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

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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 s II 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.

Keywords

MHC class II; gene expression; CIITA; antigen presentation

INTRODUCTION

The major histocompatibility complex class II (MHC-II) region encodes genes required for the presentation of antigenic peptide to CD4 T cells¹. The process results in a variety of immune responses that range from direct effector responses to those that are regulatory.

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CONFLICT OF INTEREST

The authors declare that there is no competing financial interest in relation to the work describe.

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- γ (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 organisms²⁻⁴. 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^{5, 6}. One BLS complementation group was found to be deficient for the class II transactivator (CIITA), a factor that is essential for MHC-II expression⁷.

CIITA functions as a transcriptional coactivator interacting with other MHC-II specific transcription factors bound to regions upstream of each *MHC-II* gene (reviewed in⁸). 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 manner⁹. *Ciita* promoter I (pI) is utilized by dendritic cells (DC) and macrophages exposed to IFN- γ ¹⁰, 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 ¹¹. 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)¹². Such domains have been shown for other proteins to be important for protein-protein interactions^{13, 14}. 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) proteins¹⁵⁻¹⁷. 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^{15, 18, 19}. Increasing evidence suggests that many NLRs are cytoplasmic pathogen recognition receptors, activating immune responses to intracellular pathogens¹⁹. 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 knock-out strategy to create mice lacking either pIV or both pIII and pIV^{11, 20}. Using the *Ciita* pIV targeted knock-out 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 cells¹¹. 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^{11, 21}. 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 expression²⁰. 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 \rightarrow 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 \rightarrow 0 knock-out (KO). Thus, two novel *Ciita* mouse lines were created. These mice were extensively characterized for their ability to express *Ciita* 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 \rightarrow 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 \rightarrow 0 (knock-out, 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/6J²². 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.

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⁺) compartments²³ (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* pIII²⁰; 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).

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 lineage²⁰. 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-A α mRNA levels were also similar to WT, except in the case of KO spDC, where I-A α 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 *Ciita* 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*^{24, 25}, 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*²⁴, and microarray analysis of human B cells²⁵ and mouse dendritic cells²⁶ identified *KPNA6*, *RAB4B*, and *Plxn1* as additional potential targets of *Ciita*.^{24, 25} Additional genes potentially regulated by CIITA are *Cste* and *Col1A1*^{27,28}. 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-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^{29,30}. 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.

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 thymus³¹. 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^{11,20}. 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 thymus³²⁻³⁴. 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 produced³⁵, the studies described below will use the KO model to assess the role of the pI isoform on a variety of immune responses.

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 GP₆₁₋₈₀³⁶. Mice were immunized containing either 1mg/ml GP₆₁₋₈₀ 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 [³H]-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.

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 DCs³⁷. 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).

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^{15, 19}. 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- α , 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*³⁸. 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.

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 days³⁹. 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- α 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).

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 domain¹² and its use is restricted to the myeloid and DC compartments²⁰. 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/knock-out models created here allowed this hypothesis to be tested. Surprisingly, no gross defect in immune system function or development in either the CIITApI \rightarrow III KI or CIITApI \rightarrow 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.¹² suggested that pI is a more potent transcriptional activator than the other isoforms; however, this was not confirmed by Buttice et al.²⁸, 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 protein³⁵. The half-lives of isoforms III and IV have been reported to be the same³⁵, 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*, *Psm3*, *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*²⁶, 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^{40,29,30}. 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^{41,42}, while other data suggest the opposite^{24,25,43}. 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 knock-out 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 affected¹¹. Likewise, in the double pIII/pIV KO in which a large region was deleted, expression of *Ciita* in conventional DCs appeared normal²⁰. 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.⁴⁴ 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 pI⁴⁵. 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 characterized⁴⁶⁻⁵², 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^{53,54}. 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 ⁵⁵. These regions were shown to interact with one another through chromatin conformation capture assays⁵⁵. 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.1⁴⁹, a critical regulator of B cell and myeloid cell fate, as well as STAT1⁵⁵, 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.

MATERIALS AND METHODS

Targeting vector and generation of *Ciita* mutant mice

A *Ciita* site-specific targeting construct (see 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. *ClaI* and *SaII* 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 *HindIII*, *BamHI*, or *EcoRI* digestion in combination with probes either internal or 3' external to the targeting construct. The

presence of the upstream FRT site was determined by PCR, using primers 1 and 2 (Fig. 1 and Supp 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 (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, Fig. 1 ad Supplemental 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, Supp 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, *XhoI* and D3', *ClaI*) to amplify the upstream flanking region for homologous recombination, introducing *XhoI* and *ClaI* sites for cloning into the targeting vector. Another primer pair was designed to introduce a FRT site upstream of promoter I and a *SalI* site at the start of translation (C5', *ClaI* and C3', *SalI*). 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 *XhoI* for cloning purposes. Primers for this step were: B5', *SalI*, B2-5'OL, B1-3'OL, and B2-3'. Finally, A5', *NotI* and A3', *SacII* were designed to amplify the downstream flanking region, intron 1 of pI, for homologous recombination, introducing *NotI* and *SacII* sites for cloning into the targeting vector. All primers are listed in Supplemental 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 *XhoI* site. The resulting vector was digested with *NotI* and *SacII*, 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/6 congenic strain containing our targeted locus from a 129/SvEv:C57BL/6J mixed strain background^{22, 56}. 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^{22, 56}. PCR primers used are available on the Mouse Genome Database at the Mouse Genome Informatics website⁵⁷. 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 (data not shown).

Markers used were:

D1Mit211, D1Mit19, D1Mit215, D1Mit139, D1Mit150, D2Mit5, D2Mit61, D2Mit13, D2Mit206, D2Mit304, D2Mit213, D3Mit21, D3Mit40, D3Mit351, D3Mit163, D4Mit89, D4Mit275, D4Mit31, D4Mit204, D4Mit254, D5Mit148, D5Mit20, D5Mit163, D6Mit86, D6Mit33, D6Mit146, D6Mit199, D6Mit15, D7Mit310, D7Mit237, D7Mit31, D7Mit189, D8Mit155, D8Mit191, D8Mit263, D8Mit211, D8Mit93, D9Mit2, D9Mit21, D9Mit306, D9Mit279, D10Mit51, D10Mit40, D10Mit7, D10Mit162, D10Mit180, D11Mit226, D11Mit270, D11Mit5, D11Mit38, D11Mit199, D11Mit184, D12Mit153, D12Mit3, D12Mit231, D13Mit16, D13Mit179, D13Mit193, D13Mit76, D14Mit99, D14Mit60, D14Mit192, D14Mit107, D15Mit13, D15Mit46, D15Mit29, D15Mit42, D16Mit182, D16Mit103, D16Mit64, D16Mit152, D17Mit164, D17Mit51, D17Mit205, D17Mit123, D18Mit19, D18Mit23, D18Mit33, D18Mit25, D19Mit128, D19Mit119, D19Mit91, D19Mit71, DXMit136, DXMit143, DXMit213, DXMit130, DXMit186

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. Primers used are listed in Supplemental Table 1.

Cell Collection and treatments

Dendritic cells (DC) were collected from the spleen as described in Current Protocols⁵⁸. Briefly, mice were injected i.p. with 30 ng Flt3 Ligand-Ig (Flt3-L) for 9 days⁵⁹. Flt3-L was generously provided by Dr. R. Mittler (Emory University). Following CO₂ asphyxiation, spleens were removed and injected with Dulbecco's modified Eagle's media (Cellgro, Manassas, VA) containing 10% fetal bovine serum (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 µg/ml streptomycin (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 µm cell strainer (BD Biosciences, San Jose, CA). Red blood cells were lysed with ammonium-chloride potassium-chloride (ACK) lysing buffer. 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 10 U/ml heparin. For peritoneal macrophages, mice were injected i.p. with 2.5ml 2% solution of thioglycolate four days prior to collection of the peritoneal fluid. Peritoneal exudate cells were plated 0.8-1×10⁶ cells/ml in DMEM-10 containing 0.292 mg/ml L-glutamine, 100 U/ml penicillin, 100 µg/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 and spleen cells were incubated in RPMI 1640 medium containing 10% FBS, 0.292 mg/ml L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 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.

Flow cytometry

For cell surface staining, 0.5×10^6 cells were treated as previously described³⁸. Briefly, 0.5×10^6 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*, 3×10^6 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 μ M ionomycin (Sigma-Aldrich) (positive control), or with 10^7 cells heat-killed *L. monocytogenes*. (HKLM). Brefeldin A (BFA) (Sigma-Aldrich) was added (10 μ g/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 (GP₃₃, GP₂₇₆, or GP₆₁) 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- α (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 GP₆₁₋₈₀ peptide⁶⁰. Popliteal and inguinal lymph nodes were collected 12 days later. A single cell suspension was generated, and 5×10^5 cells were incubated in a 96 well plate with increasing concentrations of peptide for 3 days, with 0.4 μ Ci/well [³H]thymidine added

during the last 24 h. Plates were harvested using a FilterMate harvester (Packard Instrument, Meriden, CT) and ³H-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 µg MOG₃₅₋₅₅ peptide emulsified in 200 µl CFA (2.5 mg/ml *M. tuberculosis*) on days 0 and 7, as previously described^{60, 61}. 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 (MOG₃₅₋₅₅) (MEVGWYRSPFSRVVHLYRNGK)⁶², LCMV epitopes GP₃₃₋₄₁ (KAVYNFATC), GP₂₇₆₋₂₈₆ (SGVENPGGYCL), and GP₆₁₋₈₀ (GLNGPDIYKGVYQFKSVEFD)⁶³ 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 LD₅₀ for C57BL/6J mice was determined to be 6×10⁶ 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 µg/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 described³⁹. Briefly, mice were infected i.p. with 2×10⁵ 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 GP₃₃₋₄₁, GP₂₇₆₋₂₈₆, or GP₆₆₋₇₇ for flow cytometric analysis of antigen-specific T cells were performed as previously described⁶⁴⁻⁶⁶.

Influenza Immunization

CIITA knockout and wild-type littermate control mice received a single immunization of 5 µg 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 naive 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^{67, 68}. Hemagglutination inhibition (HAI) titers based on the WHO protocol⁶⁹ as described previously⁶⁷. 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, Valencia, CA). Reverse transcriptase was done using 1 µg of RNA using SuperScript 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 method⁷⁰. 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 Supplemental Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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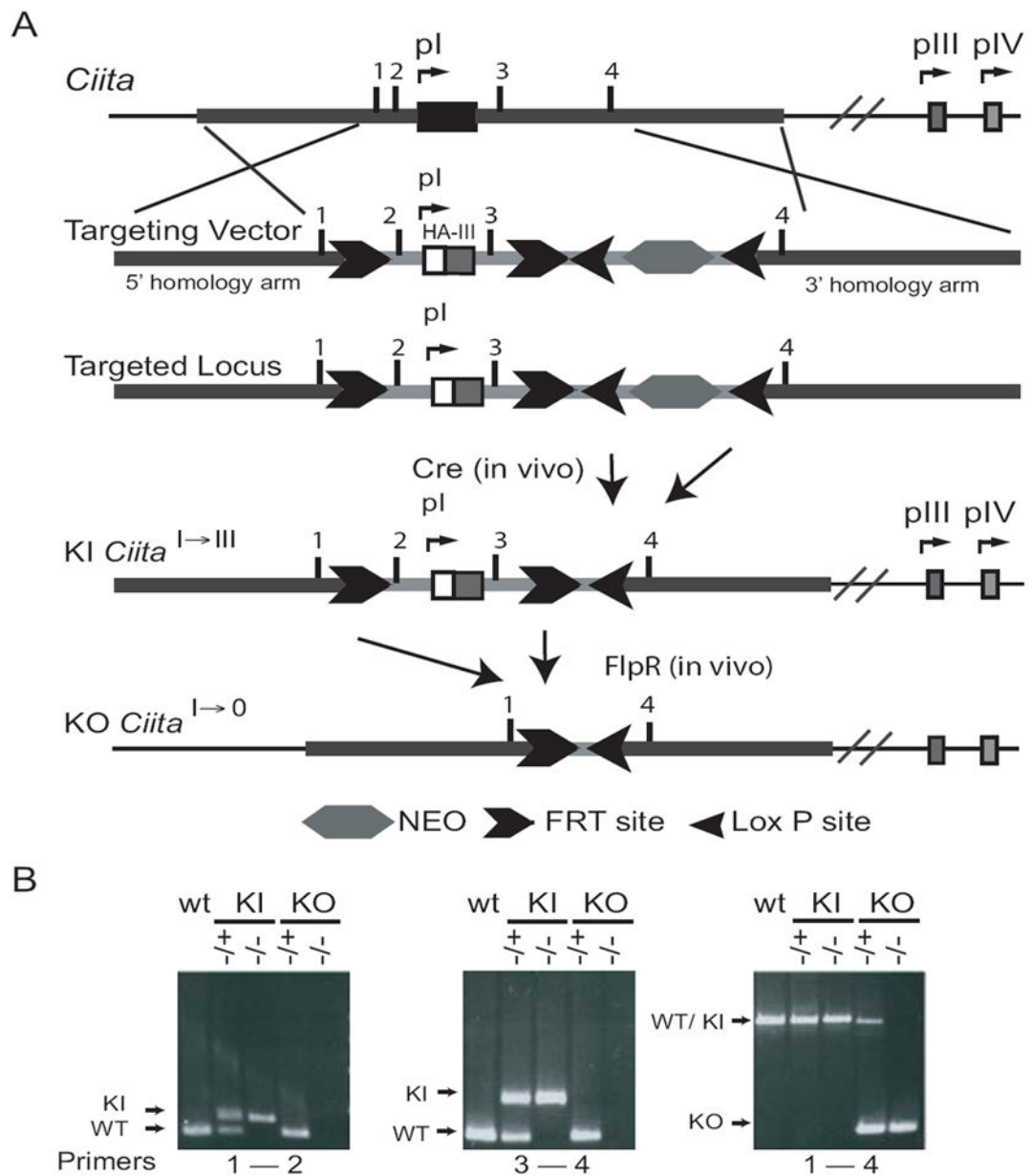


Figure 1. *Ciita* targeting construct

A. Schematic of the targeting construct used to generate *Ciita*^{I→III} (KI) and *Ciita*^{I→0} (KO). Transgene positive mice were bred 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.

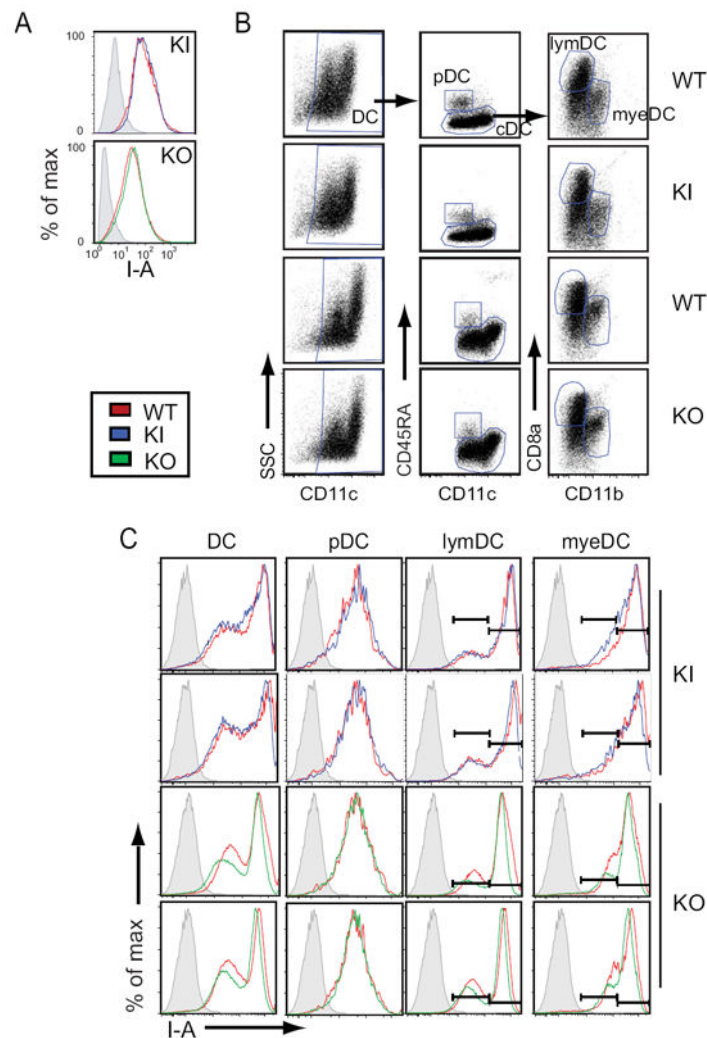


Figure 2. MHC-II surface expression on KI and KO antigen presenting cells is similar to WT
 A. Histogram analysis of flow cytometry data for I-A^b expression in KI and KO samples compared to WT littermate controls. Thioglycolate elicited peritoneal macrophages were stimulated in vitro 24 h with 500 U/ml IFN- γ . Live cells were gated on by side scatter properties; macrophages were identified as CD11c⁺. B. Flt3L elicited, CD11c⁺ sorted spleen cells were stimulated with 1 μ g/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. Dendritic cell subsets identified in B were analyzed by flow cytometry for I-A^b expression. The data generated for this figure 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.

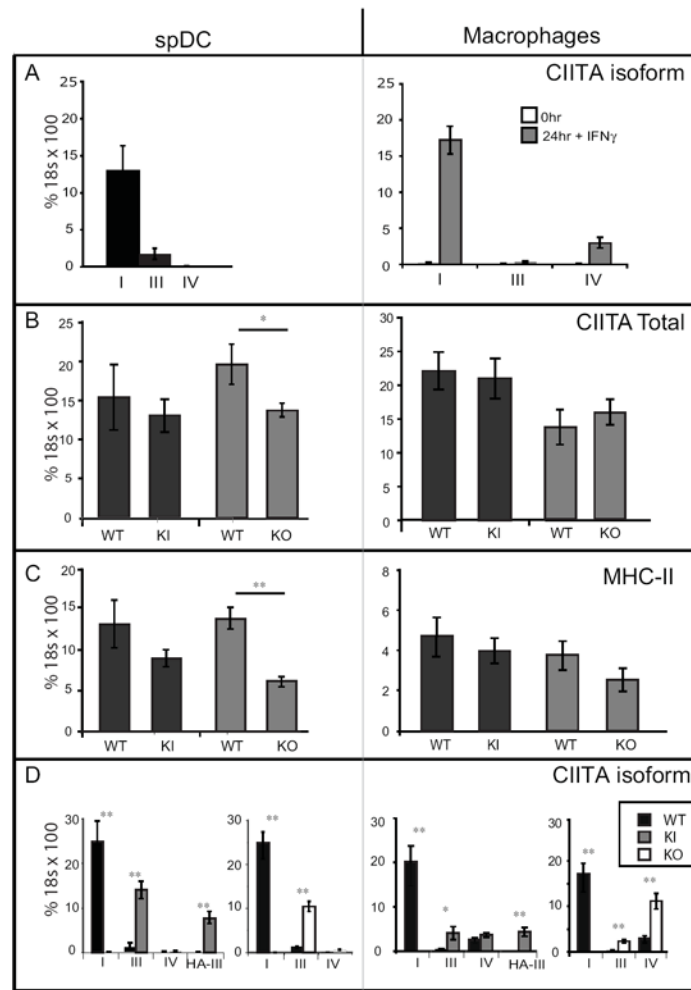


Figure 3. *Ciita* isoform I is not required for expression of CIITA or MHC-II in cells of the myeloid lineage

Ciita isoform specific, total *Ciita* mRNA, or I-A α 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-A α 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.

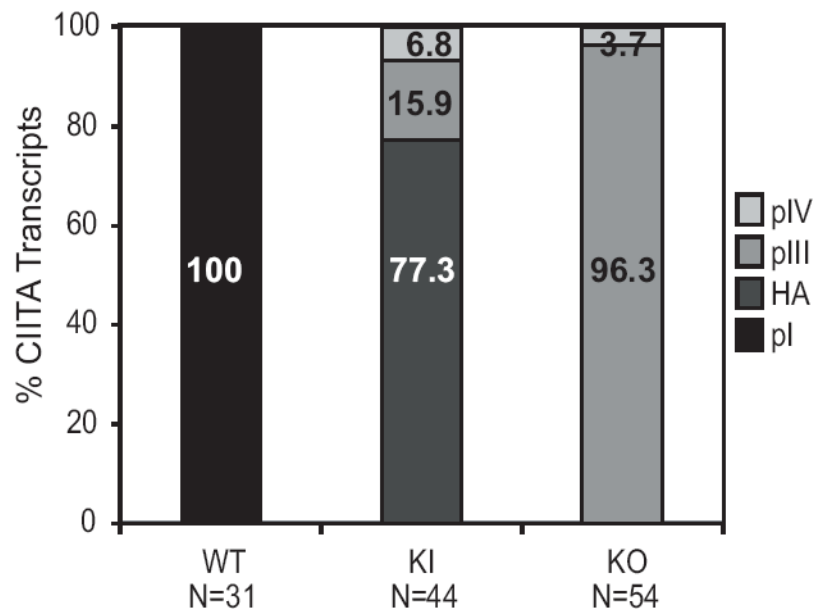


Figure 4. 5'RACE maps *Ciita* expression to promoters III and IV in KI and KO spDC
 5'RACE was carried out on mRNA from WT, KI, and KO splenic DCs. Wild-type spDC *Ciita* transcripts initiated from pI, exclusively. Both KI and KO spDC expressed *Ciita* transcripts from promoters III and IV. The majority 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.

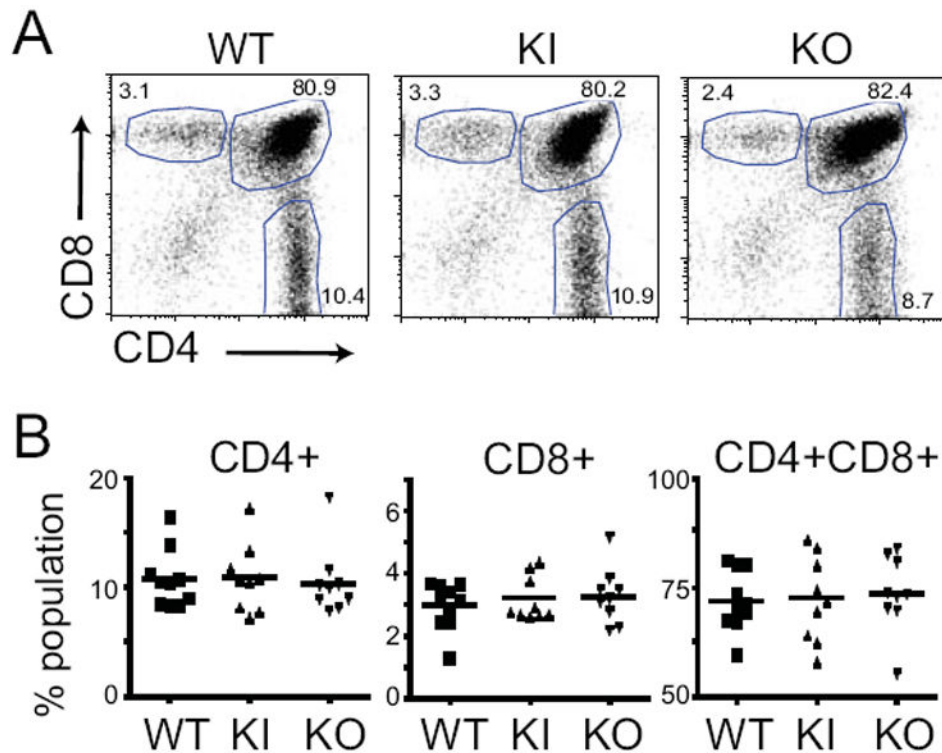


Figure 5. *Ciita* isoform I is not required for T cell development in the thymus

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.

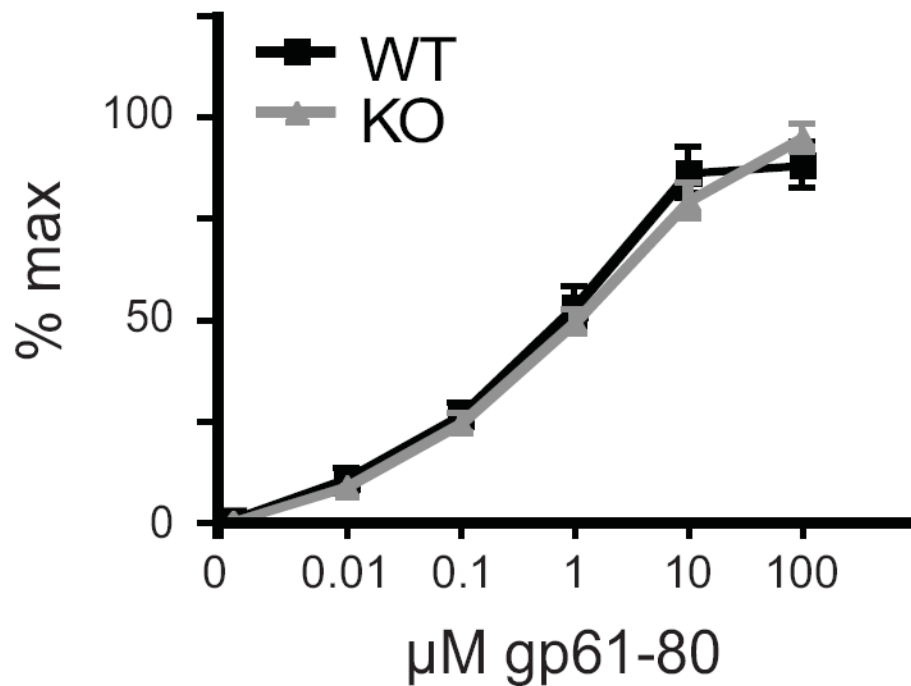


Figure 6. *Ciita* KO T cells proliferate normally in response to antigen

Mice were immunized in the footpad and the base of the tail with the 150 μl peptide/CFA emulsion containing A. 1 mg/ml or B. 0.1 mg/ml of the peptide GP₆₁₋₈₀. After 12 days, draining lymph nodes were isolated and T cell proliferation was measured by incorporation of ³H-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).

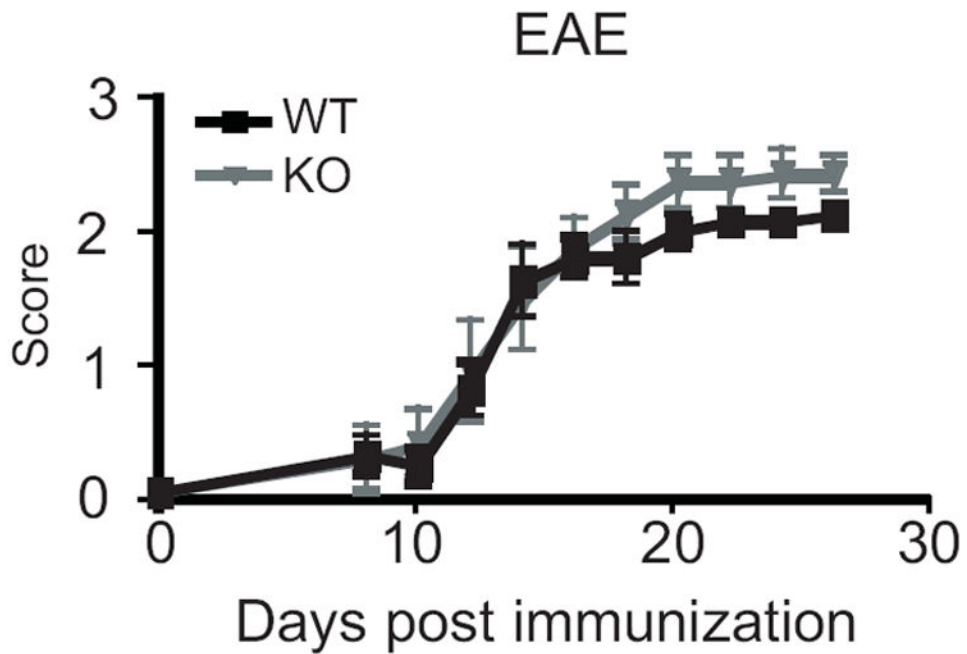


Figure 7. *CiitapI* KO mice are not more susceptible to experimentally induced autoimmune disease

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.

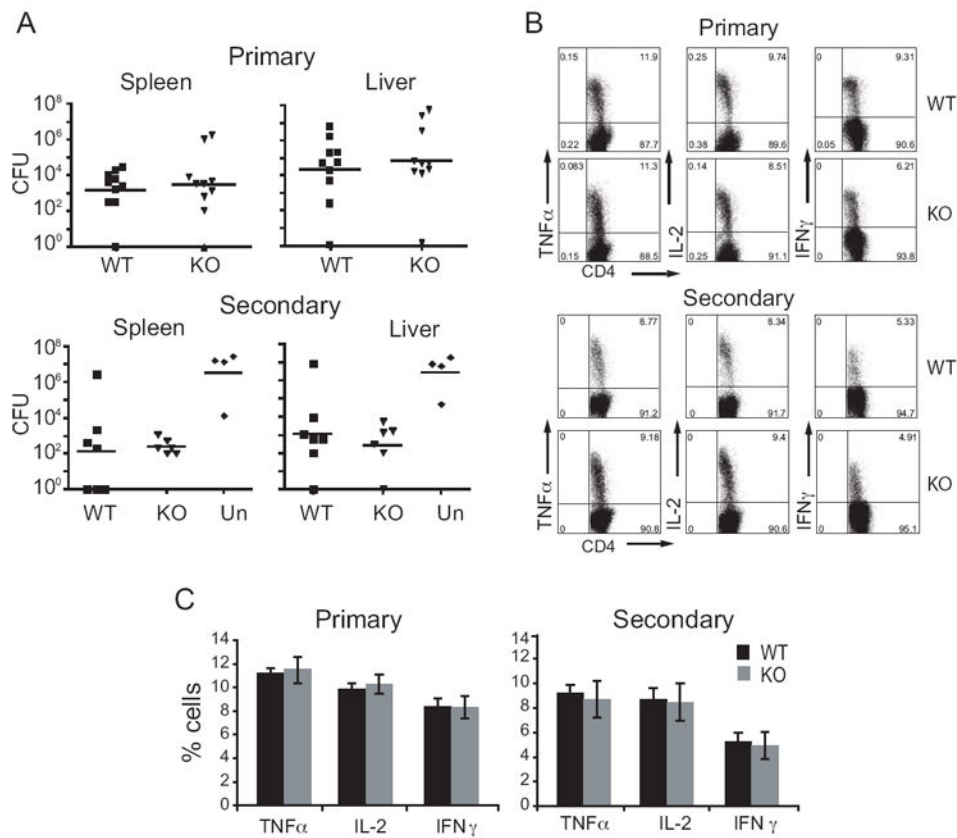


Figure 8. *Ciita* isoform I is not required for resistance to the intracellular bacteria *Listeria monocytogenes*

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 LD₅₀ *LM* and CFU were determined at day five post-infection. For secondary infection, mice were immunized with a low dose (0.1 LD₅₀), then re-infected four weeks later with a high dose (10 LD₅₀) 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 LD₅₀. For secondary infection, mice were infected with 0.1 LD₅₀, then challenged four weeks later with 6 LD₅₀. 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 TNF α , IL-2, and IFN- γ . Each graph is representative of two independent experiments using three to four mice per group. Un, unimmunized.

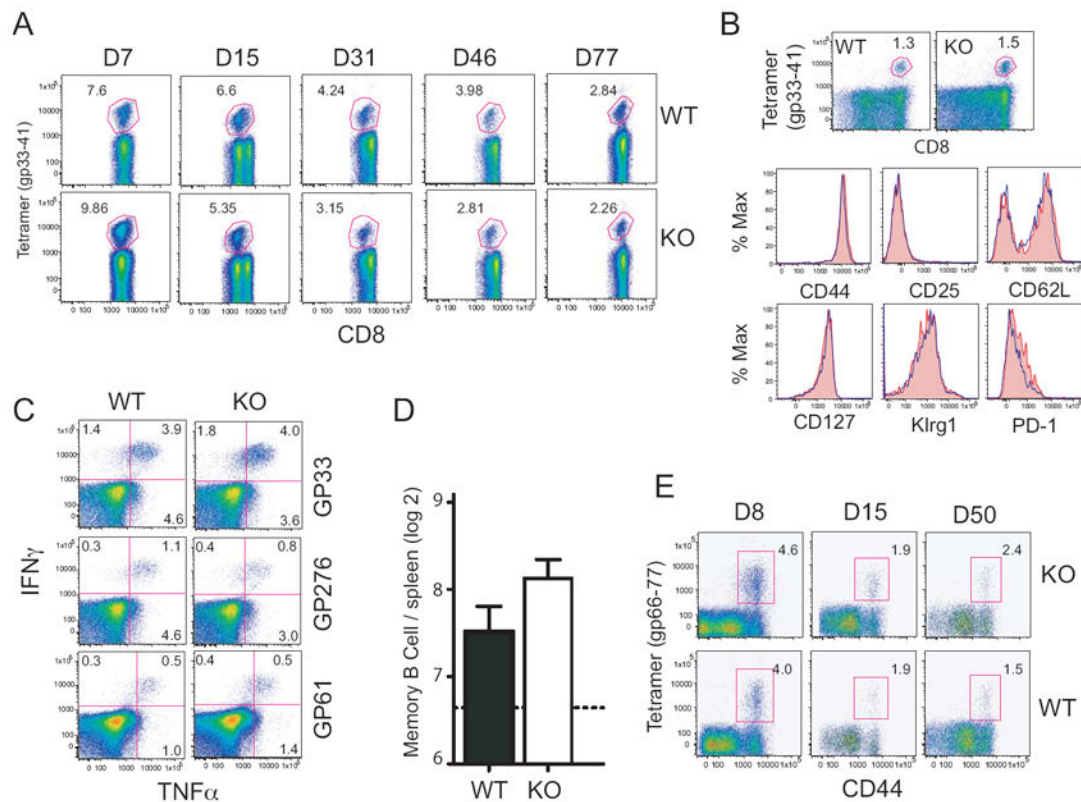


Figure 9. The adaptive immune response to an acute LCMV infection in KO mice is similar to WT mice

A. Virus-specific CD8 T cells were analyzed using fluorescently labeled tetramer specific for the GP₃₃₋₄₁ 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 (GP₃₁₋₄₀) CD8 T cells were analyzed by flow cytometry for the T cell markers CD44, CD25, CD28L, CD127, Klrp1, 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 (GP₃₃ and GP₂₇₆) and CD4 (GP₆₁) 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 GP₆₆₋₇₇ epitope of LCMV.