harton et al. 2001; Sisk, Roys et al. 2001). The GBD domain interacts with itself, the amino terminal domains (containing the AD and PST), and with the LRR domain (Linhoff, Harton et al. 2001; Sisk, Roys et al. 2001). Self-interaction at the GDB domain does not require nucleotide binding, and may promote multimer formation (Sisk, Roys et al. 2001). The PST domain may also mediate oligomerization, as N-terminally truncated CIITA oligomerizes, even in the absence of the GBD domain (Tosi, Jabrane-Ferrat et al. 2002). The acidic domain, but not the GBD, is required for self-association (Sisk, Roys et al. 2001). The LLR and GBD are primarily responsible for intra-molecular interactions, whereas oligomerization does not appear to require these domains, but does require the AD and PST domains.

Phosphorylation also mediates CIITA self-interactions. Hypo-phosphorylated CIITA preferentially interacts with the LRR domain (Sisk, Nickerson et al. 2003), promoting self-interactions between the GBD and LLR regions. Treatment with phosphatase inhibits CIITA aggregation (Tosi, Jabrane-Ferrat et al. 2002), while phosphorylation enhanced interactions between self, p300, and RFX5 (Sisk, Nickerson et al. 2003). Phosphorylation of CIITA may

enhance activity by inducing a conformational change, freeing the protein of self-association with the LRR. The GBD and LLR regions of CIITA are capable of mediating multiple interactions, which may be mediated by post-translational modification of CIITA. Exactly which interactions actually occur *in vivo* has not been determined, as most experiments on CIITA oligomerization were done with over-expressed, exogenously transcribed protein.

VII. The CARD domain of CIITA:

Isoform I of CIITA encodes an N-terminus with a unique 94 amino acids (aa) (101 in humans) (Muhlethaler-Mottet, Otten et al. 1997) and this region contains (weak) homology to a caspase recruitment domain (CARD) (Nickerson, Sisk et al. 2001). The caspase recruitment domain (CARD) was originally identified as a protein-protein interaction domain involved in apoptosis (Hofmann, Bucher et al. 1997). The CARD domain mediates recruitment of caspases to receptor complexes, and is located on a number of proteins involved in apoptotic signaling. The original sequence analysis identified the CARD domain on the human proteins RAIDD, caspase-1, caspase-2, caspase-4, caspase-9, and viral apoptosis inhibitor (c-IAP1 and c-IAP2) (Hofmann, Bucher et al. 1997). The identification of a CARD domain on isoform I of CIITA has led to the characterization of a group of proteins known as nucleotide-binding domain and leucine-rich repeat containing family (NLR) (Figure 4) (Nickerson, Sisk et al. 2001; Ting, Lovering et al. 2008). Members of this family include NOD1, NOD2, NALP1, NALP3, NAIP, and IPAF (Ting, Lovering et al. 2008; Ye and Ting 2008; Elinav, Strowig et al. 2011). NLR family members are characterized by the presence of the following domains: an N-terminal effector binding-domain (CARD, pyrin, or baculoviral inhibitory repeat domain), a nucleotide binding oligomerization domain, and a leucine-rich repeat containing domain (Nickerson, Sisk et al. 2001). The NLR family is

related to plant disease resistance proteins known as R proteins (Dangl and Jones 2001). Many NLRs, are known to mediate immune responses such as inflammation and apoptosis, and some NLR proteins are capable of pathogen detection (Inohara and Nunez 2003; Inohara, Ogura et al. 2003; Benko, Magalhaes et al. 2010). Nod2 has even been shown to play a role in adaptive immune responses (Magalhaes, Fritz et al. 2008; Shaw, Reimer et al. 2009). The CARD domains present on NLR proteins such as Nod1 and RICK have been shown to be involved in NF-kB signaling (Inohara, Koseki et al. 1999). However, even though CIITA isoform I is the founding member of the NLR family, to date no interaction partner for its CARD domain has been found.

The presence of the CARD domain has been proposed to increase the transactivation potential of CIITA isoform I compared to the other isoforms (Nickerson, Sisk et al. 2001). Constructs containing either isoform I or III were co-transfected along with the Eα-luciferase reporter. More luciferase activity was observed when the isoform I construct was used, than when the isoform III construct was used, despite the fact that protein levels of CIITA were lower when the isoform I construct was used. Mutation of a conserved leucine residue, shown to be required for CARD function, reduced transactivation activity to that of isoform III (Nickerson, Sisk et al. 2001). These experiments were done using transient transfections and reporter assays. This group also measured CIITA protein and MHC-II expression in B cells vs. DC. These experiments compared CIITA activity in different cell types. Post-translational modifications, such as ubiquitylation, are also important for mediating the activity of CIITA. Therefore, to accurately compare the activity of isoform I vs. III, it would be best to compare the activity of these isoforms in the same cell type. The method by which isoform I transactivation potential is increased was not determined.

Unlike other CARD domains, the CARD domain of CIITA does not appear to interact with other members of the caspase family or to regulate apoptosis (Nickerson, Sisk et al.

2001). CIITA does not interact with the CARD-containing proteins caspase-1, -2, -4, -9, c-IAP-1, RICK, Nod1, Nod2, ARC, Bcl-10, CARD-12, or ICEBERG (Nickerson, Sisk et al. 2001). Recently, a novel splice isoform of CIITA was discovered (Huang, Lim et al. 2010). This splice isoform was named DC-expressed caspase inhibitory isoform of CIITA (DC-CASPIC). DC-CASPIC contains an additional 142 nucleotides spliced in from intron 1. These additional nucleotides contain a stop-codon, resulting in a truncated protein that contains the CARD domain, but none of the domains present in the other isoforms of CIITA. Transfection of DC with DC-CASPIC mRNA enhanced T cell activation. DC-CASPIC also induced synthesis of nitric oxide (NO) in DC, by inhibiting caspase mediated degradation of NO synthase-2 (NOS2). DC-CASPIC was found to interact with caspase-1 and -3. These data conflict with the previous findings that CIITA does not interact with caspases, therefore further studies should be conducted to verify these findings. It has been shown that recruitment of caspase-1 to AIM2 and NLRP3 based inflammasomes is dependent on the adapter protein apoptotsis-associated speck-like protein containing a CARD (ASC) (Franchi, Eigenbrod et al. 2009; Tsuchiya, Hara et al. 2010). The presence of an adapter protein may therefore also be required for an interaction between the CARD domain of CIITA and another CARD-containing protein.

NLR Family members: Structure and function

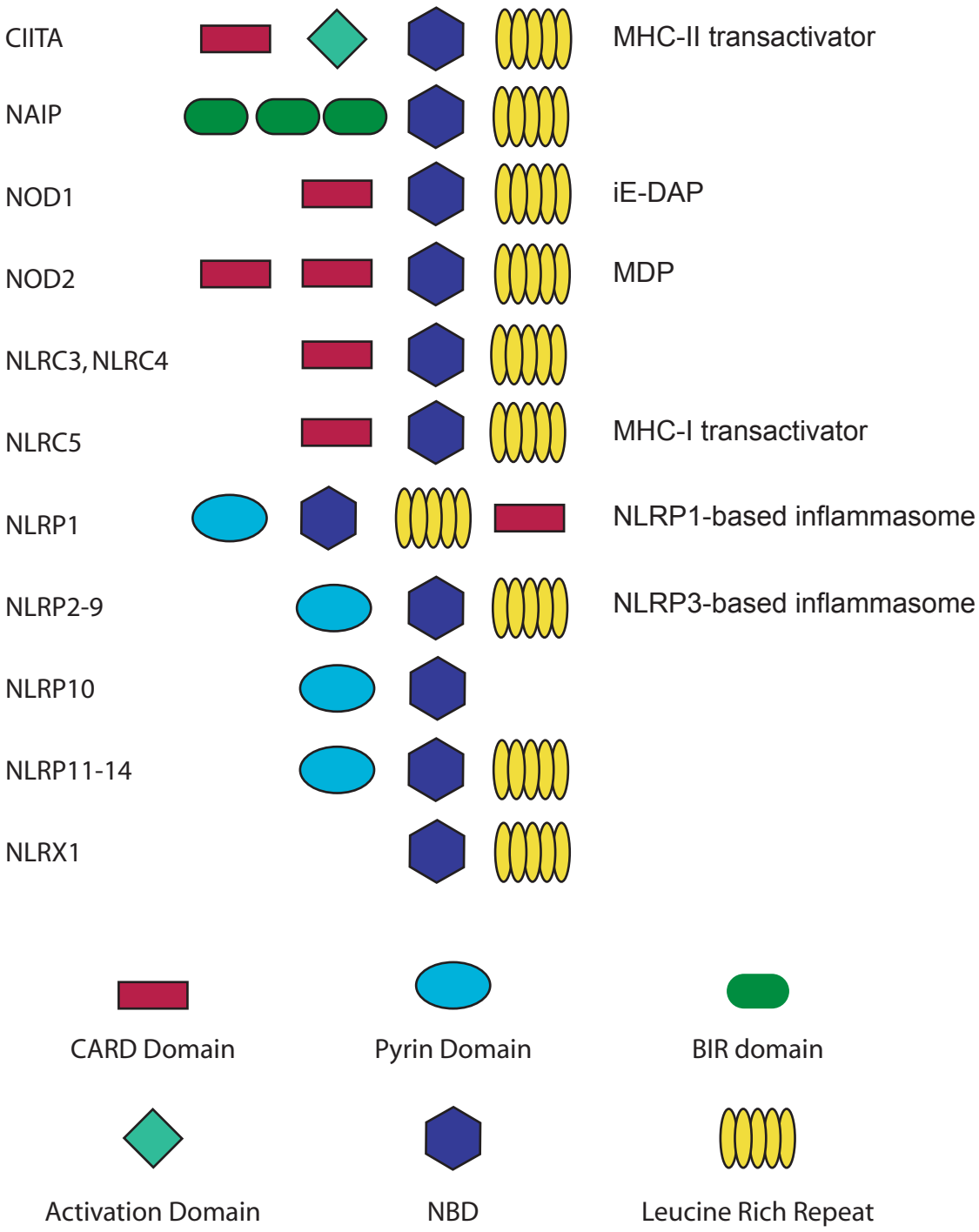


Figure 4: NLR family members. NLR family members are characterized by an N-terminal effector domain (CARD, Pyrin, or BIR), a nucleotide binding domain (NBD), and C-terminal leucine rich repeats. Known functions for some of the NLR proteins are listed in the right column. CARD: caspase recruitment domain; BIR: baculoviral inhibitory repeat.

VIII. Aims of the Study

Function of the CIITA CARD domain

The function of the CIITA CARD domain remains a mystery. Isoform I is present only in macrophages and dendritic cells, cells that are unique in that they bridge the innate and adaptive immune responses. It is tempting to speculate that the function of the CARD domain is associated with functions that are unique to these cells. CIITA is known to interact as a molecular scaffold, bringing transcription factors such as $TF_{II}B$, $TAF_{II}32$, P-TEFb, as well as histone modifying enzymes to MHC-II promoters (Figure 3). The CARD domain is known to serve as a protein-protein interaction domain. It may be the case that the CARD domain of CIITA recruits a myeloid specific transcription factor to MHC-II promoters. High levels of MHC-II molecules are critical for macrophage and DC activity, and isoform I may help increase the transactivation potential of CIITA, as suggested by Nickerson et al. (Nickerson, Sisk et al. 2001).

CIITA isoform I is similar in structure to a family of proteins including the proteins NOD1, NOD2, and Apaf-1 (Figure 4). These proteins have identified functions in cell death and microbial sensing. NOD1, NOD2, NALP3, and Ipaf all capable of detecting specific microbial signals (Fritz, Ferrero et al. 2006). NOD1 and NOD2 activate the NF-kB signaling pathway, and NALP3, Ipaf, and Naip activate the inflammasome (Fritz, Ferrero et al. 2006).

Similarity between CIITA isoform I and members of the NLR family suggest that this isoform of CIITA may have a unique function, which would be dependent the presence of the CARD domain.

CIITA function in myeloid cells

Mouse knockout studies have determined that CIITA expression from pIII and pIV is dispensable for proper expression of MHC-II in conventional dendritic cells and macrophages. Macrophages and dendritic cells play a critical role in initiation of an adaptive immune response. In particular, dendritic cells in the medulla of the thymus are thought to be required for proper negative T cell selection. Deletion of CIITA expression is known to have severe immunological consequences due to almost complete extinction of MHC-II expression. Likewise, deletion of just one or two of *CIITA*'s three promoters has specific effects on immune system function. Expression of *CIITA* from pI was found to be sufficient for MHC-II expression in cells of the myeloid lineage. Therefore, it was expected that deletion of *CIITA* pI would also result in specific immune system defects due to loss of MHC-II on a subset of cells.

A mouse knockout model in which isoform I was replaced with the coding sequence for isoform III was generated to determine whether there was specific requirement for the CARD domain. This mouse model was also designed so that the promoter region and the first exon of pI could be deleted by crossing the mouse line to a Cre-recombinase containing mouse line. Deletion of pI was expected to result in abrogation of MHC-II gene expression in specific subsets of cells, namely conventional dendritic cells and macrophages. Deletion of CIITA in myeloid cells would help elucidate the immunological function of CIITA and MHC-II expression in these cells. Chapter 3 is a detailed analysis of the functional consequence of deletion of the CARD containing isoform of CIITA.

CIITA promoter choice

The mechanism by which promoter choice is made is not fully understood. It has been proposed that CIITA is expressed from three promoters to aid in the fine-tuning of CIITA, and thus MHC-II, expression. It is critical that cells express MHC-II molecules only at appropriate times. Ectopic expression can lead to autoimmune diseases, but lack of expression would result in vulnerability to infections. Expression of *CIITA* from promoters III and IV has been well studied, and many of the required transcription factors for both constitutive and IFN-γ inducible expression are known. Several recent studies have also added to our understanding of promoter proximal elements present at pI. Some transcription factors are cell-type specific, while some may be shared among cell-types. Chapter 4 discusses in further detail *CIITA* regulatory regions, how promoter choice is made, and what factors mediate promoter choice.

Chapter 2: Materials And Methods

Targeting vector and generation of Ciita mutant mice

A *Ciita* site-specific targeting construct (see chapter 3, Fig. 1A) was generated in PGKneobpA (a gift from Dr. Grant MacGregor, Univ. California, Irvine) by standard PCR and cloning techniques. It contains, in order: a 2.8 kb fragment upstream of *Ciita* pI (3.1 kb to 0.3 kb upstream of the TSS of pI); a *FLP1* recombinase target (FRT) site; upstream sequences -298 through +85 of the pI 5'UTR; an AUG codon and HA-epitope-tag fused to *Ciita* pIII exon 1 coding sequence; 145 bp from intron 1 of pI; and a second FRT site. A *lox-P*-flanked neomycin-resistance (*neo*) cassette and 3.0 kb of *Ciita* pI intron 1 (0.5kb to 3.5 kb downstream of the TSS) completes the vector and provides the 3' targeting arm. *Cla*I and *Sal*I sites were introduced into the construct to facilitate cloning. The linearized targeting vector was transfected into 129 SvEv embryonic stem (ES) cells by electroporation, and G418-resistant clones were selected by inGenious Targeting Labs, Inc. Homologous recombinants were screened by Southern blotting using *Hin*dIII, *Bam*HI, or *Eco*RI digestion in combination with probes either internal or 3' external to the targeting construct (Figure 1). The presence of the upstream FRT site was determined by PCR, using primers 1 and 2 (chapter 3 Fig. 1 and Table 1). Correctly targeted ES cells were injected into C57BL/6 blastocysts and three chimeric males were obtained. These chimeras were crossed to C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) females and the agouti F1 heterozygote offspring were tested for the transgenic allele by PCR using primers 1 and 2 (chapter 3 Fig. 1). Mice containing the targeted allele were crossed to EIIa-cre mice (Jackson Laboratory). Offspring of these crosses were analyzed by PCR for transmission of the targeted allele and for Cre-mediated deletion of the floxed *neo* cassette (primer sets 1-2

and 3-4, chapter 3 Fig. 1 and Table 1). Mice carrying the Cre-deleted locus were then backcrossed to C57BL/6J to generate CIITApI→III knock-in (KI) mice or crossed to ACT-FLPe mice (Jackson Laboratory). Offspring of the ACT-FLPe crosses were analyzed by PCR for FLPe-mediated deletion of the DNA between the FRT sites (primer set 1-4, Table 1). Mice carrying the Cre/FLP-deleted locus were then crossed to C57BL/6J to generate CIITApI→0 knockout (KO) mice. Where indicated, C57BL/6J mice from Jackson Laboratory aged six to eight weeks were used. Animals were housed under standard conditions in a conventional mouse facility, where they remained healthy. The Emory University Institutional Animal Care and Utilization Committee approved all animal experiments and protocols.

To generate the targeting construct, we designed primers (D5'd, *Xho*I and D3', *Cla*I) to amplify the upstream flanking region for homologous recombination, introducing *Xho*I and *Cla*I sites for cloning into the targeting vector. Another primer pair was designed to introduce a FRT site upstream of promoter I and a *Sal*I site at the start of translation (C5', *Cla*I and C3', *Sal*I). The next fragment inserted into the targeting vector was generated by overlap-PCR using primers that introduced a consensus Kozak sequence and HA tag and amplified the coding region of promoter III exon 1, with the intronic sequence of promoter I exon 1, followed by a FRT site and *Xho*I for cloning purposes. Primers for this step were: B5', *Sal*I, B2-5'OL, B1-3'OL, and B2-3'. Finally, A5', *Not*I and A3', *Sac*II were designed to amplify the downstream flanking region, intron 1 of pI, for homologous recombination, introducing *Not*I and *Sac*II sites for cloning into the targeting vector. All primers are listed in Table 1. PCR was performed using genomic DNA isolated from strain 129 mouse tissues. The PCR amplicons were initially cloned into the pCR4-BluntTOPO vector (Invitrogen, Carlsbad, CA) for DNA sequence confirmation and large-scale preparation, then subcloned into

PGKneobpA at the *Xho*I site. The resulting vector was digested with *Not*I and *Sac*II, and ligated with the downstream flanking region PCR fragment.

Speed congenics

We used a marker assisted selection procedure (MASP), aka "speed congenic" strategy, to construct a C57BL/6J congenic strain containing our targeted locus from a 129/SvEv:C57BL/6J mixed strain background (Markel, Shu et al. 1997; Wakeland, Morel et al. 1997; Estill and Garcia 2000). We selected a genome-wide set of markers, spaced approximately 20 cM apart, from the MIT/Whitehead mouse map that were reportedly polymorphic between C57BL/6J and 129/SvEv (Markel, Shu et al. 1997; Estill and Garcia 2000) (Table 2). PCR primers used are available on the Mouse Genome Database at the Mouse Genome Informatics website (Blake, Bult et al. 2011). PCR products were run on polyacrylamide gels and compared to C57BL/6J and 129SvEv control DNA run in parallel. The "best" breeder of each generation was heterozygous by locus-specific PCR for our targeted mutation, and carried the most C57BL/6J alleles by genome-wide SSLP PCR analysis. By the 4th backcross, all markers tested except the one next to the targeted locus were of C57BL/6J origin (Table 2).

5'RACE

To determine the 5' ends of CIITA transcripts of KO and KI mice, rapid amplification of cDNA ends (RACE) was performed using the FirstChoice RLM-RACE Kit from Applied Biosystems (Carlsbad, CA) following manufacturer's instructions. MRNA was purified from total RNA using the oligotex mRNA mini kit according to manufacturer's instructions (Qiagen, Valencia, CA). Primers used are listed in Table 1.

Cell Collection and treatments

Dendritic cells (DC) were collected from the spleen as described in Current Protocols (Inaba, Swiggard et al. 2009). Briefly, mice were injected i.p. with 30 μg Flt3 Ligand-Ig (Flt3-L) in 200 ml phosphate buffered saline (PBS) for 9 days (Zhang, Zhang et al. 2010). Flt3-L was generously provided by Dr. R. Mittler (Emory University). Following CO_2 asphyxiation, spleens were removed and injected with Dulbecco's modified Eagle's media (Cellgro, Manassas, VA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) (DMEM-10), 1x non essential amino acids (HyClone Laboratory, Logan VT), 1 M HEPES (HyClone Laboratory), 1 mM sodium pyruvate (HyClone Laboratory), 0.292 mg/ml L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (1x PSG) (Invitrogen) and 1mg/mL collagenase D (Roche, Indianapolis, IN), cut into pieces, and incubated at 37°C for 25 min. A single cell suspension was generated by forcing cells through a 40 mm cell strainer (BD Biosciences,San Jose, CA). Red blood cells were lysed with ammonium-chloride potassium-chloride (ACK) lysing buffer containing 0.15 M NH_4CL, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4. The CD11c+ DC population was purified using CD11c MACS beads (Miltenyi Biotech, Inc., Auburn, CA) according to manufacturer's protocol. Peritoneal exudate cells were isolated in 10 ml Hanks' buffered saline solution containing 1x PSG, 10mM HEPES, 0.06% bovine serum albumin (fraction V) (Fisher Scientific), 0.0375% sodium bicarbonate (Fisher Scientific), and 10 U/ml heparin (Baxter, Deerfield, IL). For peritoneal macrophages, mice were injected i.p. with 2.5ml 3% solution of thioglycolate medium (Sigma) four days prior to collection of the peritoneal fluid. Peritoneal exudate cells were plated 0.8-1x10^6 cells/ml in DMEM-10 containing 0.292 mg/ml L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and 50 μM 2-mercaptoethanol (Sigma-Aldrich) and allowed to adhere for 2 h. Non-adherent cells were washed off, and the

adherent cells were provided fresh DMEM-10 media and treated with 500 U/ml IFN-γ (Peprotech, Inc., Rocky Hill, NJ) for the indicated time. Total primary peritoneal exudate cells (for T cell intracellular cytokine assay) and spleen cells were incubated in RPMI 1640 medium containing 10% FBS, 0.292 mg/ml L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and 10mM HEPES buffer. For the collection of B cells and T cells from the spleen or lymph nodes, a single-cell suspension was generated as described above.

The murine monocyte/ macrophage cell line J774.A1 (here after referred to as J774) and the B cell line A20 were purchased from ATCC (Manassas, Va.). J774 cells were cultured in the same media used for primary macrophages, A20 cells were cultured in RPMI containing 10% FBS, 1 mM sodium pyruvate, 1x non-essential amino acids, 10mM HEPES buffer, and 50 mM 2-mercaptoethanol.

Flow cytometry

For cell surface staining, $0.5x10^6$ cells were treated as previously described (Freeman and Ziegler 2005). Briefly, $0.5x10^6$ cells were incubated on ice for 5 min with anti-CD16/32 (FC block clone 2.4G2) prior to addition of antibodies or isotype control for 30 min. Antibodies used for surface staining were purchased from BD Biosciences: anti-CD4 (clone RM4-5 PerCP, GK1.5 PE), anti-CD8 (clone 53-6.7) anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD45R/B220 (clone RA3-6B2), anti-CD45RA (clone 14.8), anti-I-A^b (clone AF6-120.1), and appropriate isotype controls (A95-1, R35-95, G235-2356, G155-178). For secondary labeling, cells were washed once, followed by 20 min incubation on ice with Pacific Blue labeled streptavidin (Molecular Probes, Eugene, Oregon). Flow cytometry was performed using a BD FACSCalibur or LSRII flow cytometer (BD Biosciences). Fluorescence intensity was calculated in Molecules of Soluble Fluorochrome (MESF) units using Quantum

MESF beads (Bangs Laboratories, Inc, Fishers, IN) according to manufacturer's protocol. All data was analyzed utilizing FlowJo (TreeStar, Inc., Ashland, OR).

For intracellular cytokine staining after infection with *Listeria monocytogenes,* $3x10^6$ peritoneal exudate cells were cultured for 5 h at 37°C in 1 ml media either without treatment, with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) plus 1 mM ionomycin (Sigma-Aldrich) (positive control), or with 10^7 cells heat-killed *L. monocytogenes.* (HKLM). Brefeldin A (BFA) (Sigma-Aldrich) was added (10 mg/ml) after the first 30 min. After LCMV infection, 10^6 splenocytes were cultured 5-6 h in the absence or presence of the indicated peptide (GP_{33}, GP_{276}, or GP_{61}) plus BFA. For analysis by flow cytometry, extracellular antigens were stained as indicated above, followed by fixation and permeabilization using Fix and Perm cell permeabilization kit (Invitrogen) for intracellular antigens, according to manufacturer's directions. Antibodies used for intracellular cytokine detection were anti-IFN-γ (clone XMG1.2), anti-IL-2 (clone JES6-5H4), anti-TNF-a (clone MP6-XT22) and appropriate isotype controls (R3-34 and A95-1), staining was done on ice for 30 min. Live cells were gated based on light scatter parameters. The lymphocyte population was gated based on forward and side scatter parameters. All antibodies used were purchased from BD Pharmingen (Franklin Lakes, NJ).

T cell proliferation

To measure MHC-II antigen presentation efficiency, mice were injected in the footpad and base of the tail with an emulsion of Complete Freund's Adjuvant (CFA) containing 1 mg/ml heat killed *Mycobacterium tuberculosis* (Difco, Detroit, MI) and the I-A^b specific epitope of LCMV GP_{61-80} peptide (Ford and Evavold 2003). Popliteal and inguinal lymph nodes were collected 12 days later. A single cell suspension was generated, and $5x10^5$ cells were incubated in a 96 well plate with increasing concentrations of peptide for 3 days, with 0.4

mCi/well [^{3}H]thymidine added during the last 24 h. Plates were harvested using a FilterMate harvester (Packard Instrument, Meriden, CT) and ^{3}H-thymidine incorporation was measured on a 1450 LSC Microbeta TriLux counter (PerkinElmer).

Induction of EAE

Experimental autoimmune encephalomyelitis (EAE) was induced in female mice 6-8 weeks of age by subcutaneous injection in the hind flank with 200 mg MOG_{35-55} peptide emulsified in 200 ml CFA (2.5 mg/ml *M. tuberculosis*) on days 0 and 7, as previously described (Ford and Evavold 2003; Sabatino, Shires et al. 2008). On days 0 and 2 mice were given 250 ng pertussis toxin i.p. (List Biological Laboratories, Inc., Campbell, CA). Disease severity was scored according to the following scale: 0, no disease, 1, flaccid tail, 2, hind limb weakness, 3, hind limb paralysis, 4, failure to right (forelimb weakness), 5, moribund.

Peptides

Myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}) (MEVGWYRSPFSRVVHLYRNGK) (Mendel, Kerlero de Rosbo et al. 1995), LCMV epitopes GP_{33-41} (KAVYNFATC), $GP_{276-286}$ (SGVENPGGYCL), and $GP_{61–80}$ (GLNGPDIYKGVYQFKSVEFD) (Homann, Teyton et al. 2001) peptides were synthesized using standard 9-fluorenylmethyloxycarbonyl chemistry on a Prelude peptide synthesizer (Protein Technologies, Inc).

Listeria monocytogenes *infection*

A culture of *Listeria monocytogenes* (strain 10403s) was grown in brain heart infusion (BHI) to OD=0.2, then diluted in phosphate-buffered saline (PBS), the indicated dose was

administered in 200 µl. The LD_{50} for C57BL/6J mice was determined to be $6x10^6$ colony forming units (CFU) (data not shown). Infection dose was verified by plating dilutions of the inoculum and enumerating CFU. Mice were monitored daily for morbidity and mortality. Mice were sacrificed when moribund or weight loss was greater than 25% of initial weight. For enumeration of CFU in organs, spleens and livers were aseptically removed and placed in PBS. Tissue homogenizers were used to generate a single cell suspension, and 1% triton-X100 in PBS was added to final concentration of 0.5% to lyse the cells. Serial dilutions were plated on BHI plates containing 25 mg/ml streptomycin. Colonies were counted the next day, and total CFU/ organ was calculated based on the dilution plated.

LCMV

Acute LCMV infection and cellular analysis were performed as previously described (Wherry, Blattman et al. 2003). Briefly, mice were infected i.p. with $2x10^5$ pfu LCMV-Armstrong. Mice were serially bled and sacrificed as described in the figure legend. Staining with antibodies and MHC class I and II tetramers complexed with LCMV GP_{33-41}, $GP_{276-286}$, or GP_{66-77} for flow cytometric analysis of antigen-specific T cells were performed as previously described (Fuller, Hildeman et al. 2005; Wherry, Ha et al. 2007; Elsaesser, Sauer et al. 2009).

Infulenza Immunization

CIITA knockout and wild-type littermate control mice received a single immunization of 5 mg whole inactivated A/California/04/09 influenza virus and they were bled on days 7, 14 and 28. Sera were collected for ELISA and HAI assays. As a negative control group, we included 3 naïve wild-type and 2 CIITA knockout mice. Sera were individually collected, and anti-influenza specific antibody levels were determined quantitatively by enzyme-

linked immunosorbent assay (ELISA) as described (Skountzou, Quan et al. 2006; Koutsonanos, del Pilar Martin et al. 2009). Hemagglutination inhibition (HAI) titers based on the WHO protocol (2002) as described previously (Skountzou, Quan et al. 2006). The HAI titer was read as the reciprocal of the highest dilution of serum that conferred inhibition of hemagglutination. The values were expressed as the geometric mean +/- standard error of the mean.

Quantitative RT-PCR

Total RNA was isolated from cells using RNeasy kit (Qiagen). Reverse transcriptase was done using 1 mg of RNA using SuperScrpit II (Invitrogen), RNAse inhibitor (Roche), RQ1 DNase (Promega, Madison, WI), oligo $d(T)_{16}$, and random hexamers (Applied Biosystems). Real-time PCR using 1/33 of the cDNA product was used to quantify the amount of mRNA. Results were normalized to 18S rRNA or *GAPDH* mRNA levels and represented using the comparative CT method (Schmittgen and Livak 2008). Data presented represents the average of at least three independent biological replicates, and error bars represent standard error of the mean (SEM). Primers used are listed in Table 1.

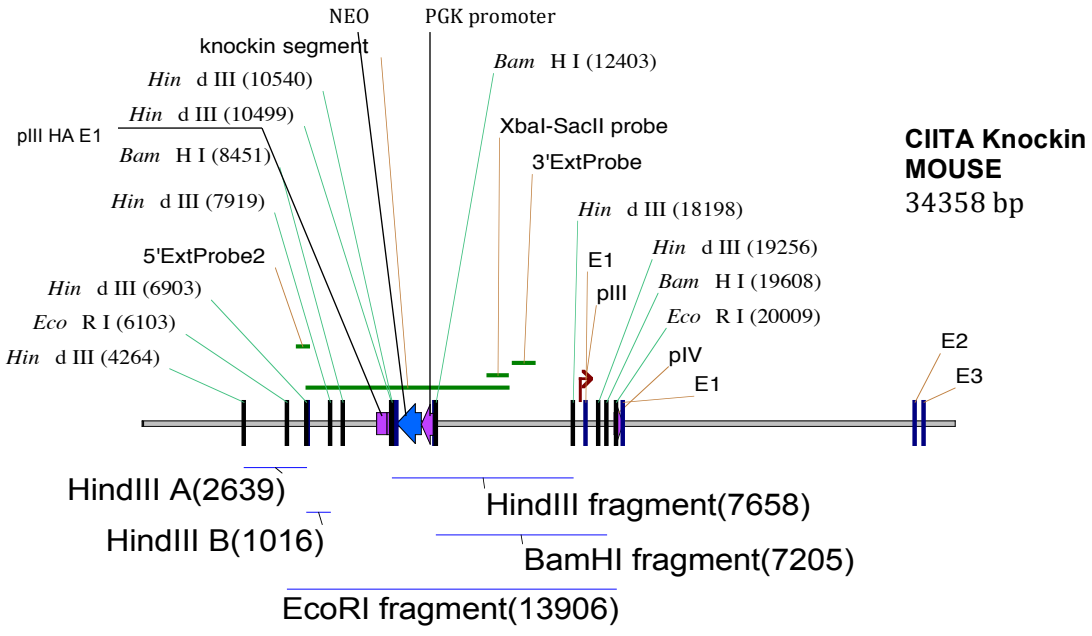
DNA methylation analysis

Four hundred ng of DNA was bisulfite converted using the EZ DNA methylation-gold kit (Zymo research, Irvine, Ca). Bisulfite DNA was amplified using primers flanking CpG sites along the CITTA locus, cloned using Topo TA cloning (Invitrogen), and sequenced. Sequences were aligned to in silico bisulfite converted genomic sequence. Alignment was done using custom scripts the Biostrings package for the R programming language (Bioconductor, Seattle, Wa.).

Figure 1: CIITA targeting construct. Schematic representation of the CIITA promoter region. The targeted locus, probes used for Southern analysis, NEO insert, and relevant restriction enzyme sites are indicated for the targeted locus (CIITA knockin) and genomic CIITA locus.

Table 1: Primer sequences used in this paper. Primer sequences for genotyping, qRT-PCR, 5'RACE, and cloning. Underlined sequences in cloning primers indicates restriction enzyme recognition sequences.

Table 2: Speed congenic markers. Markers used for speed congenic analysis are listed. Column 1 indicates the location of the marker, column 2 is the name of the marker. Column 3 is results for CIITA KI and column 4 is results for CIITA KO mouse lines, at backcross four. B denotes that locus is C57Bl/6 at both alleles, 129 indicates one allele is 129/SvEv. Markers not tested are left blank.



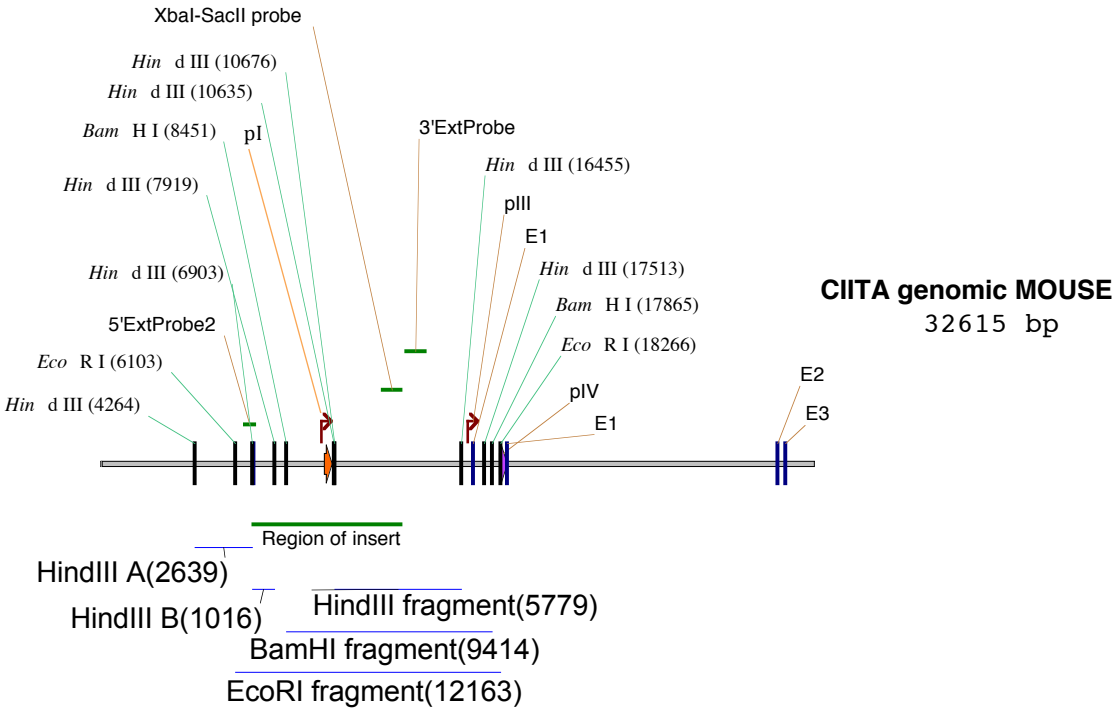

XbaI-SacII probe
Hin d III (10676)
Hin d III (10635)
Bam H I (8451)
pI
3'ExtProbe
Hin d III (16455)
Hin d III (7919)
pIII
E1
Hin d III (6903)
Hin d III (17513)
Bam H I (17865)
5'ExtProbe2
Eco R I (18266)
Eco R I (6103)
pIV
E2
Hin d III (4264)
E1
E3
CIITA genomic MOUSE
32615 bp
Region of insert
HindIII A(2639)
HindIII B(1016)
HindIII fragment(5779)
BamHI fragment(9414)
EcoRI fragment(12163)

Table 1

Name	Sequence	Reference
	GENOTYPING	
Primer 1	GTAGCCAAAGATGACCTTG	
Primer 2	GCTTTCCTTTCTGCTTTTACATTTC	
Primer 3	CTGCCGGAGTTGCAAGGTAAGCTGG	
Primer 4	GTGTCTGTTGGAAGCAGGAGTTC	
	REAL-TIME RT-PCR	
18S rRNA Fwd	GTAACCCGTTGAACCCCATT	
18S rRNA Rev	CCATCCAATCGGTAGTAGCG	
Ciita HA Fwd	CGACGGACCATGGAGTACCCATAT	
Ciita HA Rev	GGAGTTCCAGGTAGCTGCCCTCTG	
Ciita Total Fwd	AAGAGAAGGCTGGAAGGATCTTT	(Green, Yoon et al. 2006)
Ciita Total Rev	GATGTGGAAGACCTGGATCGT	(Green, Yoon et al. 2006)
Ciita I Fwd	AAGAGCTGCTCTCACGGGAAT	(Pai, Askew et al. 2002)
Ciita III Fwd	TCTTACCTGCCGGAGTT	(Pai, Askew et al. 2002)
Ciita IV Fwd	GAGACTGCATGCAGGCAGCA	(Pai, Askew et al. 2002)
Ciita Rev	GGTCGGCATCACTGTTAAGGA	(Pai, Askew et al. 2002)
Col1A1 Fwd	AAGGTGACAGAGGCATAAAGG	
Col1A1 Rev	TTGAGTCCGTCTTTGCCAG	
Cste Fwd	CCAGACCTTTGTGAATGCAG	
Cste Rev	GTCATAGCCTCCGAAAGTCAG	
H2-D1 Fwd	CCCTGACCTGGCAGTTGAATG	
H2-D1 Rev	AGCTCCAAGGATGACCACAGC	
H2-DOa Fwd *H2-DOa* Rev	CCGCAATGAGCTTCCTGAGTC TGTTCCCCGTCAAATTCGTG	
H2-DOb Fwd	TCAGGCAAGGCGGACTGTTAC	(Kang, Guo et al. 2004)
H2-DOb Rev	TCCTCTCTGGATACACTGTCACCTC	
H2-DMa Fwd	TGAAGGTCAAATCCCAGTGTCC	(Kang, Guo et al. 2004)
H2-DMa Rev	AGCGGTCAATCTCGTGTGTCAC	
H2-*DMb* Fwd	GTCCTCAGTCTGCACTGTATG	
H2-DMb Rev	CAGCACCCCAAATTCACAG	
IA alpha Fwd	CTTCCCACCTGTGATCAACAT	
IA alpha Rev	AATCTCAGGTTCCCAGTGTTT	
Ii Fwd	GTGTCTGTTTCATCGTCCCAG	(Itoh-Lindstrom, Piskurich et al. 1999)
Ii Rev	AAGGCAGCAAATGTGTCCAGC	(Itoh-Lindstrom, Piskurich et al. 1999)
Kiaa0841 Fwd	TGAAAGACACCCAGCATCG	
Kiaa0841 Rev	ACAGGTCCAAAGGTCACATC	
Kpna6 Fwd	AATCCCTACTGTGGCCTCATC	
Kpna6 Rev	GCTGTCGTCATCCTCTACACCA	
Plxna1 Fwd	CAATCCTGCTACCGTGGAGAA	(Wong, Brickey et al. 2003)
Plxna1 Rev	CCGCAGAAGTCGTCATCAAT	(Wong, Brickey et al. 2003)
Psmd3 Fwd	GTATCCAAGTCTGTGTTCCCTG	
Psmd3 Rev	CTCTACCACGATCAGAAGCTTG	
Rab4b Fwd	TGGCGACATATCCCTCCGCC	
Rab4b Rev	ACAGGTGAGCTGGTTCTGCACAT	
Tpp1 Fwd	AGGACTTTCTGACTTGCTGG	
Tpp1 Rev	CCCCACAAAATCCACATGAG	
Trim26 Fwd	TCTGAACCACTTGAACACCC	
Trim26 Rev	TGGCCCTGCTTAAATTCCG	
	5' RACE	
5'RACE inner	GGTCATAGAGGTGGTAGAGATGTAGG	
5'RACE outer	TGATGGTATCTGTGTCTGGCTCTG	

	CLONING
D5'd, *Xho*I	GGGCTCGAGCTTCCTCATATCTCAGTGAAA
D3', *Cla*I	GGGATCGATGTGATGGTTTTGCATCTGCT
C5', *Cla*I	GCCATCGATGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTTC CCCAAGTGAGCTACAGT
C3', *Sal*I	GCGGTCGACGGCAGTGCTCTGAGGCTGCCTGCT
B5', *Sal*I	GAGGTCGACGGACCATGGAGTACCCATATGACGTTCCAGACTACGCGCGCTGCCTGGTTCC TGG
B2-5'OL	CTGCCGGAGTTGC AAGGTAAGCTGGCATC
B1-3'OL	TACCTTGCAACTCCGGCAG
B2-3', *Xho*I	GCCCTCGAGGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCAAG CTTTGTGCTTGCAG
A5', *Not*I	GGGGCGGCCGCTTTCCACATTGATCAGAGCAT
A3', *Sac*II	GGGCCGCGGCACCTCAGACACCAGCCTT

Table 2

MGI cM	marker	KI Bx4	KO Bx4

MGI cM	marker	KI Bx4	KO Bx4

15	1M211	B	B
36.9	1M19	B	B
47	1M215	B	B
65	1M139	B	B
100	1M150	B	B
5	2M5		B
34	2M61	B	B
47.5	2M13		B
	2M206		B
73	2M304	B	B
105	2M213	B	B
19.2	3M21	B	B
39.7	3M40	B	B
68.5	3M351	B	B
87.6	3M163	B	B
19.8	4M89	B	B
35.7	4M275	B	B
51.3	4M31	B	B
61.9	4M204	B	B
82.5	4M254	B	B
18	5M148	B	B
52	5M20	B	B
72	5M163	B	B
0.5	6M86	B	B
25.5	6M33	B	B
38.5	6M146	B	B
68	6M199	B	B
74	6M15	B	B
18	7M310	B	B
52.8	7M237	B	
	7M31		
72.4	7M189	B	B
1	8M155	B	B
21	8M191	B	B
37	8M263		
49	8M211	B	B
72	8M93	B	B
17	9M2		
31	9M21	B	B
42	9M306	B	B
67	9M279	B	B
9	10M51		B
29	10M40	B	B
43	10M7		B
59	10M162	B	B
64	10M180	B	B

1.55	11M226	B	B
20	11M270	B	B
37	11M5	B	B
49	11M38	B	B
62	11M199	B	B
78	11M184	B	B
15	12M153	B	B
32	12M3		B
48	12M231	B	B
10	13M16	B	B
30	13M179	B	B
43	13M193	B	B
61	13M76	B	B
3	14M99	B	
15	14M60	B	
40	14M192	B	B
63	14M107	B	B
6.7	15M13	B	B
27.5	15M46	B	B
42.8	15M29	B	B
59.2	15M42	B	B
3.4	16M182	129	B
22.2	16M103		B
38	16M64		B
57	16M152	B	B
4.1	17M164	B	B
22.9	17M51	B	B
40.6	17M205	B	B
56.7	17M123	B	B
2	18M19	B	B
21	18M23	B	B
44	18M33	B	B
57	18M25	B	B
10.9	19M128	B	
27.5	19M119	B	B
47	19M91	B	B
54	19M71	B	B
2.8	x136	B	B
21.9	x143	B	B
44.4	x213	B	B
55	x130	B	B
69	x186	B	B

Chapter 3: CIITA promoter I CARD-deficient mice express functional MHC class II genes in myeloid and lymphoid compartments

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Real-time RT-PCR, flow cytometry. 5'RACE, and L. monocytogenes infections were performed by W.M. Zinzow-Kramer
EAE and T cell proliferation assays were preformed by W.M Zinzow-Kramer and K.M. Rosenthal

The manuscript was written by W.M. Zinzow-Kramer and J.M. Boss

Abstract

Three distinct promoters control the master regulator of MHC class II expression, CIITA, in a cell type specific manner. Promoter I (pI) CIITA, expressed primarily by dendritic cells and macrophages, expresses a unique isoform that contains a caspase recruitment domain. The activity and function of this isoform is not understood but has been thought to enhance the function of CIITA in antigen presenting cells. To determine if isoform I of CIITA has specific functions, *CIITA* mutant mice were created in which isoform I was replaced with isoform III sequences. Mice in which pI and the CARD encoding exon were deleted were also created. No defect in the formation of CD4 T cells, the ability to respond to a model antigen, or bacterial or viral challenge was observed in mice lacking CIITA isoform I. Although CIITA and MHC-II expression was decreased in splenic DC, the pI knockout animals expressed CIITA from downstream promoters, suggesting that control of pI activity is mediated by unknown distal elements that could act at the pIII, the B cell promoter. Thus, no critical function is linked to the CARD domain of CIITA isoform I with respect to basic immune system development, function and challenge.

Introduction

The major histocompatibility complex class II (MHC-II) region encodes genes required for the presentation of antigenic peptide to CD4 T cells (Benacerraf 1981). The process results in a variety of immune responses that range from direct effector responses to those that are regulatory. Constitutive expression of MHC-II is restricted to antigen presenting cells (APC: macrophages, B cells and dendritic cells) and thymic epithelial cells. MHC-II expression can also be induced in most cell types by exposure to interferon-g (IFN-γ). The expression of MHC-II must be tightly regulated, as aberrant expression can result in immune system dysfunctions, including: autoimmunity, increased susceptibility to cancer, and decreased resistance to infectious organisms (Ting and Trowsdale 2002; Reith, LeibundGut-Landmann et al. 2005; Kanazawa, Ota et al. 2006). Loss of MHC-II gene expression results in severe immunodeficiency, as evidenced by patients with bare lymphocyte syndrome (BLS), a disease resulting from mutations within transcription factors that regulate MHC-II expression (Reith, Steimle et al. 1995; Krawczyk and Reith 2006). One BLS complementation group was found to be deficient for the class II transactivator (CIITA), a factor that is essential for MHC-II expression (Steimle, Otten et al. 1993).

CIITA functions as a transcriptional coactivator interacting with other MHC-II specific transcription factors bound to regions upstream of each *MHC-II* gene (reviewed in (Choi, Majumder et al. 2011)). The recruitment of CIITA to *MHC-II* promoters orchestrates a set of chromatin modifications and rearrangements that are associated with and are required for *MHC-II* expression. The gene encoding human *CIITA* contains four promoters, three of which are conserved in mice (*Ciita*). *Ciita* promoters function in a cell type specific manner (Muhlethaler-Mottet, Otten et al. 1997). *Ciita* promoter I (pI) is utilized by dendritic cells (DC) and macrophages exposed to IFN-γ (Pai, Askew et al. 2002), and

appears to be myeloid specific. *Ciita* pIII is expressed in cells of the lymphoid lineage (B cells, human T cells, and plasmacytoid DCs); whereas pIV is primarily expressed in non-hematopoietic cells upon exposure to IFN-γ (Waldburger, Suter et al. 2001). Each of the *Ciita* promoters contains a unique first exon, which splices into a common second exon, resulting in three distinct CIITA isoforms. Isoform I, derived from pI, is particularly intriguing because its unique exon encodes an N-terminal domain of 93 aa that bears homology to a caspase recruitment domain (CARD) (Nickerson, Sisk et al. 2001). Such domains have been shown for other proteins to be important for protein-protein interactions (Hofmann, Bucher et al. 1997; Inohara, Chamaillard et al. 2005). The presence of the CARD domain in addition to other domains led to CIITA being the cardinal member of the family known as nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins (Harton, Linhoff et al. 2002; Ting, Lovering et al. 2008; Ye and Ting 2008). This family of proteins is related to disease resistance R genes in plants and a number of NLR family members have functions in pathogen sensing, inflammation, cell signaling, and cell death (Inohara and Nunez 2003; Ye and Ting 2008; Elinav, Strowig et al. 2011). Increasing evidence suggests that many NLRs are cytoplasmic pathogen recognition receptors, activating immune responses to intracellular pathogens (Elinav, Strowig et al. 2011). Despite being a member of the NLR family, to date, no function outside of transcriptional activation has been ascribed to CIITA. Previous studies that address cell-type specific function of CIITA have focused on promoters III and IV using a knockout strategy to create mice lacking either pIV or both pIII and pIV (Waldburger, Suter et al. 2001; LeibundGut-Landmann, Waldburger et al. 2004). Using the *Ciita* pIV targeted knockout mouse it was observed that cells of a non-hematopoietic lineage, but not macrophages or microglia, lost the ability to induce *Ciita* following exposure to IFN-γ, demonstrating a need for pIV in expression of *Ciita* in non-bone marrow derived cells (Waldburger, Suter et al. 2001). In

addition, positive selection of CD4 T cells was severely impaired due to loss of expression of MHC-II on cortical thymic epithelial cells (cTECs), though MHC-II expression on cells of the thymic medulla was unchanged (Waldburger, Suter et al. 2001; Waldburger, Rossi et al. 2003). A deletion of the regions encompassing both pIII and pIV displayed all of the phenotypes observed in the pIV KO, and in addition, resulted in loss of MHC-II expression from B cells and plasmacytoid DCs (pDC), while conventional DCs and macrophages induced with IFN-γ retained MHC-II expression (LeibundGut-Landmann, Waldburger et al. 2004). These data point towards the necessity of pI for expression of CIITA and MHC-II in cells of the myeloid lineage.

To address a role for pI and the CARD-containing isoform in regulating CIITA expression and activity, a set of mice were constructed that replaced isoform I of CIITA with the 17 aa exon of isoform III. Effectively, this CIITApI→III knock-in (KI) was designed to create a mouse that would express isoform III CIITA from pI and pIII. Using FLP-mediated recombination, an additional mouse line was created in which pI and its surrounding upstream and downstream DNA were deleted, creating a CIITApI→0 knockout (KO). Thus, two novel *Ciita* mouse lines were created. These mice were extensively characterized for their ability to express C*iita* and MHC-II gene products and response to pathogen challenge. The results showed that the KI mice expressed MHC-II at levels comparable to wild-type mice. Surprisingly, KO mice still retained *Ciita* expression in all cell types examined, including splenic DC, which typically use pI nearly exclusively. This was due to redirection of transcript initiation from pI to pIII. Thus, both KI and KO mice lack isoform I CIITA and instead express isoform III in the myeloid and lymphoid compartments where CIITA isoform I is normally expressed. T cell development and activation appeared normal in both KI and KO mice. KO mice were also able to mount normal immune responses to *Listeria monocytogenes* and lymphocytic choriomeningitis virus (LCMV) infection and

rechallenge, suggesting that the isoform I CIITA does not provide an advantage in these settings. Together, these data demonstrate that promoter I of CIITA and its corresponding CARD-containing isoform are not required for proper immune system development or function, and suggest that isoform III is capable of substituting for isoform I.

Results

Generation of Ciita promoter I isoform III knock-in and Ciita promoter I knockout mice

To examine the function of the CARD domain-containing pI isoform *in vivo*, a targeting vector that replaced the first exon of *Ciita*'s isoform I with isoform III's first exon was created using a gene targeting strategy that utilized both *Cre-loxP* and FLP-FRT mediated recombination. To maximize the utility of this animal, the replaced exon/isoform segment was flanked by FLPe recombination target sites (FRT), which would allow deletion of the entire pI promoter and its first exon *in vivo*. This includes 298 bp upstream of the transcription start site through 145 bp downstream of pI exon 1 for a total of 798 bp. Thus, the targeting vector contained a 2.5kb fragment upstream of *Ciita* pI; an HA-tagged pIII exon 1 coding sequence under control of the endogenous isoform I promoter and flanked by FRT sites; a lox-P-flanked neomycin-resistance cassette (*neo*); and a 2.5kb fragment of *Ciita* pI intron 1 (Fig. 1A). Homologous recombination was carried out in 129SvEv embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6 blastocysts and three chimeric males were obtained. These chimeras were crossed to C57BL/6J females, and the agouti F1 heterozygote offspring were examined for the transmission of the targeted allele by PCR. To delete the *neo* cassette, mice containing the targeted allele were crossed to EIIa-Cre mice (The Jackson Laboratory). Offspring of these crosses were analyzed by PCR for transmission of the targeted allele and for *Cre*-mediated deletion of the floxed DNA (Fig. 1B).

Mice carrying the *Cre*-deleted locus were then backcrossed to C57BL/6J to generate CIITApI→III (knock-in, KI) mice or crossed to ACT-FLPe mice (The Jackson Laboratory) to delete pI. Offspring of these crosses were analyzed by PCR for FLP-mediated deletion of the DNA between the FRT sites (Fig. 1). Mice carrying the *Cre*/FLP-deleted locus were then crossed to C57BL/6J to generate CIITApI→0 (knockout, KO) mice. An example of the PCR assays used to genotype the mice are shown in Fig. 1B.

Speed congenic backcrosses to C57BL/6J females using the male carrying the largest number of C57BL/6J loci were carried out for four generations. Using this speed congenic strategy, only four to five backcrosses are required to obtain a line that is 98-99% C57BL/6J (Markel, Shu et al. 1997). After two backcrosses, at least three markers on each chromosome were of C57BL/6J origin and the CIITA containing chromosome 16 was at least partially C57BL/6J (data not shown). Mice backcrossed to C57BL/6J for four to six generations were used in this study.

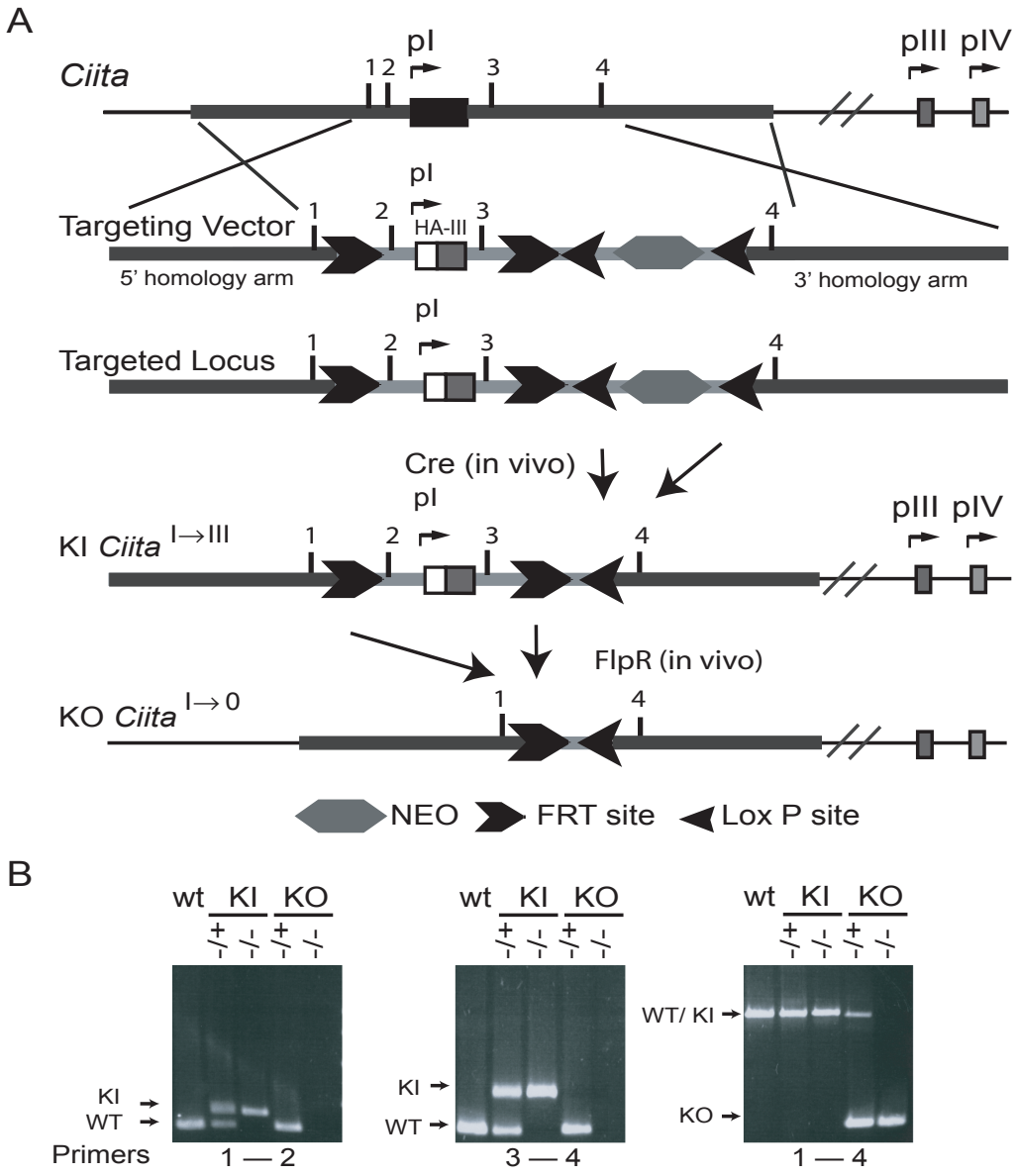


Figure 1. Ciita targeting construct. A. Schematic of the targeting construct used to generate CiitapI→III (KI) and CiitapI→0 (KO). Transgene positive mice were breed to a Cre-expressing mouse to delete the neo selective marker. KI mice were further crossed to a Flp-expressing mouse to generate the KO line. B. PCR genotyping results for transgene positive mice, KI, and KO lines.

CIITA targeted mice are not defective in MHC class II expression

As stated above *Ciita* is expressed from three promoters in mice. Promoter I has been shown to be specific to cells of the myeloid lineage and specific DC compartments. To determine if the *Ciita* targeted mice showed defects in expression of MHC-II genes, the levels of I-A^b were examined by flow cytometry on thioglycolate elicited macrophages treated with IFN-γ (Fig. 2A) and splenic DCs (spDC) (Fig. 2B and C). For each strain, wild-type (WT) control mice were compared to either their KI or KO littermates. No difference in MHC-II expression was observed between KO and KI macrophages ($CD11b^+$) compared to littermate controls.

Splenic dendritic cells were separated into two classes: conventional and plasmacytoid DCs ($CD11c^+$ or $CD11c^{dim}$, $CD45RA^+$, respectively). Conventional DCs were further divided into myeloid ($CD11b^+$) and lymphoid ($CD8a^+$) compartments (Shortman and Liu 2002) (Fig. 2B) and analyzed for MHC-II surface expression (Fig 2C). In addition, fluorescence intensity was calculated in Molecules of Soluble Fluorochrome (MESF) for each subset of dendritic cells and the values were compared between groups of mice (data not shown). In the cases where two MHC-II peaks were observed, each peak was quantitated separately (Fig. 2C). Unlike conventional DCs (cDC), plasmacytoid DCs (pDC) utilize *Ciita* pIII (LeibundGut-Landmann, Waldburger et al. 2004); therefore, it was expected that pI mutations would not affect *Ciita* expression in this subclass of cells. Indeed, neither KI nor KO strains exhibited changes in MHC-II surface expression from their littermate controls in pDC (Fig. 2C). For the KI animals this was also true of the myeloid and lymphoid DC populations. However, the myeloid and lymphoid populations from KO cells consistently displayed MHC-II at levels that were 70-76% of that seen on WT (Fig. 2C).

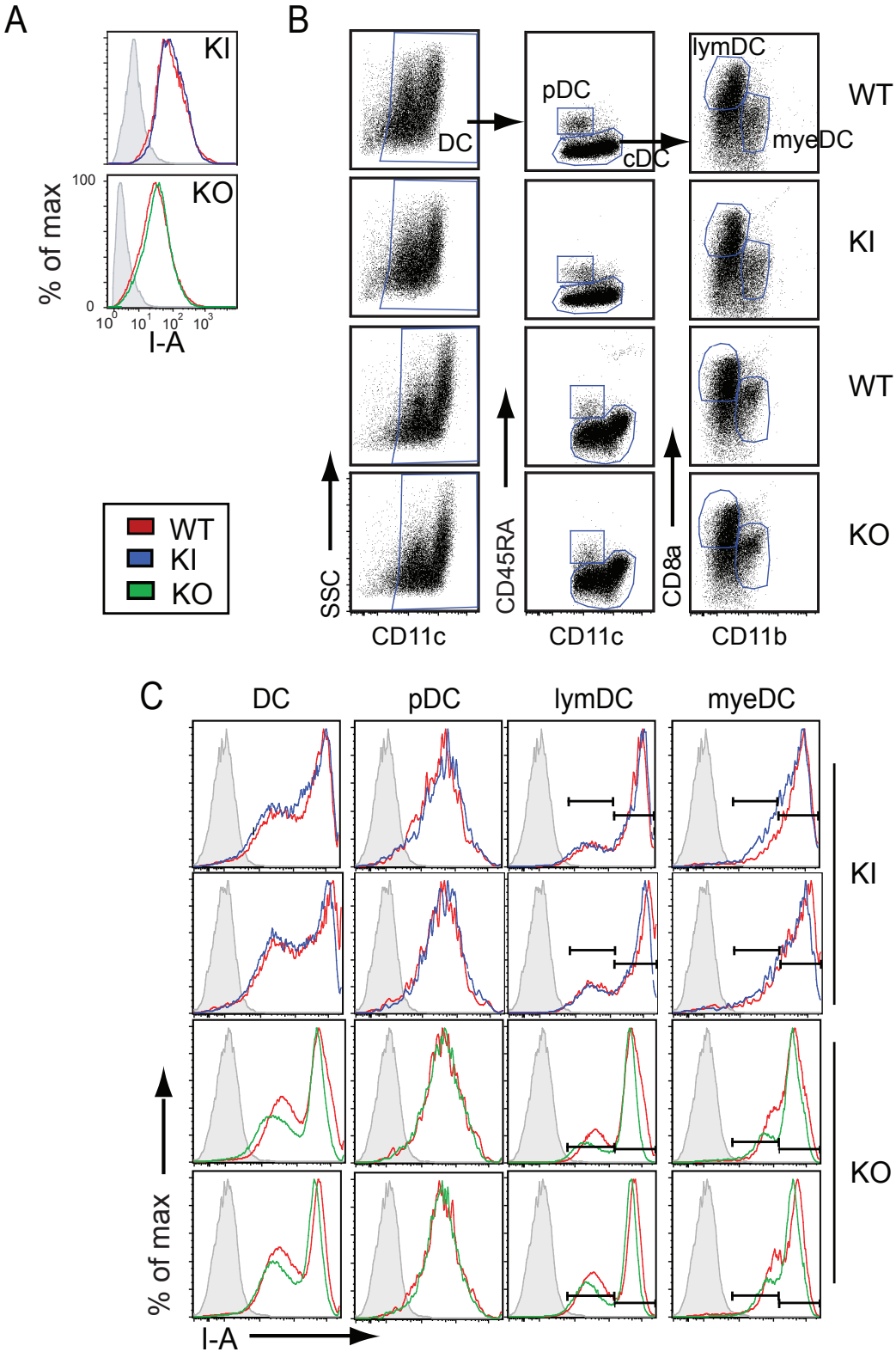

A
KI
KO
% of max
100
0
10^0 10^1 10^2 10^3
I-A
WT
KI
KO
B
DC
pDC
cDC
lymDC
myeDC
WT
KI
WT
KO
SSC
CD11c
CD45RA
CD11c
CD8a
CD11b
C
DC
pDC
lymDC
myeDC
KI
KO
% of max
I-A

Figure 2. MHC-II surface expression on KI and KO antigen presenting cells is similar to WT. A. Histogram analysis of flow cytometry data for I-A^b expression in KI and KO Figure samples compared to WT littermate controls. Thioglycolate elicited peritoneal macrophages were stimulated in vitro 24 h with 500 U/ml IFN-γ. Live cells were gated on by side scatter properties; macrophages were identified as $CD11b^+$. B. Flt3L elicited, CD11c+ sorted spleen cells were stimulated with 1 mg/ml LPS for 8 h to induce translocation of MHC-II molecules to the surface and analyzed by flow cytometry. Total dendritic cells were identified as CD11c+ and further divided into CD45RA+$CD11c^{int}$ plasmacytoid (pDC) and conventional DCs (cDCs). cDCs were divided into CD11b+ myeloid (myeDC) and CD8a+ lymphoid (lymDC) subpopulations. C. DC subsets identified in B were analyzed by flow cytometry for I-A^b expression. The data is representative of four mice for each strain. In panels A and C, WT littermate controls are shown overlaid on KI and KO samples. In panel B, WT littermate controls are shown directly above KI and KO samples.

MHC-II expression is correlated with Ciita expression in targeted mice

The expression of MHC-II on the surfaces of lymphoid and myeloid DC subsets as well as on macrophages in the KO strain was unexpected as 798 bp surrounding and including pI was deleted and this promoter was reported to control the expression of *Ciita* in cells derived from the myeloid lineage (LeibundGut-Landmann, Waldburger et al. 2004). Isoform specific quantitative real-time RT-PCR (qRT-PCR) using WT mice confirmed that the major *Ciita* transcript expressed in splenic DC (spDC) and macrophages treated with IFN-γ was isoform I (Fig. 3A). Isoform III was expressed at significant levels in spDC but not macrophages; whereas some isoform IV was detected in macrophages, but only after treatment with IFN-γ (Fig. 3A).

To investigate the transcriptional basis for cell surface MHC-II gene expression observed on the KI and KO spDC and macrophage cell populations presented in Fig. 2, RNA was also isolated from these samples to perform analyses of *Ciita* transcripts by qRT-PCR. Cell purity was greater than 95% as measured by CD11c expression on spDC and CD11b expression on macrophages (Fig 2B and data not shown). Except in the case of KO spDC, in which *Ciita* expression was reduced by 30%, *Ciita* levels were similar in both wild-type and mutant cells (Fig. 3B). This correlated with the observed decrease in surface MHC-II expression observed only on cDC from the KO line (Fig. 2C). I-Aα mRNA levels were also similar to WT, except in the case of KO spDC, where I-Aα was decreased by 55% (Fig. 3C, left panel).

Isoform specific qRT-PCR was used to determine the origin of the *Ciita* transcripts that were being detected (Fig. 3D). Again, wild-type cells principally use pI and produce isoform I C*iita* transcripts. KI cells were found to express isoform III CIITA, however, this could be a combination of expression from both pI and pIII as the primers cannot distinguish between the two. Using a primer specific for the HA-tag confirmed that KI spDC

and macrophages express the KI, HA-tagged-isoform III CIITA allele. Unexpectedly, spDC derived from KO cells expressed high levels of transcripts containing pIII exon 1, producing CIITA isoform III, and low, but detectable levels of isoform IV. Furthermore, in the case of KO derived macrophages, CIITA isoform III, as well as isoform IV, were expressed at significant levels in response to IFN-γ (Fig. 3D, right panel). These results suggest that there may be a change in promoter utilization in the *Ciita* pI KO mice.

CIITA has been found to be responsible for the regulation of multiple MHC-II associated genes, including *H2-D1*, *H2-DMa*, *H2-DMb, H2-DOa, H2-DOb,* and *Ii* (Nagarajan, Bushey et al. 2002; Krawczyk, Seguin-Estevez et al. 2008), as well as genes outside of the MHC locus. ChIP-chip experiments on human dendritic and B cells identified the genes *KIAA0841, PSMD3, RAB4B*, *TPP1*, and *TRIM26* (Krawczyk, Seguin-Estevez et al. 2008), and microarray analysis of human B cells (Nagarajan, Bushey et al. 2002) and mouse dendritic cells (Wong, Brickey et al. 2003) identified *KPNA6, RAB4B,* and *Plxna1* as additional potential targets of *Ciita.* (Nagarajan, Bushey et al. 2002; Krawczyk, Seguin-Estevez et al. 2008)Additional genes potentially regulated by CIITA are *Cste* and *Col1A1* (Yee, Yao et al. 2004) (Buttice, Miller et al. 2006). To determine if the CARD-containing isoform of CIITA was differentially influential in the expression of these genes, qRT-PCR was carried out on RNA isolated from spDCs and macrophages treated with IFN-γ. For the most part, no significant differences in expression for the above genes were observed between KO and wild-type macrophages or dendritic cells (Supplemental Fig. 1A and 1B). There were however two exceptions. In concordance with the level of total CIITA mRNA expressed in KO derived splenic DC, *Ii* was reduced to a similar extent as $I\text{-}A^b$ (Supplemental Fig. 1A and Fig. 3C). The second exception involved *H2-DOb* expression. In contrast to the other CIITA regulated genes in which IFN-γ treatment of wild-type macrophages either had limited effect or induced their expression, IFN-γ treatment of wild-type macrophages reduced the

levels of *H2-DOb* mRNA by ~7 fold (Supplemental Fig. 1C). Intriguingly, *H2-DOb* expression only decreased by about half in the IFN-γ treated KO-derived macrophages (Supplemental Fig. 1C). As a point of reference, *H2-Ob* was expressed at very low levels in both stimulated and unstimulated macrophages, while the expression was much higher in spDC (data not shown). This agrees with previous data that *H2-Ob* is expressed primarily by B cells, subsets of thymic epithelial cells, and subsets of dendritic cells, but not monocytes or macrophages (Hornell, Burster et al. 2006; Fallas, Yi et al. 2007). Therefore, for the most part, expression of non-MHC, as well as MHC genes regulated by CIITA, was not dependent on the CARD domain of CIITA for induction.

spDC use alternate promoters in the absence of pI

Because cells from KO mice expressed transcripts that appeared to derive from promoters III and IV, 5'RACE was conducted to determine the transcriptional start sites of the CIITA transcripts in spDC from KO and KI mice (Fig. 4 and Supplemental Fig. 2). One hundred percent of the transcripts from wild-type spDCs originated at pI. In the KI spDCs, 77, 15.9, and 6.8% initiated their *Ciita* transcripts from pI, pIII, and pIV, respectively. The majority of transcripts in the KO derived spDCs were initiated in the proximity of pIII (96.3%), with the remaining transcripts being derived from pIV. Thus, spDCs derived from the KO mouse have switched the utilization of their *Ciita* promoter from pI to pIII.

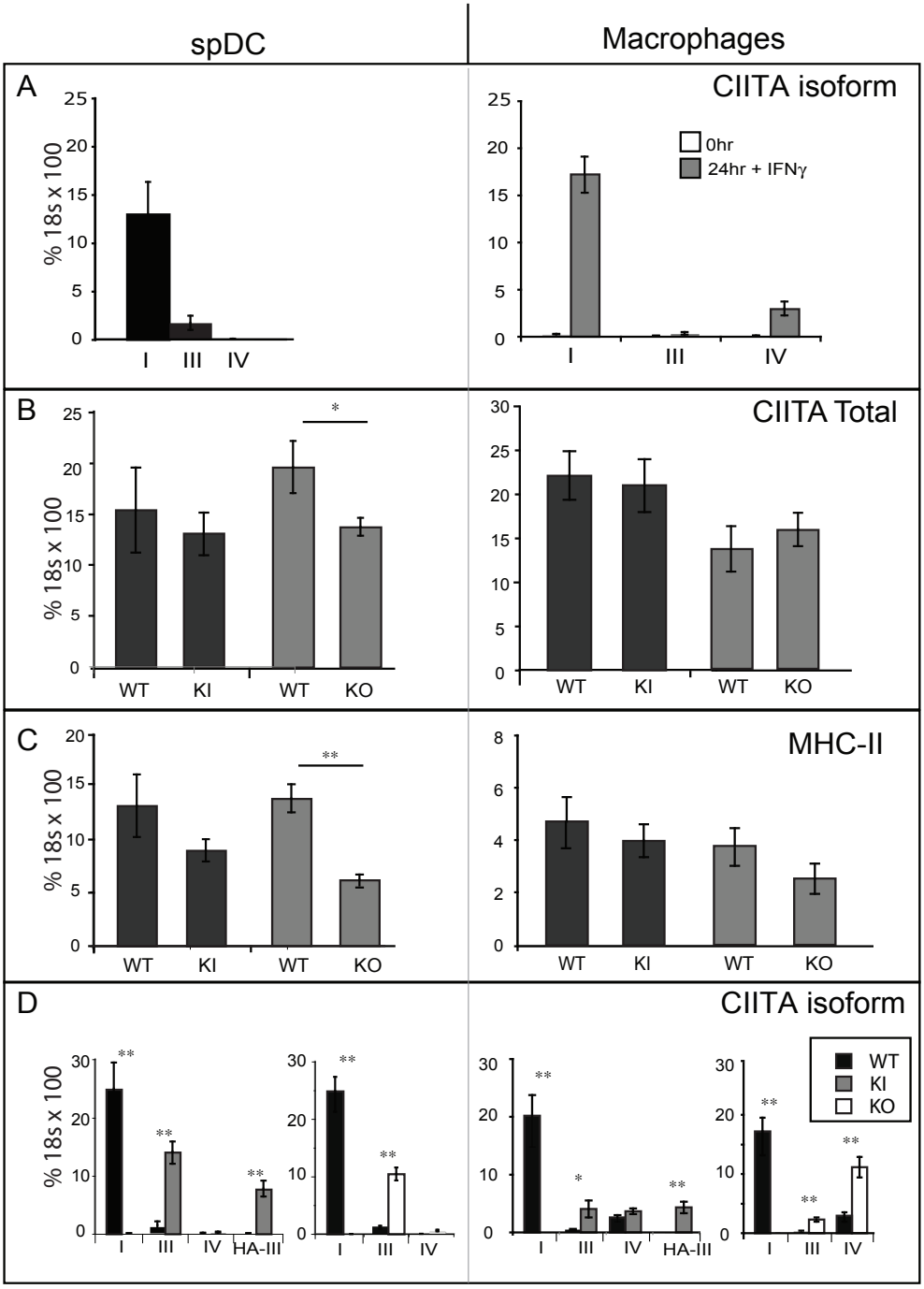

spDC
Macrophages
A
CIITA isoform
% 18s x 100
0hr
24hr + IFNγ
I
III
IV
B
CIITA Total
*
WT
KI
KO
C
MHC-II
**
D
HA-III

Figure 3. *Ciita* isoform I is not required for expression of CIITA or MHC-II in cells of the myeloid lineage. Ciita isoform specific, total Ciita mRNA, or I-Aa mRNAs were quantified in Flt3L-elicited splenic DCs (left panels) or thioglycolate-elicited peritoneal macrophages (-/+IFN-γ) (right panels) were analyzed by qRT-PCR with the primer sets indicated. Flt3L elicited spDC were purified with CD11c+ MACs beads and were >95% $CD11c^+$. Thioglycolate-elicited, peritoneal cavity macrophages were purified by adherence to tissue culture plates and were >95% CD11b+. A. Isoform specific PCR of CIITA in WT splenic dendritic cells (spDC) and macrophages treated for 24h with IFN-γ. B. Total *Ciita* mRNA levels in KI, KO, and WT littermate controls. C. I-Aa mRNA levels in KI, KO, and WT (littermate controls) spDC or macrophages treated with IFN-γ. D. Isoform specific expression of *Ciita*. HA-III represents primers specific for the Knock-in allele, HA-tagged isoform III derived from promoter I. Data were averaged from four mice for each genotype, with each representing one biological replicate. Significance was calculated using the students *t*-test. *$p<0.05$, **$p<0.005$.

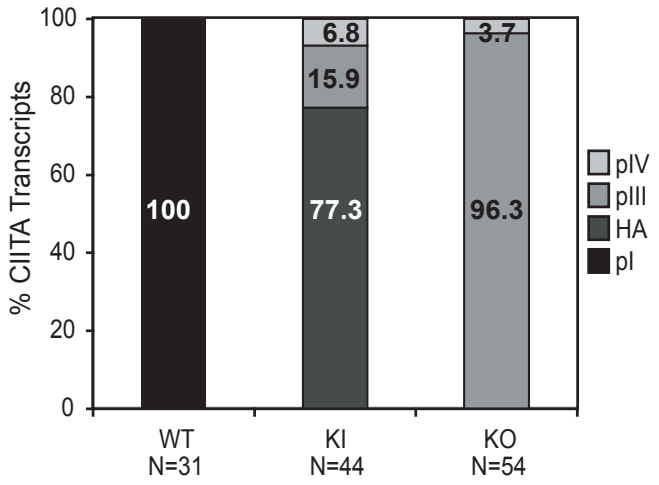


Figure 4. 5'RACE maps *Ciita* expression to promoters III and IV in KI and KO spDC. 5'RACE was carried out on mRNA from WT, KI, and KO splenic DCs. Wild-type spDC *Ciita* transcripts initiated from pI, exclusively. Both KI and KO spDC expressed *Ciita* transcripts from promoters III and IV. The majority of *Ciita* transcripts in KI cells were the HA-tagged isoform III, and were expressed from promoter I (HA-pI). N refers to the number of cloned sequences analyzed.

CIITA Isoform I is dispensable for proper T cell development

Positive and negative thymic selection of the naïve T cell repertoire is in part controlled by the specific expression of peptide-MHC complexes by specific cell types in the thymus (Klein, Hinterberger et al. 2009). Negative T cell selection is maintained in both *Ciita* pIV and pIII/pIV knock out mouse models, suggesting that bone marrow derived thymic medullary cells, which are required for negative selection, express CIITA from promoter I and therefore CIITA isoform I (Waldburger, Suter et al. 2001; LeibundGut-Landmann, Waldburger et al. 2004). Thus, any specific need for CIITA isoform I's CARD domain might be revealed by a change in the percentage of single positive CD4 T cells when comparing the KI and KO with wild-type thymocytes. It has been shown that impairing negative selection, either by deletion of dendritic cells or knocking down CIITA in mTECs, results in an increased percentage of CD4 T cells in the thymus (van Meerwijk, Marguerat et al. 1997; Ohnmacht, Pullner et al. 2009; Hinterberger, Aichinger et al. 2010). Flow cytometry of CD4 and CD8 single and double positive populations in the thymi of wild-type and *Ciita*-targeted mice showed that there was no perturbation of the CD4+, CD8+, or CD4+CD8+ T cell compartments when CIITA isoform I was lacking (Fig. 5). These data suggest that CIITA isoform I is not required for the regulation of MHC-II expression to the appropriate levels to ensure proper T cell development in the thymus. Because the KI line has a higher percentage of transcripts derived from pIII and pIV (Fig. 4) and the fact that the HA-tagged allele may add to the stability of the CIITA protein produced (Schnappauf, Hake et al. 2003), the studies described below will use the KO model to assess the role of the pI isoform on a variety of immune responses.

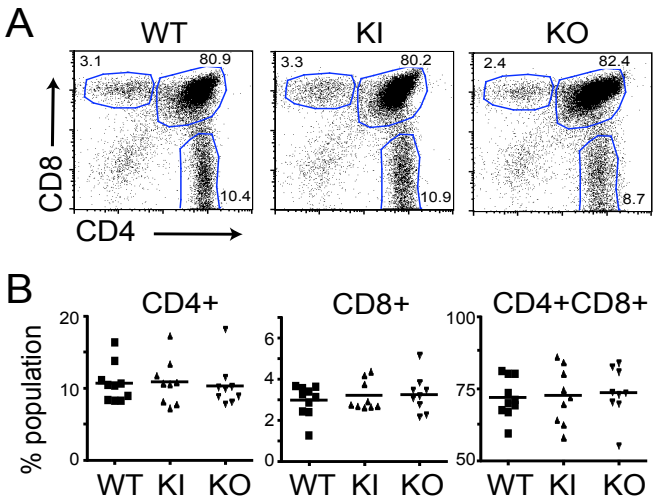


Figure 5. *CIITA* isoform I is not required for T cell development in the thymus. Thymocytes from five-week old mice were analyzed for CD4+, CD8+, and CD4+CD8+ populations using flow cytometry. A. Representative dot plots from individual thymi, gated on live cells and plotted as CD4 vs. CD8. Gates used to define CD4+, CD8+, and CD4+CD8+ populations are shown. B. The results from three experiments with 3-4 mice per group are combined (N=10 WT, N=9 KI and KO). None of the samples were found to differ from the wild type by a p value of <0.05. using a student's t test.

The CARD domain is not required for antigen presentation in vitro

To determine whether CIITA isoform I's CARD domain is required in antigen presentation, a lymphocyte proliferation assay was used to compare T cell activation between WT and KO mice. Lymph nodes were collected from mice primed with the LCMV, CD4 T cell specific antigen, peptide GP_{61-80} (Oxenius, Bachmann et al. 1995). Mice were immunized containing either 1mg/ml GP_{61-80} in CFA or a ten-fold dilution of peptide to determine whether immunization with suboptimal doses would reveal subtle differences in T cell response. Single cell suspensions were incubated with increasing concentrations of peptide *ex vivo* and the proliferation of T cells was measured by incorporation of [^{3}H]-thymidine. CD4 T cells derived from the draining lymph nodes of WT and KO mice displayed similar responses to the peptide antigen when immunized with either dose (Fig. 6). Thus, despite loss of isoform I CIITA, there was no defect in activation of T cells *in vitro*, indicating no defect in antigen presentation in KO APCs.

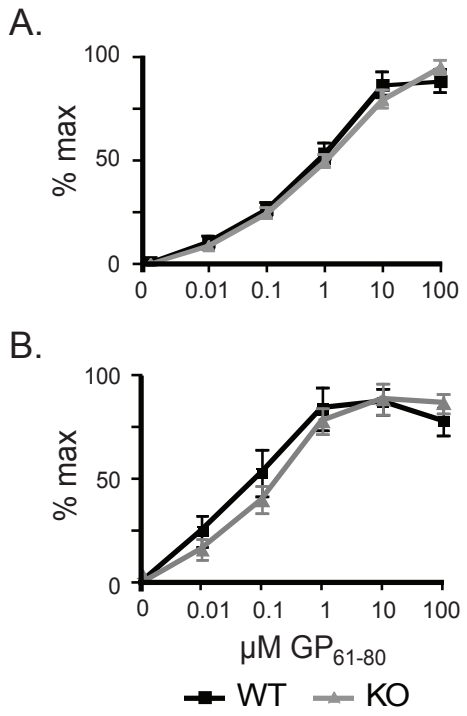


Figure 6. *Ciita* pI KO T cells proliferate normally in response to antigen. Mice were immunized in the footpad and the base of the tail with the 150ml peptide/CFA emulsion containing A. 1 mg/ml or B. 0.1 mg/ml of the peptide GP_{61-80}. After 12 days, draining lymph nodes were isolated and T cell proliferation was measured by incorporation of ^{3}H-thymidine in response to increasing concentrations of the antigenic peptide. Averaged data from multiple independent experiments are graphed as normalized counts per minute (CPM,) as a function of increasing concentration of peptide. (A. n=9 WT and 8 KO, B. n=6 WT and 7 KO).

CIITA Isoform I does not contribute to the development of experimental autoimmune encephalomyelitis (EAE)

EAE is an inflammatory demyelinating disease that is used as a model of multiple sclerosis. EAE is mediated by CD4 Th1 cells that recognize self-antigens associated with MHC-II molecules. CIITA isoforms 1 and 4 were found in the brain and spinal cord of mice with acute EAE, along with infiltrating DCs (Suter, Malipiero et al. 2000). Suter et al. found the majority of *Ciita* was expressed from pI in infiltrating DCs, while astrocytes express isoform IV in response to IFN-γ, and microglia express low levels of both isoforms 1 and 4 in response to IFN-γ. To examine if this model could reveal a need for isoform I, EAE was induced in WT and KO mice, and disease scores were recorded for 4 weeks. Disease severity was compared between the two groups over the entire four weeks, as well as in the last week alone, as this was when the disease scores stabilized. KO mice appeared slightly more sensitive than wild-type mice, with a maximum disease score of 2.5 vs. 2.0 for the WT group, although overall disease severity (combined disease index), disease severity in the fourth week only, and day of onset, were not found to be statistically different between the two groups (Fig. 7).

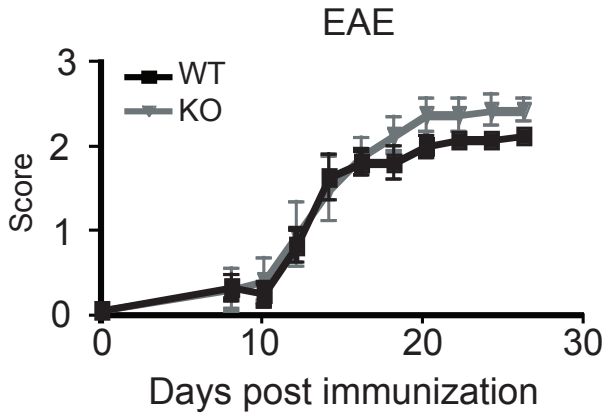


Figure 7. *Ciita* pI KO mice are not more susceptible to experimentally induced autoimmune disease. Experimental autoimmune encephalomyelitis was induced in groups of three to five females by injecting an emulsion of MOG and CFA. The experiment was performed three times and results were combined (n=11 WT and 10 KO). No difference was observed in onset or severity of disease. Severity was measured both for the entire four weeks of observation and for week four only. The max score was 2.0 for WT mice and 2.5 for the KO group ($p<0.05$). Error bars represent standard error. Statistics were done using student's *t*-test (onset) or Mann-Whitney (total disease score, max score) using Prism software.

Ciita KO mice are not more susceptible to L. monocytogenes

Listeria monocytogenes is an intracellular pathogen that targets macrophages. Several members of the NOD family have roles in *L. monocytogenes* recognition including NOD1, NOD2, and NLRP3 (Ye and Ting 2008; Elinav, Strowig et al. 2011). To determine whether the CARD-containing CIITA plays a role in resistance to *L. monocytogenes*, mice were infected and bacterial colony forming units (CFU) were counted in the spleen and liver five days after primary infection, or seven days after secondary infection (Fig. 8A). No difference was seen in bacterial burden in spleens or livers of KO mice compared to WT littermate controls after either primary or secondary infection, and all mice cleared the primary infection by day 10 (data not shown). To further dissect the immune response to *L. monocytogenes*, splenocytes were collected at the peak of the primary and secondary T cell response (ten or six days post infection or rechallenge, respectively), and intracellular cytokine staining for TNF-a, IL-2, and IFN-γ was performed in CD4+ T cells. It has previously been shown that CD4 lymphocytes express a Th1-type cytokine profile after infection with *L. monocytogenes* (Freeman and Ziegler 2005). There was no difference in cytokine expression in KO CD4+ spleen cells compared to WT control (Fig. 8B, C). These results indicate that KO mice are not deficient in immune response to *L. monocytogenes* infection and that the other CIITA isoforms can substitute for isoform I.

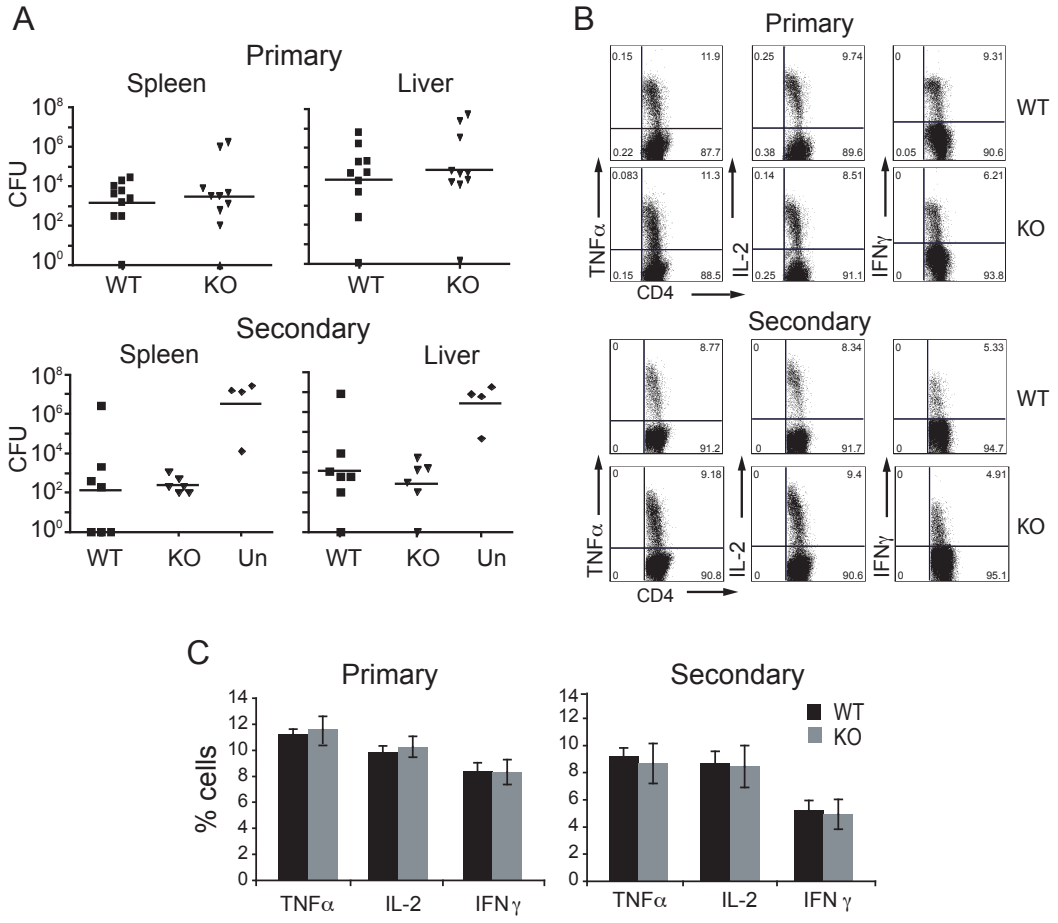

A
Primary
Spleen
Liver
CFU
WT
KO
Secondary
Un
B
Primary
Secondary
WT
KO
TNFα
IL-2
IFNγ
CD4
C
Primary
Secondary
% cells
TNFα
IL-2
IFN γ

Figure 8. *CIITA* isoform I is not required for resistance to the intracellular bacteria *Listeria monocytogenes*. WT and CIITA KO mice were infected with *LM*, and colony-forming units (CFU) were determined in the spleens and livers. A. For primary infection, mice were infected with 0.5 LD_{50} *LM* and CFU were determined at day five post-infection. For secondary infection, mice were immunized with a low dose (0.1 LD_{50}), then re-infected four weeks later with a high dose (10 LD_{50}) of *LM*, CFU was determined 3 days after re-challenge. Data represent two independent experiments. N=10 WT, 11 KO primary, 7 WT, 6 KO secondary. B. Representative flow cytometry plots of intracellular cytokine expression from CD4 T cells in peritoneum. In primary infection, cells were collected 10 days after infection with 0.8 LD_{50}. For secondary infection, mice were infected with 0.1 LD_{50}, then challenged four weeks later with 6 LD_{50}. Peritoneal exudate cells were collected six days later and CD4 T cell intracellular cytokine levels were measured by flow cytometry. C. Average with standard deviation of the percentage of CD4 T cells (from B) expressing cytokines TNFα, IL-2, and IFN-γ. Each graph is representative of two independent experiments using three to four mice per group. Un, unimmunized.

Ciita pI KO mice mount an effective immune response to lymphocytic choriomeningitis virus (LCMV)

To examine if the CARD domain containing CIITA isoform was required for specific responses to a viral infection, mice were infected with the Armstrong strain of LCMV. This viral strain generates an acute infection that is cleared after eight days (Wherry, Blattman et al. 2003). Following infection, WT and KO mice were analyzed for a number of parameters depicting a successful immune response to virus challenge and memory. The percentages of virus-specific CD8 or CD4 T cells following initial infection were similar between WT and KO mice (Fig. 9A and E). Virus-specific CD8 T cells from the spleen were analyzed for T cell specific memory markers CD44, CD25, CD62L, CD127, KLrg1, and PD-1 (Fig. 9B). No difference was observed in the expression of these markers as well. When CD4 and CD8 T memory cells were stimulated *ex vivo* for the production of cytokines, TNF-a and IFN-γ were also found to be similar (Fig. 9C). The LCMV-specific memory B cell pool, as measured by an antigen specific ELISPOT assay revealed higher values for the *Ciita* pI KO mice, but this value was not found to be statistically significant (Fig. 9D). Together these data indicate an effective adaptive immune response is generated in response to infection with LCMV in the absence of CIITA isoform I.

LCMV and *L. monocytogenes* elicit primarily a T cell mediated response. In order to test the B cell response in the CIITApI null model, mice were immunized with influenza and antibody titers were measured at various time points post-immunization. No differences were seen between the wild-type and KO mice in antibody responses as measured by hemagglutinin inhibition (HAI) titers or IgG, IgG1, or IgG2a titers in the serum at 8, 14, or 28 days after infection (Supplemental Fig. 3).

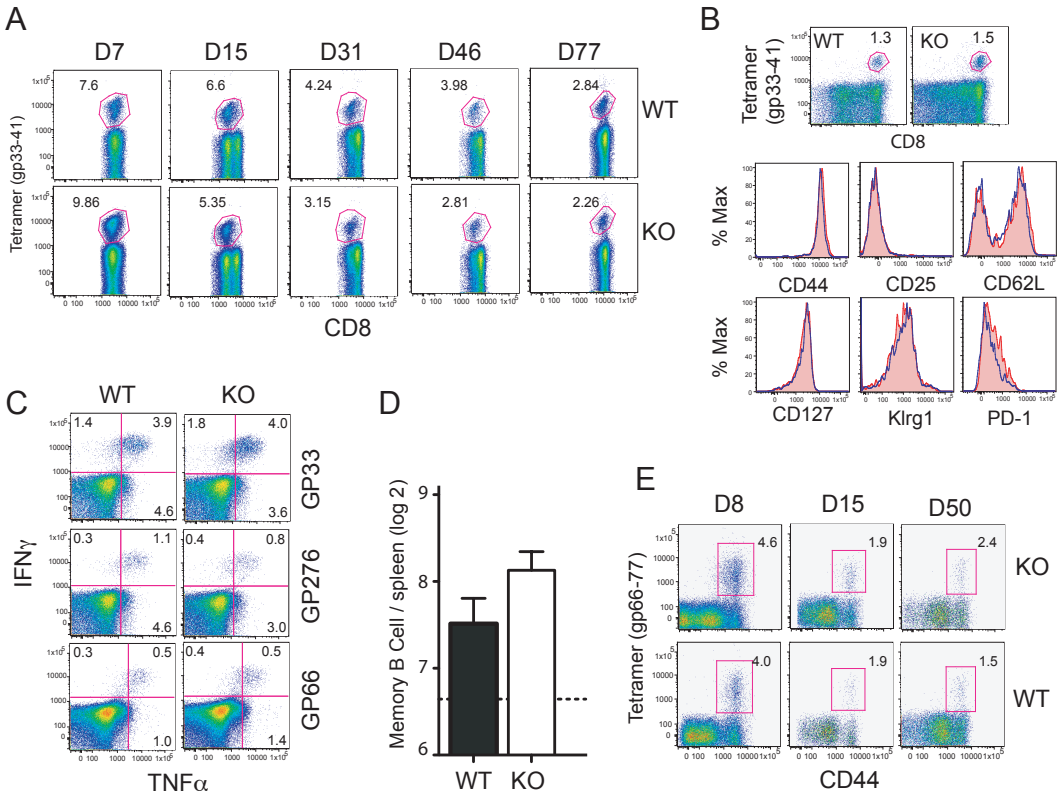

A
D7
D15
D31
D46
D77
Tetramer (gp33-41)
7.6
6.6
4.24
3.98
2.84
WT
9.86
5.35
3.15
2.81
2.26
KO
CD8
B
WT
1.3
KO
1.5
Tetramer (gp33-41)
CD8
% Max
CD44
CD25
CD62L
CD127
Klrg1
PD-1
C
WT
KO
IFNγ
1.4
3.9
4.6
1.8
4.0
3.6
GP33
0.3
1.1
4.6
0.4
0.8
3.0
GP276
0.3
0.5
1.0
0.4
0.5
1.4
GP66
TNFα
D
Memory B Cell / spleen (log 2)
9
8
7
6
WT
KO
E
D8
D15
D50
Tetramer (gp66-77)
4.6
1.9
2.4
KO
4.0
1.9
1.5
WT
CD44

Figure 9. The adaptive immune response to an acute LCMV infection in KO mice is similar to WT mice. A. Virus-specific CD8 T cells were analyzed using fluorescently labeled tetramer specific for the GP_{33-41} epitope of LCMV. Mice were serially bled and the percent of LCMV-specific CD8 T cells in PBMCs was determined by flow cytometry analysis at the given time points. B & C. Acutely infected mice were sacrificed at the maintenance stage of T cell memory differentiation, and phenotypic and functional analyses were performed on the LCMV-specific lymphocytes. B. Tetramer specific (GP_{31-40}) CD8 T cells were analyzed by flow cytometry for the T cell markers CD44, CD25, CD28L, CD127, Klrg1, and PD-1. Red filled histograms represent wild-type, blue histograms represent KO CD8 T cells. C. T cell receptor mediated cytokine expression was assessed by culturing splenocytes for 5h in the presence of peptides for the CD8 (GP_{33} and GP_{276}) and CD4 (GP_{61}) dominant epitopes of LCMV. D. The absolute number of LCMV-specific memory B cells was determined using an LCMV-specific ELISPOT analysis of memory B cells responses. E. Virus-specific CD4 peripheral blood mononuclear T cells were analyzed for CD44 expression and with tetramer specific for the GP_{66-77} epitope of LCMV.

Discussion

Expression of MHC-II genes is regulated at the level of transcription by the presence or absence of the master regulator CIITA. The expression of CIITA from pI was of particular interest because this isoform has homology to a CARD domain (Nickerson, Sisk et al. 2001) and its use is restricted to the myeloid and DC compartments (LeibundGut-Landmann, Waldburger et al. 2004). Thus, it seemed that this isoform would have unique properties that might be revealed by either substitution or deletion of the isoform. The knock-in/knockout models created here allowed this hypothesis to be tested. Surprisingly, no gross defect in immune system function or development in either the CIITApI→III KI or CIITApI→0 KO was observed. In the case of the KO model, total levels of *Ciita* transcript were not reduced in macrophages treated with IFN-γ, and were not reduced by more than 30% compared to WT in spDC. Surface MHC-II expression reflected *Ciita* mRNA levels. No obvious defect in T cell development was observed either, as the percentage of CD4+ T cells in the thymus was normal. Moreover, T cells from the mutant mice responded normally to *in vitro* stimulation and showed no significant differences to lower doses of antigen. KO mice were only marginally more susceptible than wild-type in one model for autoimmunity (EAE), but all mice stabilized with the same level of disease. KO mice were equally immune competent (compared to wild-type) when challenged with a viral or bacterial agent (LCMV or *L. monocytogenes,* respectively), and produced antibodies at levels comparable to wild-type after vaccination to influenza. It remains possible that the decreased levels of *I-A* and *Ii* observed in KO spDC have a subtle effect on immune system function not detected by the experiments presented here. Thus, we conclude that in the models tested, no unique function can be ascribed to the CARD-containing isoform of CIITA.

Interestingly, expression of MHC-II was decreased to a greater extent than *Ciita*. Nickerson et al. (Nickerson, Sisk et al. 2001) suggested that pI is a more potent

transcriptional activator than the other isoforms; however, this was not confirmed by Buttice et. al. (Buttice, Miller et al. 2006), who found no difference in transactivation activity between the various isoforms. The reason Nickerson et al. observed greater activity from isoform I may be related to the CARD sequence, which could potentially provide increased stability of the protein (Schnappauf, Hake et al. 2003). The half-lives of isoforms III and IV have been reported to be the same (Schnappauf, Hake et al. 2003), but to date there is no data on the half-life of isoform I. Data presented here argue that equal levels of CIITA either from pI (endogenous) or from the KI lead to similar levels of I-A transcripts, suggesting that CIITA levels are the limiting determinant. Similarly, isoform I is not specifically required for induction of a number of genes identified as CIITA targets, including *I-A^b*, *H2-D1*, *H2-DOa*, *H2-DM*, *Ii, Col1A1, Cste, Kiaa00841*, *Kpna6*, *Plxna1*, *Psmd3, Rab4b, Tpp1,* and *Trim26,* as differences in expression of these genes was not observed in mice expressing only isoform III. With the exception of *Plxna1* (Wong, Brickey et al. 2003), which is specific to DC, all of these genes are also expressed in B cells. KO macrophages treated with IFN-γ had higher levels of *H2-DOb* mRNA than wild-type macrophages. This result was unexpected for a few reasons. First, *H2-DO* is strongly expressed in B cells, but it is poorly expressed by macrophages *(Karlsson, Surh et al. 1991)* (Hornell, Burster et al. 2006; Fallas, Yi et al. 2007). Second, the levels of H2-DOb mRNA were reduced in wild-type but not KO IFN-γ treated macrophages. Lastly, the regulation of *HLA-DOB/H2-DOb* in B cells by CIITA has been controversial, some data suggest that *HLA-DOB/H2-DOb* is not regulated by CIITA in B cells (Chang, Guerder et al. 1996; Taxman, Cressman et al. 2000), while other data suggest the opposite (Nagarajan, Bushey et al. 2002; Nagarajan, Lochamy et al. 2002; Krawczyk, Seguin-Estevez et al. 2008). These data may indicate that CIITA isoform III, but not isoform I, is important for the proper regulation of *H2-Ob*. Thus, with the possible exception of H2-DOb,

the CARD domain of isoform I does not appear to be required for expression of any of the genes identified as targets of CIITA regulation.

Previous mouse knockout models showed no evidence for cross-talk between the promoters. In the *Ciita* pIV KO mouse, expression of *Ciita* from myeloid and lymphoid cells was not affected (Waldburger, Suter et al. 2001). Likewise, in the double pIII/pIV KO in which a large region was deleted, expression of *Ciita* in conventional DCs appeared normal (LeibundGut-Landmann, Waldburger et al. 2004). Therefore, it was unexpected that the deletion of pI would have an effect on downstream promoters. The fact that deletion of the 798 bp surrounding and including pI alters expression from other promoters suggests that the mechanisms that control expression from pI also operate on alternate promoters, but are prevented from doing so in cells of the myeloid lineage. Thus, it is most likely that there is an unidentified regulatory region that normally promotes expression of *Ciita* from promoter I in cells of the myeloid lineage. Expression of *Ciita* from pIII in the dendritic cells of mice in which pI was deleted suggests that *Ciita*'s "myeloid specific" regulatory region can direct expression from any (or perhaps the nearest available) promoter. Alternatively, pI and pIII may share transcription factors necessary for expression and that some other mechanism prevents pIII expression in spDC. Previous models that deleted pIV or pIII/pIV failed to identify this possibility, as there was no promoter switching observed in these models and little is known about the regulation of *Ciita* pI. Recently, Smith et al. (Smith, Wright et al. 2011) characterized the promoter proximal region and identified binding sites for the positive regulatory factors Pu.1 and IRF8, and PRDM1 as a negative regulatory factor. Choi et al. identified two STAT5 binding sites upstream of pI (Choi, Yu et al. 2009). Our data demonstrate that deletion of the proximal promoter region, which includes the regions studied in these reports, is insufficient to silence *Ciita* expression in cells that primarily use pI.

Transcription factors regulating *Ciita* pIII and pIV have been well characterized (Muhlethaler-Mottet, Di Berardino et al. 1998; Ghosh, Piskurich et al. 1999; Piskurich, Linhoff et al. 1999; Morris, Beresford et al. 2002; van der Stoep, Quinten et al. 2002; van der Stoep, Quinten et al. 2004; Yoon and Boss 2010), and using these as a guide, some suggestions of what may occur at pI and promoter choice can be hypothesized. Several years ago it was found that DNA methylation of the CpG dinucleotides encompassing pIV prevented expression in response to IFN-γ in tumors or cell types refractory to this induction (Morris, Spangler et al. 2000; van Eggermond, Boom et al. 2011). Analyses of the chromatin structure of pIV in human cells showed that other mechanisms may be at play and, by analogy such mechanisms may function with pI as well. In HeLa cells, five regions spread across a 110 kb span that contained the *CIITA* gene were identified that could influence expression of pIV in response to IFN-γ (Ni, Abou El Hassan et al. 2008). These regions were shown to interact with one another through chromatin conformation capture assays (Ni, Abou El Hassan et al. 2008). Intriguingly, one of these regions was upstream of pI and was also identified as a DNase hypersensitive site in B cells. This region, termed HSS1, binds PU.1 (Yoon and Boss 2010), a critical regulator of B cell and myeloid cell fate, as well as STAT1 (Ni, Abou El Hassan et al. 2008), a factor activated when cells are induced by IFN-γ. HSS1 lies three kb upstream of pI and was not deleted from our constructed mouse strains. Thus, the possibility exists that HSS1 could be a pI and pIII control region. However, it was only one of several elements in the long-range control of pIV and one of the other elements, or an as yet undiscovered element could control the use of pI. Thus, whichever element(s) controls expression in myeloid cells, our data suggest that this element targets pI first as opposed to the other *Ciita* promoters.

Thus, although CIITA in cells of the myeloid lineage express a unique isoform with a CARD homologous domain, this specific domain does not appear to have a unique function

with respect to MHC-II expression and the ability of cells using this promoter to mount successful immune responses. Instead, the system appears to be designed to specifically control CIITA and subsequently MHC-II gene expression in a strict tissue specific fashion by having distinct promoters and regulatory mechanisms that restrict expression to each of the promoters.

Acknowledgements

We thank Dr. Joseph Sabatino for help with some of the experiments presented. We also thank the Emory University School of Medicine Core Facility for Flow Cytometry and the NIH tetramer core. This work was supported by the National Institutes of Health grants RO1AI43000 and R56AI34000 (to JMB), PO1 AI080192-01 (to JMB and RA), HHSN266 200700006CMD8 (to IS), and the American Cancer Society postdoctoral fellowship PF-09-134-01-MPC (to BY).

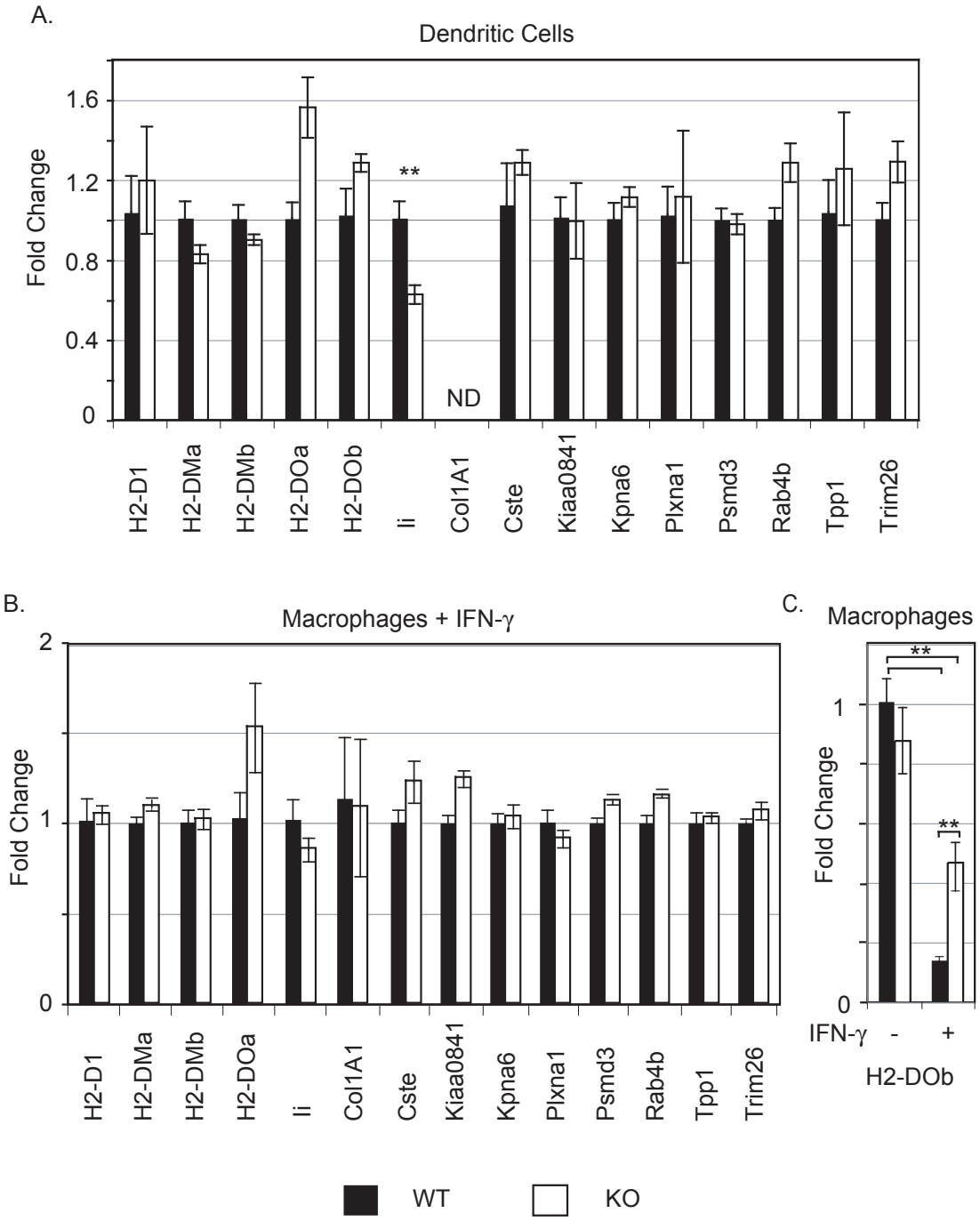
Supplemental figure legends

Supplemental Fig. 1. CIITA regulated genes do not require CIITA isoform I for expression. A. Splenic dendritic cells and B. Macrophages treated with IFN-γ 24 h were analyzed by qRT-PCR for the indicated genes identified previously as being regulated by CIITA. Ct values were normalized to the values for actin mRNA and graphed as fold over wild-type. ND: Col1A1 was not detected at significant levels in dendritic cells. C. RNA from control and IFN-γ treated macrophages (24 h) was assayed for the steady state levels of *H2-DOb* mRNA levels as above. Values are represented as fold over untreated wild-type. Data in this figure were averaged from four independent biological replicates (i.e., four mice), and the statistical significance was calculated using students *t*-test, ** $p<0.01$.

Supplemental Fig. 2. 5'RACE sequence results from splenic dendritic cells. Sequences obtained from 5'RACE analysis are shown relative to the predicted transcriptional start sites for each promoter. Colored lines represent transcribed sequence and thicker lines represent translated sequence. The numbers of transcripts sequenced in each mouse line are shown in the right hand columns.

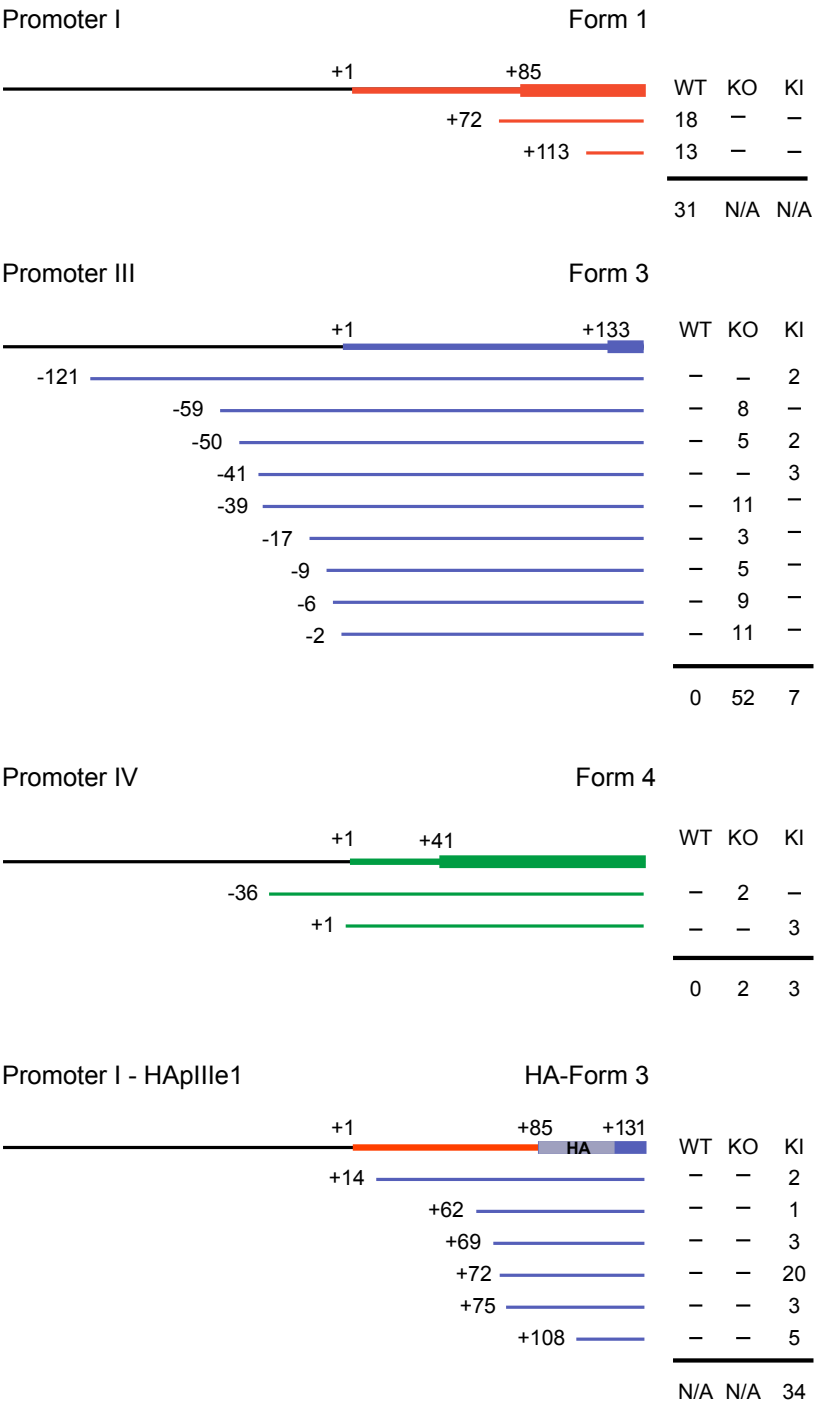
Supplemental Fig. 3. CIITA mice form antibody secreting B cells in response to immunization with influenza. Mice were immunized and serially bled as described in materials and methods. HAI titer and IgG, IgG1, or IgG2a concentration is plotted for each mouse and each time point.

Supplemental Figure 1

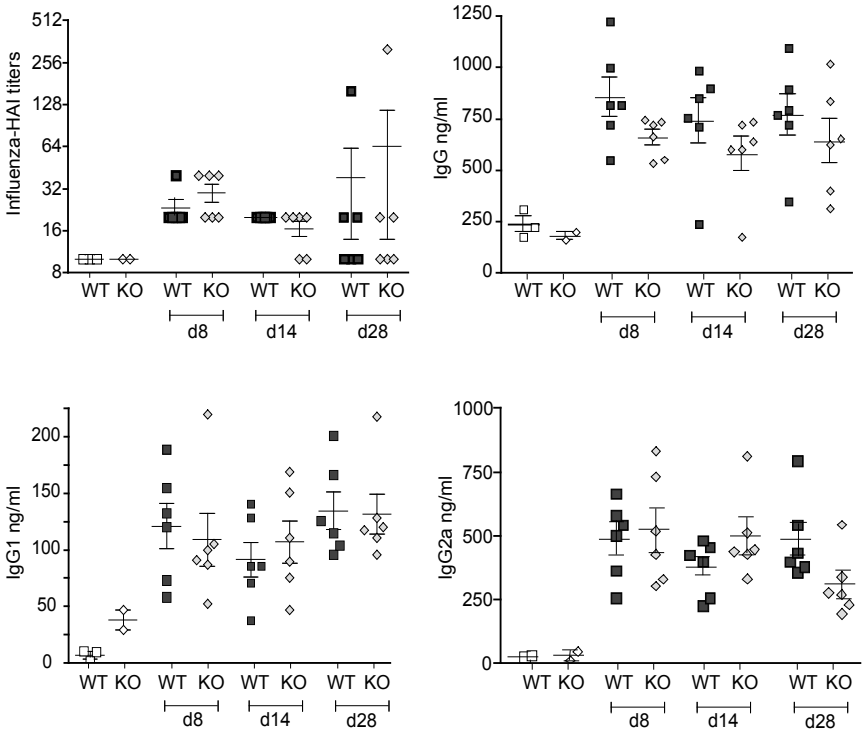


Supplemental Figure 2

5'RACE Results from splenic Dendritic Cells



Supplemental Figure 3



Chapter Four: Regulation of CIITA Promoter choice

This chapter presents current knowledge of CIITA promoter regulation and presents data on DNA methylation at the CIITA locus.

This Chapter was written by W.M. Zinzow-Kramer

Data for this chapter were contributed by: W.M. Zinzow-Kramer, James W. Austin, Benjamin Barwick, Sarah Lohsen, and Christopher Scharer.

W.M. Zinzow-Kramer and S. Lohsen did 5-azacytidine treatments and subsequent CIITA qRT-PCR analysis.

Splenic dendritic cells, primary peritoneal macrophages, and J774 macrophages were isolated and prepared by W.M. Zinzow-Kramer.

Introduction

The three promoters of the gene encoding CIITA (*CIITA* in humans, *Ciita* in mice) have cell type specific patterns, and as such, have been labeled the myeloid, lymphoid, or IFN-γ responsive promoters to reflect this characteristic. Promoter specific analysis has shown that most antigen presenting cells (APCs) express *CIITA* primarily from one promoter: pI in myeloid cells, and pIII in lymphoid cells. Promoter IV is inducible in most cell types, including APCs, after stimulation with IFN-γ. Studies of *CIITA* gene expression have thus far focused mainly on individual promoters and the regions directly surrounding those promoters. The mechanisms by which non-expressed promoters are silenced or are otherwise inactive are not known. Our recent findings in *Ciita* $pI^{-/-}$ mice indicate that in wild-type dendritic cells (DC), promoters III and IV are transcriptionally competent, but inactive. The predominant isoform expressed by DC is transcribed from pI. If pI is deleted, *Ciita* expression in DC is redirected to downstream promoters. There therefore must be some mechanism by which dendritic cells "choose" to express *Ciita* primarily from pI. This "promoter choice" relies on more than availability of transcription factors or activation of specific enhancers. Expression of pIII and pIV in *Ciita* $pI^{-/-}$ mice indicates that activating transcription factors and enhancers must already be present and functional. Factors that influence promoter choice may do so by influencing DNA binding of transcription factors. These factors might include DNA methylation, histone modifications, distal enhancer elements, and higher order chromatin structure, such as chromatin looping. The first half of this chapter will discuss current knowledge of cell type specific activation of the various *CIITA* promoter regions. The second half of the chapter presents preliminary data on DNA methylation and how it may function in CIITA promoter choice by altering transcription factor binding and chromatin structure.

Many *cis*-acting transcription factors have been identified that bind *CIITA* promoters. Some of these factors are capable of binding multiple *CIITA* promoters, but binding is cell type specific (van der Stoep, Quinten et al. 2002; van den Elsen, Holling et al. 2004; Ito, Nishiyama et al. 2009; Smith, Wright et al. 2011). The fact that transcription factor binding is cell type and context dependent means that factors other than DNA sequence must determine factor binding. In fact, this is a common theme with DNA binding proteins. Not only must the DNA sequence match some consensus sequence, but the chromatin must also be accessible to the protein. In many cases, multiple cooperatively binding proteins must also be present. Thus, DNA-protein interactions are highly complex and require a number of factors for favorable binding (Carey 1998). In this instance, *CIITA* promoters share transcription factors, and availability of these factors alone cannot determine which promoters will be active.

To date, regulation of *CIITA* has been studied mostly by examination of approximately 300 base pairs upstream of the transcriptional start site of each promoter. Indeed, the 300 promoter-proximal base pairs were determined to be sufficient to maintain cell type specificity of pIII and pIV (Lennon, Ottone et al. 1997; Muhlethaler-Mottet, Otten et al. 1997; Piskurich, Wang et al. 1998; O'Keefe, Nguyen et al. 2001). Promoter-reporter assays using only the promoter-proximal region of pIII or pIV demonstrated that cell type specificity is maintained in some cell types. A reporter construct containing 322 bp upstream of pIII transcriptional start site (TSS) was active in the B cell line Raji, but not the melanoma cell line Me67.8, even after induction with IFN-γ. Likewise, a construct containing 461 bp upstream of pIV was active in Me67.8 cells induced with IFN-γ, but not Raji cells (Muhlethaler-Mottet, Otten et al. 1997). This is consistent with endogenous expression of *CIITA* in these cell lines: Raji cells express *CIITA* exclusively from pIII, whereas Me67.8 cells express *CIITA* from pIV, but only after induction with IFN-γ. The fact that the

promoter regions of pIII and pIV maintain cell type specificity suggests that there are cell type specific factors required for expression from these promoters. When these factors are missing, transcription does not occur. However, our recent studies with *Ciita* pI knockout mice demonstrate that, at least in myeloid cells, the promoter proximal regions are not sufficient to confer cell type specificity. It is evident that pIII and pIV can be expressed in cells of the myeloid lineage as well, meaning the transcription factors required for expression from these downstream promoters are present in myeloid cell types (specifically conventional dendritic cells and macrophages). The first step to understanding *CIITA* promoter regulation is to characterize promoter proximal regions and the transcription factors required for expression from each promoter.

Although *CIITA* pI is the primary promoter utilized by myeloid cells (DC and macrophages), detectable levels of transcripts from downstream promoters are present. This expression from pIII and pIV in myeloid cells is weak unless pI is deleted, which allows for increased expression from pIII and pIV. In the *Ciita* pIV knockout model, it was reported that expression from *Ciita* pI was not affected in DC or B cells. Both of these cell types express very little pIV-derived *Ciita*. Unfortunately, no comparison was made of pI expression in macrophages induced with IFN-γ. Macrophages express both pI and pIV in response to IFN-γ. Deletion of pIV did not result in decreased MHC-II expression, even though it has been shown that MHC-II expression is quantitatively linked to *CIITA* expression (Otten, Steimle et al. 1998). It is therefore of interest to know if expression from pI increased in $pIV^{-/-}$ macrophages, thereby compensating for loss of pIV-derived *Ciita*. Recent data from *Ciita* $pI^{-/-}$ mice indicates that expression from downstream promoters can compensate for loss of pI, though mechanism by which this promoter switch occurs is not known.

Comparison of cis*-elements and transcription factor binding*

Multiple *cis*-acting elements have been identified for each of the promoters. Though many papers have been published detailing *CIITA* gene expression from pIII and pIV, there are only a few papers detailing pI (Choi, Yu et al. 2009; Kitamura, Yokoyama et al. 2011; Smith, Wright et al. 2011). In dendritic cells, PU.1 seems to be a critical factor for *CIITA* pI expression, though IFN regulatory factor 4 (IRF-4), IRF-8, the p65 NFκB subunit, and Sp1 also bind the promoter and are required for optimal *CIITA* expression (Kitamura, Yokoyama et al. 2011; Smith, Wright et al. 2011). A cartoon of the promoter proximal region for pI, along with identified DNA-binding proteins, is depicted in Figure 1.

Promoter III is perhaps the most well studied *CIITA* promoter. The promoter region of pIII, known *cis*-elements, and transcription factors known to bind in specific cell types, are illustrated in Figure 1. In vivo genomic footprinting was used to compare promoter occupancy at *CIITA* pIII in both B and T cells (van der Stoep, Quinten et al. 2002). Some regions were occupied in both cell lines, while other regions were more highly occupied in just one cell line. Expression of *CIITA* pIII in B cells is dependent on five elements, that together recruit PU.1, E47, SP1, cAMP-responsive element binding protein (CREB), activating transcription factor (ATF), NF1, Oct1, AML2, and IRF4 (Ghosh, Piskurich et al. 1999; van der Stoep, Quinten et al. 2002; van der Stoep, Quinten et al. 2004; Green, Yoon et al. 2006). The transcription factors CREB and ATF are ubiquitously expressed, and even bind *CIITA* pIII in non-CIITA pIII expressing cells (van der Stoep, Quinten et al. 2002). In contrast, interferon regulatory factor 4 (IRF-4) interacts with PU.1 and E47 to activate *CIITA* pIII only in B cells, even though IRF-4 binds the *CIITA* promoter in monocyte cell lines as well (van der Stoep, Quinten et al. 2002; van der Stoep, Quinten et al. 2004). Though many of the factors bound at *CIITA* pIII are shared between cell types, a few factors have been shown to bind to and activate *CIITA* in a cell type specific manner.

As mentioned previously, *CIITA* pIV is primarily active in response to IFN-γ. A promoter proximal region of only about 150 bp is required for IFN-γ induced activation of pIV. This region contains three regulatory elements: a GAS box, an E box, and an IFN-regulatory factor element (IRF-1 or IRF-E) (Figure 1) (Muhlethaler-Mottet, Otten et al. 1997; Fisher and Scott 1998; Muhlethaler-Mottet, Di Berardino et al. 1998; Dong, Rohn et al. 1999; Piskurich, Linhoff et al. 1999; Morris, Beresford et al. 2002). STAT 1 and USF-1 cooperatively bind the GAS element and the E box (Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002). The IRF-1 site is bound by IRF-1 and IRF-2 (Muhlethaler-Mottet, Di Berardino et al. 1998; Xi, Eason et al. 1999; Morris, Beresford et al. 2002). These three regions are sufficient for IFN-γ induced *CIITA* pIV expression in both bone marrow derived and non-bone marrow derived cells. On the other hand, thymic epithelial cells express *CIITA* pIV independently of IFN-γ signaling (Reith, LeibundGut-Landmann et al. 2005), and the factors required for *CIITA* expression in these cells are not known. Because *CIITA* pIV is inducible in most cell types, but only upon IFN-γ induction, it is likely that pIV is transcriptionally competent, but inactive due to lack of necessary transcription factors.

Results

DNA methylation at CIITA promoters

DNA methylation is a common mechanism for silencing of *CIITA* gene expression. Fetal throphoblasts do not express MHC-II molecules, even after stimulation with IFN-γ. This has been shown to be the result of silencing of *CIITA* by hypermethylation of *CIITA* promoters (Morris, Riley et al. 1998; Morris, Spangler et al. 2000). Silencing of *CIITA*, and thus MHC-II gene expression, is also commonly seen in tumor cell lines. (reviewed in (van den Elsen, Holling et al. 2003)). Promoter III, which is expressed in human T cells, is methylated in

mouse T cells, and therefore silenced (Chang, Hong et al. 1995; Holling, van der Stoep et al. 2002; Wong, Ghosh et al. 2002; Schooten, Klous et al. 2005). Thus, methylation is one mechanism that may mediate promoter choice.

Although DNA methylation has been studied in a number of cell types, no comprehensive analysis has been done across all three *CIITA* promoters in typical antigen presenting cells. Therefore, bisulfite sequence analysis in a variety of murine cell lines was used to determine the methylation status of *Ciita* promoters I, III, and IV (Figures 2 and 3). Primary splenic dendritic cells (spDC), peritoneal macrophages, and the macrophage cell line J774 were chosen to represent myeloid cells. The cell line J774 was also chosen because *Ciita* is expressed exclusively from pI, in contrast to other myeloid cell lines such as Raw264.7, which expresses *Ciita* from all three promoters (data not shown). IFN-γ induction was used to stimulate *Ciita* expression from the macrophages, as basal expression levels in these cells are low (Chapter 3 Fig. 3 and data not shown). Primary splenic B cells were chosen as representative *Ciita* pIII expressing lymphoid cells. For comparison, non-*Ciita* expressing murine embryonic fibroblasts (MEFs), primary plasma cells, and the plasma cell line P3X, were also analyzed. The bisulfite sequencing data is summarized in Figure 2. Eight CpG sites were analyzed for promoter I, six sites for pIII, and 14 sites for pIV. The single pie chart presented represents average methylation across all of the CpG sites at that promoter, with black representing methylated, and white representing unmethylated CpGs.

The highest degree of methylation was observed at pI, while pIII and pIV were largely unmethylated. SpDC and primary macrophages, which express the highest amounts of pI, display the least amount of methylation at this promoter. The plasma cell line P3X also displays low amounts of methylation at *Ciita* pI, though this appears to be specific to this cell line, as primary plasma cells are significantly methylated at pI. Primary splenic B

cells, which do not express *Ciita* pI, are highly methylated at pI. The cell line J774 is highly methylated across the entire *Ciita* locus. In all of the cells tested, except J774 and MEF pIII, promoters III and IV are highly unmethylated. This finding is consistent with the observation that promoters III and IV are capable of transcription in spDC and macrophages from *Ciita* pI-/- mice (Chapter 3, Fig. 3 and 4).

To determine which specific CpG sites are differentially methylated, individual CpG sites at *Ciita* pI were examined (Figure 3). The largely unmethylated pIII is also presented. As in Figure 2, each pie chart represents the percentage of methylation at each individual CpG site. The first three CpG sites at pI are mostly unmethylated in SpDCs, macrophages, and plasma cells. Strikingly, these three CpGs are almost entirely methylated in B cells. This is consistent with *Ciita* promoter usage in these cell types: *Ciita* is expressed from pI in spDC and macrophages, but not B cells. Silencing of *Ciita* in plasma cells is mediated by mechanisms other than DNA methylation. Binding of B lymphocyte-induced maturation protein I (BLIMP-1) at pIII of the *Ciita* locus during the B cell to plasma cell transition represses *Ciita* expression (Piskurich, Lin et al. 2000). Also, the *Ciita* locus in P3X cells is characterized by repressive histone marks (Green, Yoon et al. 2006). In contrast to *Ciita* pI, all of the CpG sites at pIII are largely unmethylated in spDC, macrophages, plasma cells, and B cells. Importantly, even though some methylation is observed at pIII, spDC and macrophages do not appear to be more methylated at this locus than B cells. Thus, our data show that methylation does not appear to be a mechanism for silencing of *Ciita* pIII in myeloid cells, but may play a role in promoter choice by silencing *Ciita* pI in B cells.

To determine whether methylation at *Ciita* pI does indeed represses transcription, cells were treated with the DNA methylation inhibitor 5-azacytidine (5-aza). Cells must be actively dividing in order for 5-aza mediated de-methylation to occur, therefore for cell lines were chosen instead of primary cells, which divide poorly in culture. The macrophage cell

line J774 was chosen because *Ciita* pI was highly methylated in these cells. The A20 cell line was chosen because it is a *Ciita* pIII expressing B cell line. Treatment with 5-aza resulted in low but detectable expression of *Ciita* pI from A20 B cells. Expression from pI increased by as much as 60-fold in A20 cells treated with 5-aza (Figure 4A). Expression of *Ciita* pI increased by as much as 100-fold in A20 cells when the 5-aza treatment was continued for a longer period of time (data not shown). J774 cells, which express *Ciita* from pI when induced with IFN-γ, further increased expression of pI by two-fold after treatment with 5-aza (Figure 4B). As predicted, methylation does not seem to be required for silencing of *Ciita* pIII and pIV in these cell types. No change was seen in expression from *Ciita* pIII or pIV in either J774 or A20 cells. Even though J774 cells are heavily methylated at pI and pIV, only expression of pI was affected by demethylation. 5-azacytidine is a global demethylating agent; therefore it is possible that the increase in *Ciita* pI expression is indirect. Treatment with 5-aza could result in increased expression of *Ciita* pI specific transcription factors. To rule out this possibility, it will be necessary not only to determine what transcription factors are required for expression of *Ciita* pI, but also whether expression of these factors were increased by 5-aza treatment. Nonetheless, these data are consistent with the hypothesis that demethylation results in de-repression of expression from *Ciita* pI.

Discussion

Many factors contribute to gene regulation. These include availability of transcription factors, chromatin accessibility, histone modifications, and DNA methylation. All of these factors help to define higher order chromatin structure. Myeloid cells express *CIITA* primarily from pI. Our recent data in *Ciita* pI-/- mice suggest that the inactivity of pIII and pIV in myeloid cells is not due to lack of availability of transcription factors or enhancer

activity. Therefore, promoter choice in myeloid cells may be in large part dependent on chromatin structure and the ability of enhancer elements and transcription factors to interact with specific promoters. On the other hand, promoter choice in lymphoid cells seems to be mediated, at least in part, by DNA methylation specifically at *Ciita* pI, which silences this promoter.

Regulatory elements often bind transcription factors and are capable of interacting with multiple promoter regions. Additionally, distal regulatory elements may interact with other elements to help define chromatin structure and alter chromatin accessibility. Although most *CIITA* activating transcription factors identified to date have been shown to bind within 300 bp of the promoter, a few important factors have been found at distal regions. For example, STAT1 is required for IFN-γ induced expression from both pIII and pIV. While STAT1 does bind CIITA pIV, remote binding sites are also important. In contrast to pIV, STAT 1 does not bind pIII, but binding at a distal element 6kb upstream of pIII is important for IFN-γ induction of this promoter (Piskurich, Linhoff et al. 1999). STAT1 also binding at multiple sites upstream of pIV is important for IFN-γ induced *CIITA* expression in HeLa cells (Ni, Abou El Hassan et al. 2008). Another transcription factor that is of particular interest is the ETS family transcription factor PU.1, found in all hematopoietic lineages (reviewed in (Fisher and Scott 1998; Carotta, Wu et al. 2010). PU.1 binding sites are present at all three CIITA promoters, and PU.1 binding at each of these promoters has been detected *in vivo* by chromatin immunoprecipitation (Piskurich, Wang et al. 1998; Ito, Nishiyama et al. 2009; Yoon and Boss 2010; Kitamura, Yokoyama et al. 2011; Smith, Wright et al. 2011). PU.1 binding at a distal regulatory region in B cells was shown to be required for optimal expression of *Ciita* (Yoon and Boss 2010). Thus, to date, at least two distal enhancer elements have been shown to regulate *CIITA* expression from multiple promoters.

DNA methylation plays an important role in gene regulation because it can alter DNA-protein binding dynamics. For example, DNA methylation has been shown to prevent binding of the only known mammalian insulator CTCF (Bell and Felsenfeld 2000). In addition to its role as an insulator, CTCF also plays an important role in forming higher order chromatin structure by mediating chromatin looping. The *CIITA* locus contains multiple potential CTCF binding sites. Two CTCF binding sites located in the *CIITA* gene are bound by CTCF in B cells (data not published) and contain multiple CpG sites. Methylation at these two CTCF binding sites was determined using bisulfite sequencing (Figure 2). Methylation at CTCF sites prevents CTCF binding (Mukhopadhyay R, Yu W et al. 2004). Therefore, differential methylation of CTCF sites at the *CIITA* locus could be a mechanism to control promoter choice by altering chromatin looping and thereby changing the interactions between *CIITA* promoters and enhancer elements. To determine the role of CTCF in promoter choice, it will be necessary to define CTCF binding and loop formation in multiple antigen presenting cells. It will be of interest to determine whether altered methylation status or mutations at CTCF binding sites results in different CTCF binding profiles, and consequently alters chromatin looping, between various cell types.

Another mechanism by which DNA methylation can alter chromatin dynamics is by preventing transcription factor binding at promoters or regulatory elements. Methylation at *Ciita* pI corresponds well with expression in primary cells. The high degree of methylation observed at *Ciita* pI in B cells might prevent binding of some necessary transcription factor. Intriguingly, pIII and pIV are largely unmethylated in spDC and macrophages. Methylation does not seem to be a mechanism utilized by wild-type myeloid cells for silencing of *Ciita* pIII or pIV. In contrast, methylation at *Ciita* pI may be important for keeping this promoter silenced in lymphoid cells, allowing pIII to be the dominantly expressed promoter. Accessibility of pI may be a determining factor in promoter choice. If

CIITA pI is methylated, and therefore not accessible to enhancer elements (due to the inability of transcription factors to bind or altered chromatin conformation), expression may default to pIII. In addition to methylation, histone modifications also contribute to chromatin accessibility. *CIITA* silencing during the transition from B cell to plasma cell is characterized by replacement of active histone marks with repressive histone modifications at the *CIITA* gene (Green, Yoon et al. 2006). Thus, one model of CIITA promoter choice would be that myeloid cells express CIITA from pI by virtue of having a transcriptionally competent promoter. B cells and pDC express pIII because they have silenced pI and thus transcription factors and enhancer elements are freed to interact with the next available promoter: pIII. This model is consistent with the observation that the presence of *Ciita* pIII transcription elements does not de facto lead to pIII expression (as is the case in dendritic cells). It is clear that multiple transcription factors are required for CIITA expression. Some of these factors are cell type specific, while many are found not only in multiple cells types, but are able to bind multiple promoters. Some transcription factors bind distal regulatory regions, which may allow interaction with multiple CIITA promoters. Promoter methylation may be one (of many) mechanisms utilized to control promoter choice in primary antigen presenting cells.

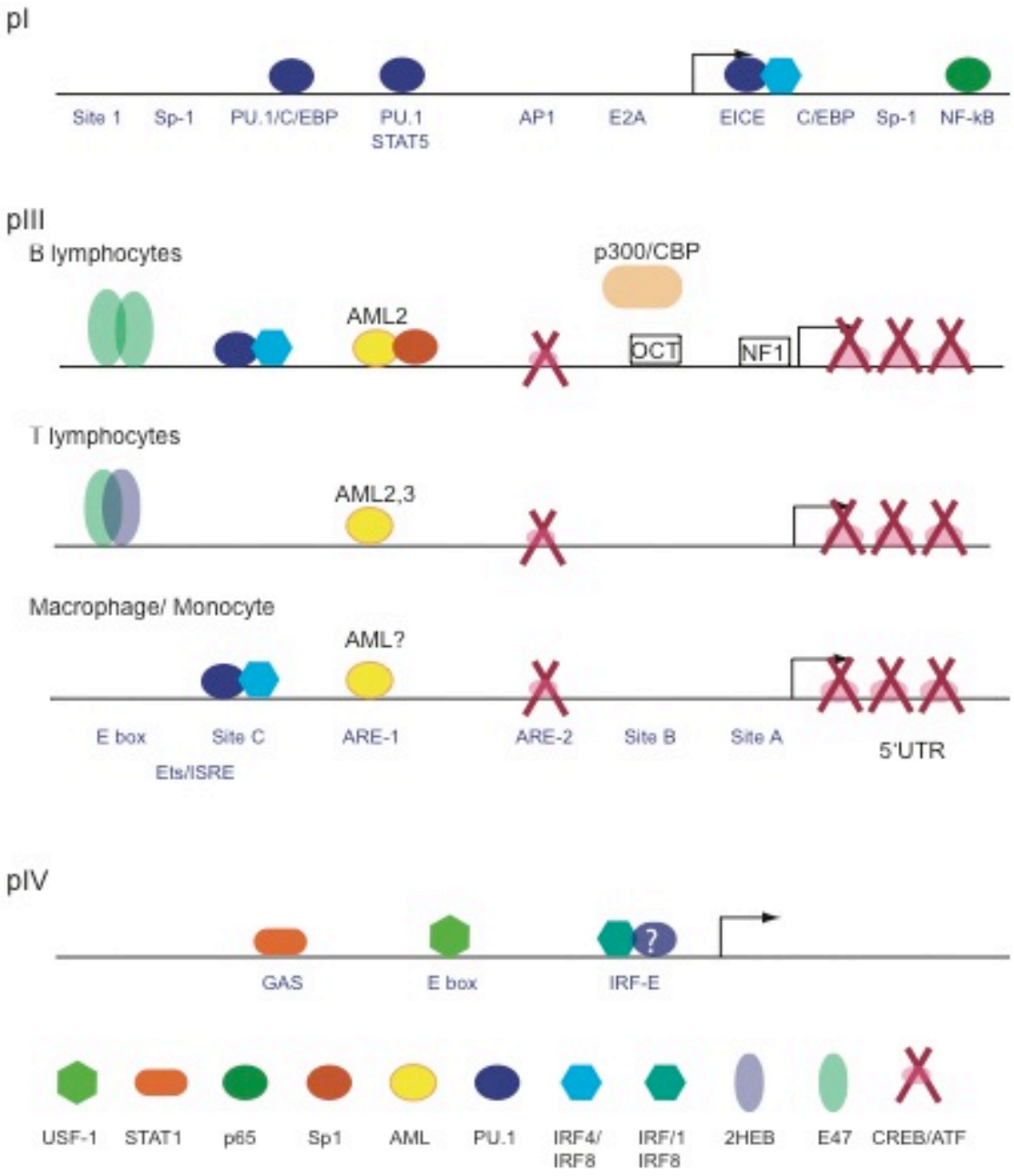


Figure 1: Promoter proximal regions of *CIITA*. Known *cis*-regulatory regions and factors known to bind these regions are depicted for each *CIITA* promoter. Binding of factors at pIII is reproduced from van den Elsen (van den Elsen, Holling et al. 2004), with the addition of Sp1 binding (Green, Yoon et al. 2006). PU.1 binding at pIV was demonstrated by ChIP in bone marrow derived mast cells (Ito, Nishiyama et al. 2009).

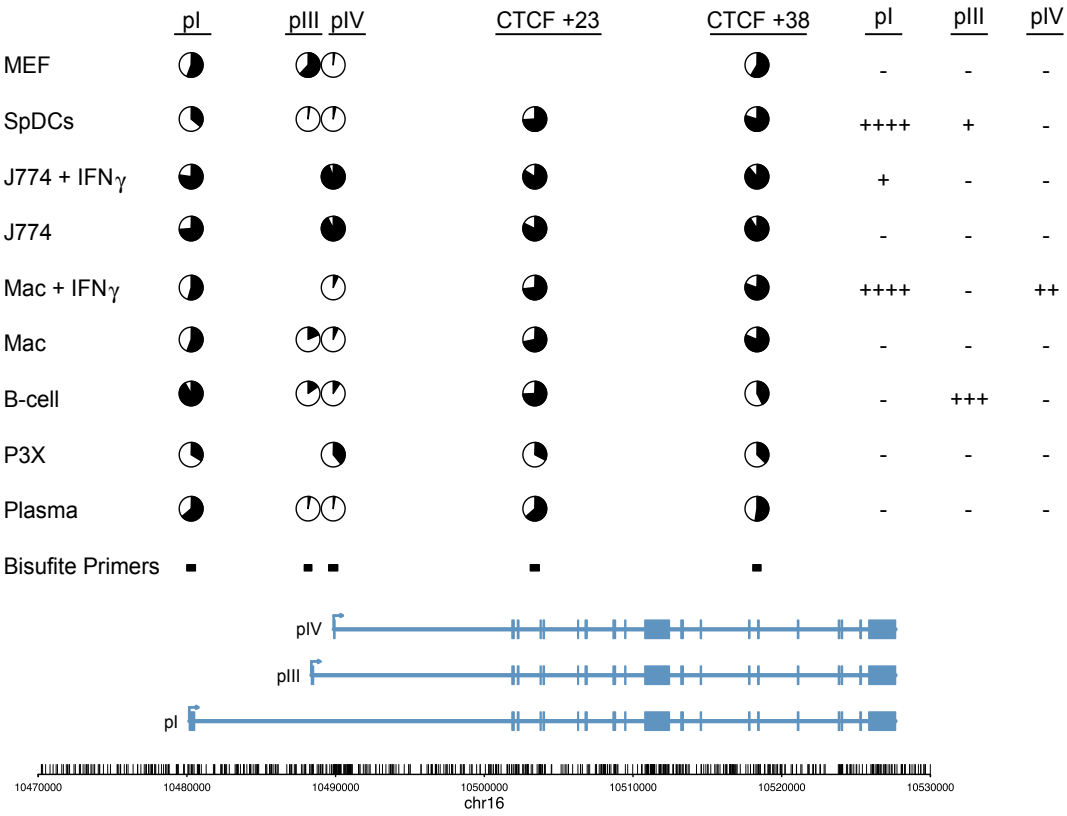


Figure 2: Methylation across the CIITA locus. The methylation status of CpGs at CIITA pI, pIII, and pIV and at two downstream CTCF sites (+23 and +38 with respect to CIITA pI) was determined using bisulfite sequencing. Each pie chart represents the percentage of methylation at each locus; black represents methylation. Black bars represent the locations of primers used to amplify regions of interest. Each isoform of the Ciita gene is drawn in blue at the bottom. Black bars in the bottom row represent individual CpG sites located along the Ciita gene. The last three columns depict relative expression from each promoter. For each location, the number of CpG sites queried is: pI, 7; pIII, 6; pIV, 14; CTCF +23, 3; and CTCF +38, 9. MEF: murine embryonic fibroblasts, Mac: macrophages.

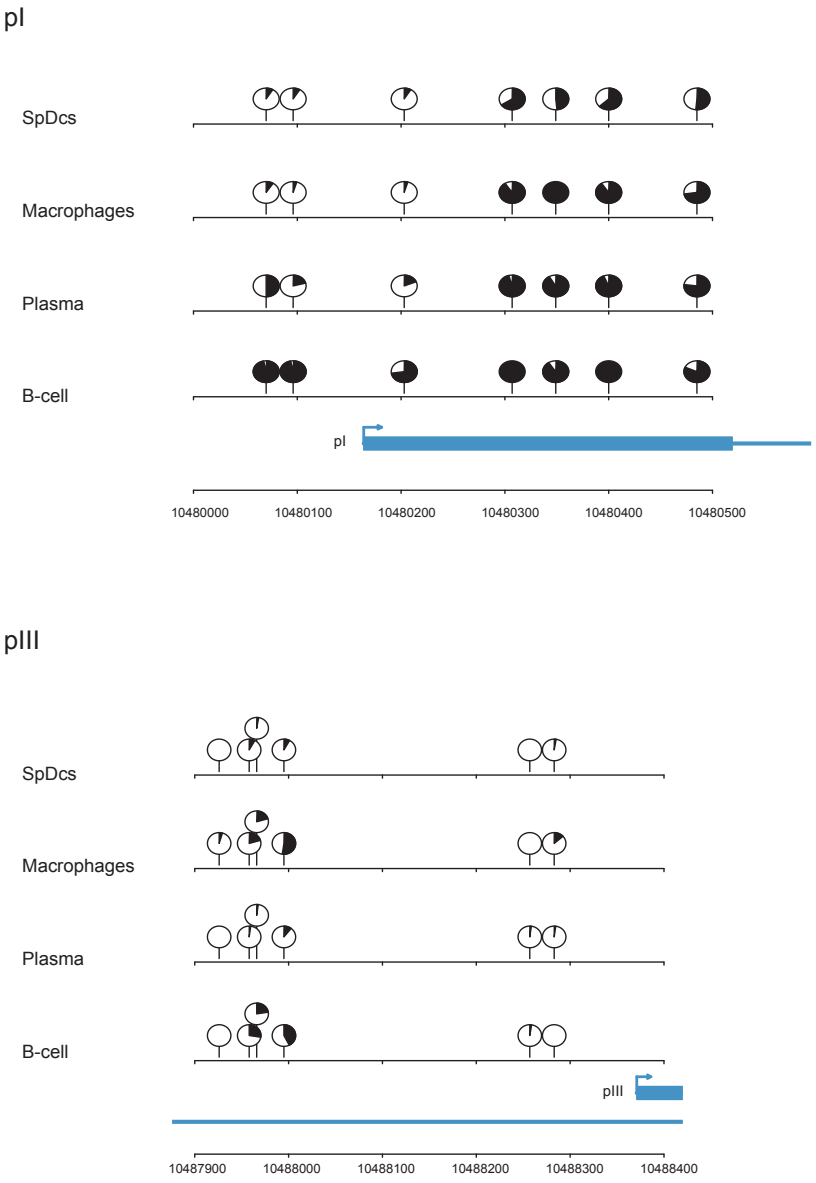


Figure 3: Methylation at CIITA pI and pIII. The percentage of methylated CpGs at individual CpG sites at CIITA pI and pIII in splenic dendritic cells (spDC), macrophages, plasma cells, and B cells are represented by pie charts. Black represents the percentage of CpGs that are methylated at that site.

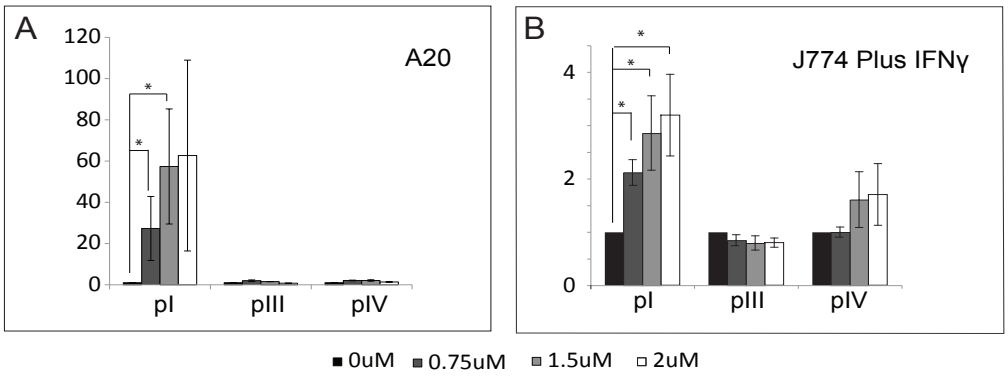


Figure 4: 5-azacytidine treatment of A20 and J774 cell lines. Cells were treated every 8hrs for 48hrs with 5-aza. CIITA promoter specific expression was graphed relative to expression in untreated cells.

Chapter 5: Discussion

This Chapter was written by W.M. Zinzow-Kramer

CTCF ChIP-seq data presented in this chapter was contributed by

Christopher Scharer and Parimal Majumder

Major histocompatibility genes are required for initiation of adaptive immune responses. MHC-II molecules are primarily responsible for the presentation of foreign antigen to CD4 T cells, while MHC-I molecules present self and foreign antigen to CD8 T cells. MHC-II expression changes in response to developmental and environmental stimuli. How MHC-II gene expression is controlled is biologically relevant because inappropriate expression can lead to autoimmune disorders. Likewise, lack of MHC-II expression results in severe vulnerability to infections. Moreover, the targeting of MHC-II expression is one mechanism utilized by some pathogens to escape immune system surveillance. Similarly, MHC-II expression has also been correlated with poor prognosis in specific cancers such as melanomas and T-cell leukemias (Goodwin, Xi et al. 2001; Holling, Schooten et al. 2004). Thus understanding how MHC-II genes are regulated may lead to improved treatments for pathogens and cancers that have been identified to manipulate MHC-II expression. Because expression of MHC-II genes is controlled primarily at the level of transcription by the master regulator of MHC-II (the MHC-II transactivator, CIITA), understanding *CIITA* gene regulation is central to understanding MHC-II gene expression. This dissertation examined the regulation of CIITA in a specific subset of antigen presenting cells: dendritic cells and macrophages. These cells are the primary sentinels of immune responses, as they are the first to come in contact with antigen, especially those from pathogens.

I. The CARD domain of CIITA is not required for proper immunological responses

All isoforms of CIITA share four domains; only isoform I stands out by the presence of a fifth domain. In chapter 3, the immune system function was tested in mice lacking the CARD- containing CIITA isoform I with a variety of assays. Isoform I was found to be dispensable for basic immune system function. Specifically, *Ciita* pI$^{-/-}$ mice responded like

wild-type mice to bacterial and viral challenges. They also had normal percentages of CD4 T cells in the thymus, and there was no evidence of a defect in positive selection or susceptibility to autoimmunity. It was reported that CIITA isoform I is a more potent transactivator, due to the presence of the CARD domain (Nickerson, Sisk et al. 2001). In addition to a *Ciita* pI$^{-/-}$, a mouse line was created in which isoform III was expressed in place of isoform I. In these CIITA pI→III mice, expression from the MHC-II gene *I-A^b* was not different than wild type. This indicated that isoform III was capable of inducing MHC-II expression at levels that are comparable to isoform I. The possibility remains that the CARD domain may have some function in modulating immune response, even though it is not essential for basic immune function.

One possibility is that the CARD domain mediates CIITA activity by modulating CIITA posttranslational modifications. Immature dendritic cells express high amounts of *CIITA*. However, surface expression of MHC-II is low because MHC-II molecules are stored in intracellular compartments (Pierre, Turley et al. 1997). After activation, DC rapidly silence *CIITA*, and therefore MHC-II gene expression (Landmann, Muhlethaler-Mottet et al. 2001). Existing MHC-II molecules are stabilized and transported to the surface. This process is important so that DC will preferentially present antigen encountered at the source of infection. Although it is known that DC rapidly silence MHC-II genes by silencing transcription of *CIITA* (Landmann, Muhlethaler-Mottet et al. 2001), existing CIITA protein might also be degraded. Utilization of CIITA isoform I by DC might contribute to the rapid silencing of MHC-II genes by allowing for rapid CIITA protein degradation. In macrophages, it was discovered that LPS stimulation promoted ubiquitylation of CIITA. Transient monoubiquitylation increased the transcriptional activity of isoform I, though subsequent polyubiquitylation led to rapid degradation (Drozina, Kohoutek et al. 2006). Phosphorylation of a serine near one of the degrons in CIITA was linked to ubiquitylation

and subsequent degradation. LPS stimulation of DC also results in a transient increase in MHC-II gene expression, followed by rapid downregulation of both *CIITA* and MHC-II genes (Landmann, Muhlethaler-Mottet et al. 2001). Although N-terminal modifications of CIITA have been shown to alter CIITA stability, no direct comparison of protein stability has been made between isoform I and any of the other isoforms (Schnappauf, Hake et al. 2003). Therefore, it may be that the presence of the CARD domain alters the kinetics by which CIITA is ubiquitylated or phosphorylated in comparison to the other isoforms. Expressing CIITA isoform I by dendritic cells and macrophages might allow for rapid and transient CIITA activity, followed by rapid CIITA protein degradation and silencing of MHC-II gene transcription after exposure to activating stimuli such as LPS. Determining the half-lives of CIITA in mouse DC, the system that was explored in this dissertation, was attempted but was not possible due to the poor reactivity of antibodies to murine CIITA; thus, this possibility remains.

II. CIITA promoter I is not required for MHC-II expression in myeloid cells

Transcription factors and DNA looping

Though most studies of CIITA regulation focus on regions that are near the promoters, a few studies have identified distal regulatory regions required for *CIITA* pIII or pIV expression. Of particular interest is one region, located 3 kb upstream of pI, (called HSS1 for DNase "hypersensitivity site 1") that was determined to be important for expression of *Ciita* pIII in murine B cells and *CIITA* pIV in IFN- γ induced HeLa cells (Ni, Abou El Hassan et al. 2008; Yoon and Boss 2010). HSS1 is bound by PU.1 in B cells and STAT1 in IFN-γ induced HeLa cells. The ETS family transcription factor PU.1 is present in all hematopoietic lineages, and is important for *CIITA* pI, PIII, and potentially pIV expression in

these cell types (Ito, Nishiyama et al. 2009; Yoon and Boss 2010; Kitamura, Yokoyama et al. 2011; Smith, Wright et al. 2011). STAT1, which is activated by IFN-γ stimulation, is required for IFN-γ induced expression of *CIITA* in both macrophages and cells of the non-hematopoietic lineage (Meraz, White et al. 1996; Morris, Beresford et al. 2002). Because HSS1 binds both PU.1 and STAT1, it may also play an important role in CIITA expression in both dendritic cells and macrophages.

Looping interactions between enhancers and promoters are mediated by proteins bound at these sites and promote efficient recruitment of required transcription factors to the promoter (Dean 2006; Kim, Bresnick et al. 2009; Nolis, McKay et al. 2009). HSS1 was shown by chromatin conformation capture assay (3C) to interact with pIII in (murine) B cells, and pIV in (human) HeLa cells (Ni, Abou El Hassan et al. 2008; Yoon and Boss 2010). PU.1, which binds both HSS1 and pIII, may mediate the interaction observed between HSS1 and pIII. Two potential looping scenarios based on PU.1 binding in B cells are depicted in Figure 1. 3C data from Yoon et al. (Yoon and Boss 2010) indicates that pI interacts with pIII; therefore pI may be involved in the looping interactions between HSS1 and pIII in B cells. Even if pI does interact with HSS1 in B cells, our DNA methylation data suggest that pI is not transcriptionally active due to heavy methylation. In a similar manner, looping between HSS1 and pIV in IFN-γ induced HeLa cells may be mediated by STAT1 binding at both these sites. IFN-γ stimulation activates STAT1, which in turn induces the expression of IRF-1 and IRF-4 (Gough, Levy et al. 2008). STAT1 and IRF-1, once activated, are recruited to the *CIITA* locus and stabilize existing interactions between pIV and regulatory regions (Ni, Abou El Hassan et al. 2008). A simplified diagram of looping based on STAT1 and IRF-1 binding at HSS1 and the *CIITA* pIV locus in IFN-γ stimulated cells is presented in Figure 1. PU.1 may also mediate looping interactions between HSS1 and pI in dendritic cells and macrophages. In addition, stimulation of macrophages with IFN-γ increases *CIITA* expression from both pI

and pIV. No IFN-γ regulatory elements have been identified at pI, therefore IFN-γ induction of pI may be due to increased binding of STAT1 along the CIITA locus. PU.1, STAT1, IRF-4, and IRF-8 together may promote looping interactions between HSS1, pI, and pIV (Figure 1). Therefore, because HSS1 binds transcription factors necessary for expression from all three promoters, and has already been shown to be required for expression from pIII and pIV, HSS1 has the potential to be a master regulatory element for *CIITA*.

Though HSS1 seems to be important for *CIITA* gene expression from multiple promoters, it is not the only distal regulatory element present. The study by Ni et al. found five regions over 100kb that were required for expression of *CIITA* pIV in IFN-γ induced HeLa cells (Ni, Abou El Hassan et al. 2008), indicating that multiple enhancers are required for proper expression of *CIITA* pIV. Although PU.1, and HSS1 are featured in the proposed looping interactions presented in Figure 1, these interactions likely require multiple transcription factors and regulatory elements. Future studies will identify *CIITA* pI distal regulatory elements and how these elements interact with *CIITA* promoters.

DNA Methylation

DNA methylation, by altering chromatin accessibility, is a well-established mechanism for gene regulation. Chapter 4 begins our analyses of DNA methylation across the *CIITA* locus. The results found that methylation at *CIITA* pI is highly correlated with expression. In contrast, pIII and pIV are largely unmethylated in all primary cells. Preliminary results indicated that methylation at *Ciita* pI reduced expression; i.e., treating B cells and macrophages with the demethylating agent 5-aza increased expression of *Ciita* pI in the two cells lines tested. In myeloid cells, the interaction between *CIITA* pI and an enhancer region might serve two purposes. The first is, of course, to promote expression of *CIITA* from pI. The second might be to serve as an "enhancer trap". Interaction with pI

could prevent the enhancer from interacting with downstream promoters. The insertion of a transcription factor-binding site between the IFN-β promoter and its enhancer blocks gene expression by interacting with the enhancer, diverting binding of the enhancer away from the promoter (Nolis, McKay et al. 2009). A similar mechanism at the *CIITA* locus may help explain why myeloid cells express *CIITA* primarily from pI, even though all three CIITA promoters are transcriptionally competent.

In addition to mediating protein interactions at promoters and enhancers, methylation can alter chromatin structure by mediating CCCTC binding factor (CTCF) binding (Bell and Felsenfeld 2000). CTCF has been shown to mediate multiple looping interactions at the MHC-II locus (Majumder and Boss 2010). There are many CTCF binding sites located along the MHC-II locus, allowing for multiple potential CTCF-CTCF, and therefore looping, interactions. The *CIITA* locus also contains multiple potential binding sites for the insulator protein CTCF. Results showing CTCF binding along the *CIITA* locus in B cells as determined by chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-seq) is depicted in Figure 2 (Scharer, Majumder, Boss, unpublished data). Depending on how these CTCF sites interact with each other, they might allow for a number of different chromatin conformations, which may impact *CIITA* gene expression. Future studies in our lab will characterize CTCF binding along the *CIITA* locus in a variety of cell types and whether differential CTCF binding leads to altered chromatin configurations.

Histone modifications

Histone modifications also regulate chromatin accessibility and specific modifications are characteristic of enhancer regions. The histone modification H3K4me3 is associated with active chromatin and specifically is found at transcriptional start sites (TSS) (Schneider, Bannister et al. 2004; Heintzman, Stuart et al. 2007). H3K4me3 is present at

CIITA pIII in B cells, and pIII and pIV in IFN-γ induced HeLa cells (Figure 2) (Green, Yoon et al. 2006; Heinz, Benner et al. 2010; Yoon and Boss 2010). Low H3K4me3, along with high amounts of H3K4me1 is highly correlated with enhancer activity (Heintzman, Stuart et al. 2007; Ghisletti, Barozzi et al. 2010; Heinz, Benner et al. 2010). While there are many H3K4me1 sites along the *CIITA* locus, H3K4me3 is present almost exclusively at TSS (Figure 2). In macrophages and B cells, PU.1 binding is significantly correlated with H3K4me1, and at some loci PU.1 binding is even required for maximal H3K4me1 binding (Ghisletti, Barozzi et al. 2010; Heinz, Benner et al. 2010). Because PU.1 and H3K4me1 binding is characteristic of enhancers in both macrophages and B cells, these features can be used to identify potential regulatory regions along the *CIITA* locus. Indeed, many of the PU.1 bound sites along the *Ciita* locus in macrophages and B cells are also bound by H3K4me1 (Figure 2). Both PU.1 and H3K4me1 bind some sites, such as HSS1, in both B cells and macrophages. Other sites, such as the PU.1 bound regions at pI and just downstream of pI in macrophages, appear to be cell type specific. Future experiments are needed to identify potential regulatory regions along the *Ciita* locus based on PU.1 and H3K4me1 binding and to test these regions for enhancer activity in a variety of cell types.

III. Concluding remarks

MHC-II molecules have a well-established role in the maintenance of health. As the master regulator of MHC-II, CIITA is also required to maintain a healthy and functional immune system. Understanding *CIITA* gene regulation, and thus MHC-II gene regulation, will improve our understanding of immune system responses. In particular, understanding how *CIITA* is regulated may help inform us on how particular pathogens manipulate *CIITA* expression to their benefit. The three different promoters of *CIITA* help to fine-tune control in a cell type and context dependent manner. Work presented in this dissertation examined

a specific isoform of CIITA: the CARD-domain containing isoform I. Here we show that expression of CIITA isoform I, and therefore the CARD domain, was not required for a variety of basic immune system responses. These results were surprising because a similar effect was not observed in *Ciita* pIV or pIII/pIV knockout models (Waldburger, Suter et al. 2001). We also show that when *Ciita* pI was deleted, transcription was directed to downstream promoters, resulting in no loss of total *Ciita* expression. In addition, MHC-II expression was not decreased in *Ciita*$^{-/-}$ myeloid cells, indicating alternate isoforms transactivate MHC-II genes just as efficiently as isoform I. Transcriptional regulation of *CIITA* pI is poorly understood. Whereas regulation of pIII and pIV has been studied for years, it is only recently that promoter proximal elements for pI have been mapped (Kitamura, Yokoyama et al. 2011; Smith, Wright et al. 2011). Data from *Ciita* pI$^{-/-}$ mice suggest that a critical, as yet unknown, regulatory element(s) is present and is located outside of the *Ciita* pI promoter region.

Preliminary experiments presented in this dissertation elucidate some of the mechanisms regulating promoter choice. Data from bisulfite sequencing and 5-aza treatments suggest that methylation may be one factor mediating promoter choice by silencing *Ciita* pI in B cells. Though methylation of pIII and pIV have been shown to result in silencing of these promoters in a variety of cell types, to our knowledge this is the first time that methylation has been demonstrated to be a mechanism by which *Ciita* pI is silenced. The data presented here expands on our current knowledge of *Ciita*, specifically expression and regulation of *Ciita* pI and the unique isoform encoded by this promoter.

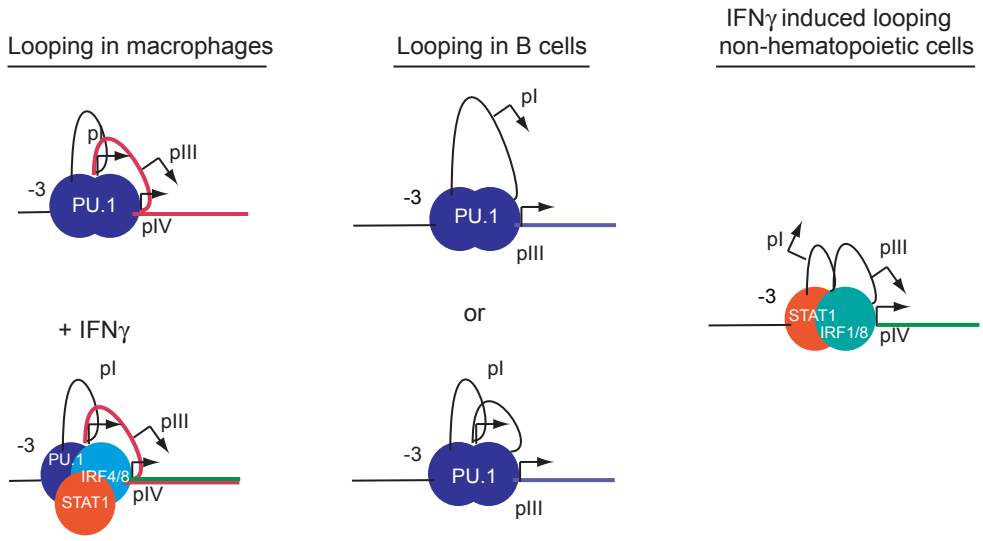


Figure 1: Looping at the *CIITA* locus. Possible looping scenarios in macrophages, B cells, and IFN-γ induced non-hematopoietic cells are presented. Two looping configurations are presented for macrophages: before and after induction with IFN-γ. IFN-γ induction leads to the activation of STAT1 and IRFs, which are recruited to and bind along the CIITA locus, stabilizing (as presented in macrophages) or inducing (as presented in non-hematopoietic cells) interactions between enhancer regions and CIITA promoters. Two possible looping conformations are also presented for B cells. Colored lines represent transcripts produced: red for transcription from CIITA pI, blue for pIII, and green for pIV. The looping interactions presented for B cells and IFN-γ induced non-hematopoietic cells are based on known DNA-protein interactions from ChIP, ChIP-seq, and chromatin conformation assays.

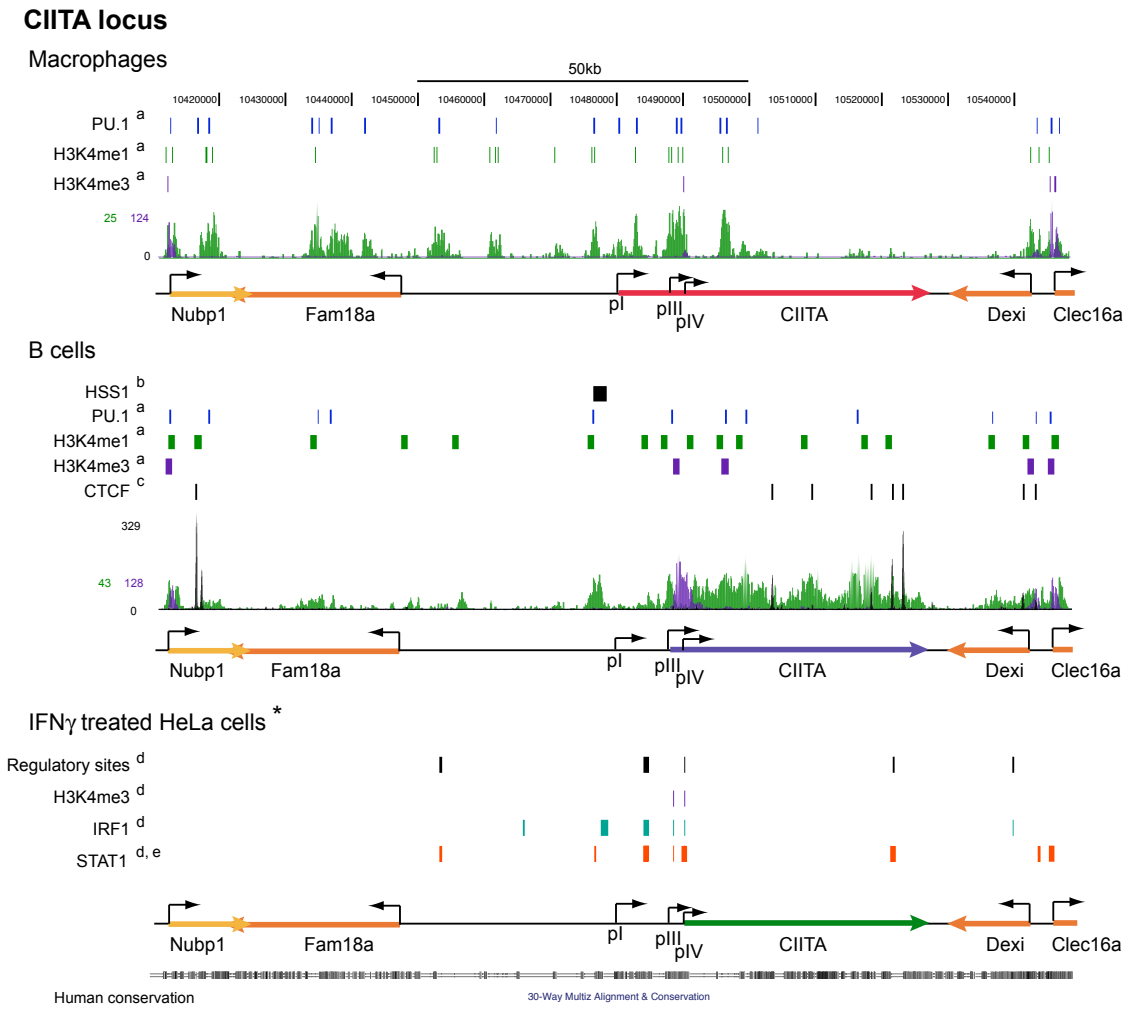


Figure 2: Histone modifications and transcription factor binding at the *CIITA* locus. ChIP-seq analysis in macrophages, B cells, and IFN-γ treated HeLa cells for PU.1 (blue), H3K4me1 (green), H3K4me3 (purple), CTCF (black), IRF1 (aqua), and STAT1 (orange) are presented for a 100kb region surrounding the *Ciita* locus in mice. Also represented are HSS1, and the regulatory regions identified in IFN-γ treated HeLa cells. *Coordinates were converted from human to mouse for peaks present at conserved regions. Data are from: Heinz et al. 2010[a], Yoon et al. 2010 [b], unpublished data, our lab[c], Ni et al. 2008[d], and Robertson et al. 2007[e]

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