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Histone Deacetylase 7 Licenses Innate Effector Development in
iNKT Cells

by

Intelly Lee

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Dedication

This thesis is dedicated to the persevering spirit of all scientists who have ever triumphed through unexpected obstacles in pursuit of that flighty but uncompromising temptress, discovery.

Acknowledgements

The complete list of people I would have to thank for all of their support during my thesis would take just as long to read through as the rest of this document, so here I can attempt to highlight only select individuals whom I feel deeply indebted to, though there are many others whom I will not be able to mention in this abbreviated section that seems inadequately titled to express the full depth of my gratitude. First and foremost, my training as a scientist has been most profoundly influenced and undeniably improved by having Eric Verdin as my mentor and principal investigator – so much of my current outlook and future directions will indelibly be, if not controlled, then at least haunted by his words, guidance, scientific style and attitude towards discovery. Within the first few months of joining his lab, I remember feeling inspired and perhaps just a smidge intimidated by Eric's constant exhortations to "do courageous science" – in the face of unreasonable opposition, unknown pitfalls and unforeseen roadblocks, whether financial, stylistic, personal or social in nature. Fast forward five years, and now whenever I feel confronted by an overwhelming problem, or feel discouraged and self-defeating in the face of upcoming adversity, I simply muster and channel Eric's élan and verbally force myself to repeat, out loud, his mantra that "Things are never as easy as you hoped, but never as difficult as you feared," much like even the messianic Paul Atreides has to whisper "Fear is the mind-killer" to himself over and over again in the darkest moments. Eric's unrelenting optimism, his can-do spirit and his quest to uncover the root explanations behind the most compelling questions that interest him are all traits I hope to nuture myself in the future, in all capacities from physician scientist to family member to treasured friend.

As deeply as Eric's scientific mentorship may have etched into the crevices of my bones, I do think that many graduate students at UCSF enjoy the benefits of having a great scientific mentor – the list of high-quality principal investigators and inspiring scientists on campus could fill reams of single spaced pages. Far fewer of those students and other trainees, though, could say they have as supportive and welcoming of a lab environment as the Verdin lab has proven to be over the past half-decade I have spent at the Gladstone Institutes. My lab members constantly motivate with me with their diligence and curiosity, comfort and console me during moments, however daunting and seemingly never-ending, of temporary failure, and remind me that the outside world is far grander and more adventurous than I typically give it credit for given my myopic delusions of grandeur. The entire spirit of the floor, at the Gladstone Institutes of Virology and Immunology, is one of open-hearted acceptance and reciprocal encouragement, and that has proven to be much more important of a resource and boon than I could have ever expected before starting graduate school. In particular, I feel intensely grateful to those friends who shook me out of the prison of my own consciousness and to whom I turn to for advice and comfort at any major life juncture, especially I. Ali, K. Fontaine, W. He, M. Jeng, V. Saykally and M. Wang. The natural state of human being, as David Foster Wallace once so poignantly captured, is one of feeling "uniquely, completely, imperially alone day in and day out" – and without genuine friendship with other people to nurture me, I expect I could have easily succumbed to this preternatural loneliness.

After my first couple decades on this planet I've grown inured to pithy mantras like "It takes a village," but finishing this graduate degree has, somewhat annoyingly, proven the value and truth behind these oft-repeated aphorisms. I used to harbor idle fantasies of being a cultured, Renaissance man of knowledge and refinement, but then within the first year of working with H. Kasler I quickly gave up that private dream on account of how much more extensive and compelling his body of general knowledge proved. Still something to aspire towards, I guess. Anytime I hear about a first year rotation student or summer intern breaking a

machine, contaminating some cells, or generally being a nuisance in the lab environment, I think back to my first lab rotation under H. Lim’s guidance and wonder how he ever refrained from strangling me with his bare hands out of immunology and teaching frustration. I will always be impressed by his work ethic and skilled bench qualities. The advice and feedback, both scientific and personal, that I received my core thesis committee members A. Abbas, A. Chawla, and D. Sheppard has also proven invaluable as I look towards my postgraduate training. My fellow students and peers, both in the Medical Scientist Training Program and in the Tetrad Graduate Program, have alternately and simultaneously proven to be sources of comfort, unequivocal support, inimitable leadership, and unwavering kindness and generosity. Administrative help, at the Gladstone Institutes and with various affiliated program offices at this University, has made my life far easier and manageable given my seeming inability – and probably innate aversion – to bureaucratic paperwork and event planning. Close personal friends I made and knew before coming to San Francisco – most notably J. Foster from Parkway Central, and S. Cardick, J. Swenson, S. Chang Reale and W. Bates from Washington University in St. Louis – have been both wings to help me soar beyond my greatest aspirations, and chains to keep me grounded to reality.

And finally, I must acknowledge the love, care and unwavering support that my family, particularly my immediate family in St. Louis, has provided me over the past years. After finishing my undergraduate studies as a biochemistry major in the Midwest, I was eager to move to a new region and rediscover, maybe reinvent, a different kind of life on the West Coast. There is no small irony that through a transformative move farther away, I would end up growing closer with my parents and sister in the ensuing years. Much as I cherish where I am now, there are certain comforts from their mere physical proximity that I will never be able to find suitable replacement for in California, and I hope circumstances will conspire for us to be brought back together in the not too distant future. Strange and wondrous it seems, after so many years of long phone conversations and befuddled responses to the question of “How much longer do I

have before I finish/graduate/move on?” that I would look back and be amazed at how quickly this all passed, how the interminable years and frustrated nights would accumulate, grind and build into a seemingly impenetrable limestone monument, only for it to transfigure, at the very end when it all comes together, into something ephemeral, unnoticeable, and ungraspable, vanishing once again back into the ether.

Histone Deacetylase 7 Licenses Innate Effector Development in iNKT Cells

Intelly Lee

Innate effector lymphocytes, including invariant natural killer T-cells (iNKT), are conserved and integral components of the vertebrate immune system that orchestrate the early host response to infection, yet the mechanisms by which developing thymocytes acquire either a naïve or innate effector identity remain unclear. Here we report that histone deacetylase 7 (HDAC7), a highly conserved signal-dependent transcriptional corepressor abundantly expressed in thymocytes, is a crucial regulatory factor that licenses innate effector development in iNKT cells. In a gain-of-function setting where HDAC7 is constitutively nuclear localized, innate effector development is blocked and iNKT cells become diverted to extremely rare, naïve-like T-cells with limited cytokine production and propensity to recirculate. Conversely, in a loss-of-function setting where HDAC7 is removed via conditional genetic deletion, naïve T-cell development is impeded and more thymocytes acquire an innate effector identity, particularly in an Eomesodermin-expressing CD8 peripheral subset that resembles so-called "innate memory" T-cells. Regulation of this fate decision hinges on the ability of HDAC7 to antagonize the transcriptional activity of Promyelocytic Leukemia Zinc Finger (PLZF), a signature innate effector transcription factor of innate effector development, which we demonstrate occurs in part through upstream transcriptional repression and direct physical binding. Finally, we find that in mice with a gain-of-function *HDAC7* transgene which spontaneously develop tissue-specific autoimmunity directed mainly against the hepatobiliary tissues and gastrointestinal mucosa, restoring iNKT cells *in vivo* can mitigate tissue destruction and reduce mortality rate. These studies identify HDAC7 as an important epigenetic licensing factor that controls naïve versus innate effector development in thymocytes and implicates a heretofore underappreciated role for innate effectors in protecting against autoimmune disease.

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CHAPTER 1

INTRODUCTION

In addition to specializing as well-described naïve T-cells, developing thymocytes may alternatively complete their thymic development as innate effector T-cells. Whereas naïve T-cells are circulating components of the adaptive immune response that generate immunological memory after a lengthy priming period, requiring recognition of cognate antigen in the periphery and costimulation provided by dendritic cells, innate effectors function instead as frontline first-responders in the early phase of pathogen invasion, providing sentinel protection at mucosal barrier surfaces and directly in tissue parenchyma (Kang and Malhotra, 2015). As such, despite possessing a fully-rearranged T-cell receptor and having expressed RAG recombinase during development, innate effectors are ascribed the term "innate" to denote their functional overlap with traditional innate immune cells such as monocytes and macrophages. They differ dramatically from naïve T-cells in phenotype and function: for instance, innate effectors often express T-cell receptors (TCRs) that bind ubiquitous, non-peptide microbial products presented on nonclassical major histocompatibility complex (MHC) molecules, as in the case of iNKT cells that recognize CD1d-presented glycolipids or MR1-restricted mucosal associated invariant T-cells (MAIT) that bind to Vitamin B_{12} and riboflavin metabolite-derived bacterial products (Kronenberg, 2014). They exit the thymus bearing an antigen-experienced memory phenotype, typically expressing high levels of the surface marker CD44. Morphologically, they are larger and have a better-developed Golgi apparatus than naïve T-cells, rapidly elaborating copious quantities of cytokines after brief TCR stimulation, within hours or even minutes of stimulation, in contrast to naïve T-cells that require days-long priming and maturation before egressing back to the site of peripheral inflmmation. Finally, after thymic egress, innate effectors directly migrate to reside in peripheral tissues with limited ability to recirculate through the blood and secondary lymphoid organs (Brennan et al., 2013; Chandra and Kronenberg, 2015; Kang and Malhotra, 2015).

Altogether, these differences suggest that innate effectors undergo an alternative maturation process in the thymus that parallels multi-day activation of naïve T-cells by primed dendritic cells in the periphery. Indeed, unlike proliferatively inert conventional thymocytes, iNKT cells undergo an extensive, hundred-fold thymic expansion to generate the vast majority of their population in the periphery (Benlagha et al., 2002), akin to naïve T-cell blasting from a single clone, while the basal rate of proliferation of conventional, peptide-binding thymocytes is extremely low. Despite the crucial role for innate effectors in maintaining tissue homeostasis and orchestrating the early immune response to pathogen invasion, how developing thymocytes are instructed to specialize along this bifurcating branch point as naïve T-cells or innate effectors remains poorly understood.

We previously described how naïve T-cell development is regulated by the class IIA histone deacetylase HDAC7, a conserved transcriptional corepressor abundantly expressed in thymocytes (Dequiedt et al., 2003; Kasler and Verdin, 2007). The activity of HDAC7 is controlled by signal-dependent nuclear-cytoplasmic shuttling depending on the phosphorylation status of N-terminal serine residues (Verdin et al., 2003), and in thymocytes, strong TCR stimulation results in pronounced HDAC7 nuclear exclusion due to protein kinase D-induced phosphorylation of those serines (Parra et al., 2005). When HDAC7 is lost through genetic deletion, conventional thymocytes experience a block in positive selection and are impeded from completing naïve T-cell development (Kasler et al., 2011). Conversely, in a gain-of-function setting where HDAC7 is constitutively nuclear localized via the mutation of those N-terminal serines into alanines to prevent phosphorylation, negative selection of naïve T-cells becomes impaired, leading to the escape of autoreactive clones and subsequent development of severe, multifocal autoimmunity directed against gastrointestinal and hepatobiliary tissues (Kasler et al., 2012). Thus, HDAC7 functions as a critical regulatory switch that directs conventional thymocyte development following TCR stimulation, with the majority of activity in a narrow TCR signaling axis threshold encompassing the boundary and positive and negative selection.

Recent studies have suggested that many innate effector subtypes, including iNKT cells and CD8αα$^+$ intraepithelial lymphocytes, receive stronger-than-normal TCR signals during their thymic education (Klein et al., 2014; Moran et al., 2011) as befitting their mature, memory-phenotype status and function. This alternate developmental pathway is termed agonist selection (Stritesky et al., 2012), as the received TCR signal strength exceeds that of conventional positive selection, which induces thymocytes to become naïve T-cells, and approaches the signaling strength found in negative selection. As we had previously uncovered regulatory roles for HDAC7 in both positive and negative selection at this “boundary” zone, we next wanted to investigate whether HDAC7 might additionally influence agonist-selected innate effector development.

RESULTS AND DISCUSSION

A Gain-of-Function HDAC7 Mutant Abrogates iNKT Development

As a test case, we focused on iNKT cells, which can be reliably, easily and sensitively identified by staining with α-galactosylceramide-loaded (PBS-57) tetramer. We first examined gain-of-function *HDAC7-ΔP* mice, expressing a thymus-specific transgene encoding for a constitutively nuclear HDAC7 that fails to export following TCR stimulation due to mutation of its N-terminal serines (Kasler et al., 2012). Strikingly, *HDAC7-ΔP* mice exhibit a near complete absence of peripheral iNKT cells (Tet^{+} $TCR\beta^{+}$) in circulation through the spleen and in tissue residence in liver (Figures 1A and 1B), where iNKT cells are especially abundant. Nearly all thymocytes in *HDAC7-ΔP* also failed to stain with tetramer (Figures 1A and 1B), suggesting that the iNKT absence was due to a developmental block in thymic ontogeny.

Despite the overall profound reduction in the iNKT numbers (95-99%), occasional *HDAC7-ΔP* mice had detectable numbers of tetramer-binding T-cells that could be assessed by flow cytometry. We found these rare iNKT cells in the periphery were $CD24^{lo}$ but failed to upregulate either CD44 or NK1.1 (Figure 1C). Thus, using the conventional staging system for iNKT development (Stritesky et al., 2012), *HDAC7-ΔP* iNKT cells are Stage 1 developmentally arrested. Interestingly, most naïve T-cells exit the thymus at an equivalent Stage 1 surface phenotype as developing iNKT cells, namely by downregulating CD24 while also maintaining limited expression of CD44 and NK1.1; the stage 1 to stage 2 transition characterized by upregulation of CD44 is typically considered the beginning of innate effector development. Thus, because these extremely rare iNKT cells from HDAC7-ΔP mice egress with a naïve-like surface phenotype, one possibility to explain their absence is due to a block on innate effector development when HDAC7 remains nuclear-localized.

The Developmental Block in HDAC7-ΔP is Cell Autonomous

We surmised that this block could have resulted from defects in any early aspect of iNKT development, including failure of DP thymocytes to present CD1d-bound glycolipids or aberrant

costimulatory signaling through the SAP family of coreceptors (Bendelac et al., 2007). To help distinguish between these several possibilities, we next generated irradiated mixed bone marrow (BM) chimeras reconstituted with a mixture of wild-type (WT) and *HDAC7-ΔP* bone marrow. As previously seen (Kasler et al., 2012), the *HDAC7-ΔP* transgene did not grossly disrupt conventional thymic development in these chimeras. Although a significant reduction in *HDAC7-ΔP*-derived thymocytes at the immature single positive (ISP; TCRβ$^-$CD8$^+$CD4$^-$) stage was noted, their prevalence was restored back to wild-type levels at subsequent double positive (DP) and single positive stages (Figure 2A). The distribution of naïve and memory T-cells in peripheral CD4$^+$ and CD8$^+$ subsets were equivalent as well, at least in chimeras analyzed early (6-8 weeks) post-reconstitution (Figures 2B).

However, despite conventional thymic development being left intact, *HDAC7-ΔP* bone marrow failed to produce iNKT cells (Figure 2C) in appreciable quantities in chimeras. Across thymus, spleen and liver, there was a pronounced loss of iNKT cells from *HDAC7-ΔP* bone marrow compared to the population of WT origin (Figure 2D). Because providing WT thymocytes in the same congenic host failed to rescue iNKT development from *HDAC7-ΔP* precursors, we ruled out a role for HDAC7 in regulating CD1d-mediated glycolipid presentation and costimulatory signaling from *trans*-acting DP thymocytes during iNKT selection. Thus, a gain-of-function, constitutively nuclear HDAC7 preserves conventional thymic development but induces a specific, cell-autonomous block in iNKT development.

Constitutively Nuclear HDAC7 Diverts iNKT Cells into Naïve-like T-Cells

We next wanted to ascertain if HDAC7 controlled TCR chain usage and expression, preventing DP thymocytes from recognizing glycolipids during agonist selection, or if it was disrupting innate effector development and preventing iNKT precursors from dividing through thymic proliferation (Benlagha et al., 2002). Therefore, we took advantage of a Vα14-Jα18 TCRα transgene ("*Vα14*"), encoding for the specific, invariant TCR α-chain that allows iNKT cells to bind self-glycolipids with high affinity, and crossed it with *HDAC7-ΔP* to determine if

tetramer-binding iNKT cells could be restored. As expected, *Vα14*-only mice had many more iNKT cells in thymus and spleen compared to WT mice (Figures 3A and 3B). When *HDAC7-ΔP* was additionally introduced in *Vα14 x HDAC7-ΔP* crosses, iNKT numbers were robustly restored in the thymus to a level comparable to *Vα14* alone, yet brought back to only WT levels in the periphery (Figures 3A and 3B). These results suggest HDAC7 has limited to no effect on TCR rearrangement and expression, allowing *Vα14* to strongly rescue glycolipid-binding thymocytes even when combined with *HDAC7-ΔP* and explaining the prominent rescue of iNKT numbers in the thymus as *Vα14 x ΔP* mice (significantly more than the WT frequency), but continues to block innate effector development and halt the 100-fold thymic proliferation of developing iNKT cells, leading to the near complete absence of iNKT cells in the periphery in *HDAC7-ΔP* mice and only partial rescue in the periphery of *Vα14 x ΔP* mice, whose circulating frequency of iNKT cells is essentially equivalent to the WT frequency.

In further support of this strong HDAC7 activity repressing innate effector development, rescued iNKT cells in *Vα14 x HDAC7-ΔP* mice arrested at Stage 1 (Figure 3C) and failed to upregulate CD44 and NK1.1, as was the case of *HDAC7-ΔP* mice alone (Figure 1C), while a majority of *Vα14*-only derived iNKT cells had post-Stage 1 $CD44^{hi}$ expression (Figure 3C). Fortunately, by crossing *HDAC7-ΔP* with *Vα14*, we restored a peripheral iNKT population in sufficient numbers to allow for additional functional assessment. In particular, we wished to examine if these aberrant *HDAC7-ΔP* derived iNKT cells, with a naïve surface profile, functionally resembled innate effector or naïve $CD4^+$ T cells.

When briefly stimulated *ex vivo* with phorbol 12-myristate 13-acetate (PMA) and ionomycin, WT and *Vα14* iNKT cells produced enormous quantities of IFNγ and IL-4, two signature effector cytokines, as is typical of most innate effector types. In contrast, *Vα14 x HDAC7-ΔP* iNKT cells were severely stunted in their secretory capacity (Figures 3D and 3E), similar to naïve T-cells that require days of additional development following their initial peripheral stimulation to secrete cytokines. Additionally, iNKT cells typically express high levels

of the integrin LFA-1, which allows them to tightly adhere to endothelial cells and remain localized in tissue-specific vascular beds such as hepatic sinusoids (Thomas et al., 2011). We confirmed splenic WT iNKT cells had high LFA-1 expression, yet *Vα14 x HDAC7-ΔP* iNKT cells exhibited far lower expression levels(Figure 3F), comparable to that seen in circulating non-tetramer-binding $CD4^+$ (mainly naïve) T-cells (Figure 3G).

Furthermore, *Vα14 x HDAC7-ΔP* iNKT cells were found at comparable frequency in spleen to WT iNKT cells, yet failed to concentrate in peripheral tissues such as the liver (Figure 3H). Whereas WT iNKT cells are typically 10-15 fold more abundant in liver than spleen when assessed as a proportion of all $TCR\beta^+$ cells, *Vα14 x HDAC7-ΔP* iNKT cells were only modestly two fold enriched (Figure 3I). This suggests *Vα14 x HDAC7-ΔP* iNKT cells are predisposed to recirculate through blood and secondary lymphoid organs rather than reside in peripheral tissues. Thus, constitutively nuclear HDAC7, encoded by *HDAC7-ΔP*, prevents iNKT precursors from initiating innate effector development. As they have low CD44 expression, produce few cytokines after brief restimulation, and freely recirculate, they appear to become diverted into functional naïve-like T-cells.

The discovery and characterization of innate effector lymphocytes has transformed our understanding of T-cell receptor signaling, barrier protection at mucosal surfaces, and the evolutionary origins of the vertebrate immune system, yet the identification of key nodal factors that control naïve versus innate effector development in thymocytes has remained elusive. Herein we have demonstrated that the conserved epigenetic modifier HDAC7 serves as a crucial regulator of this developmental fate decision. When HDAC7 is strengthened, as in the case of constitutively nuclear HDAC7-ΔP, PLZF-dependent innate effector development is blocked and iNKT cells become diverted to naïve T-cells. Thus, the timely nuclear export of HDAC7 represents an important licensing step that permits the acquisition of alternative cell fates in both negative selection (Kasler et al., 2011) and agonist selection following strong TCR signaling.

In this study we focused on iNKT cells due to their relatively high abundance and ease of identification using available tetramers, but in unpublished work we find that *HDAC7-ΔαP* does not abrogate development of all innate effector subtypes. In particular, another well-described innate effector type, $CD8\alpha\alpha^+$ IELs localized in small intestine (Mayans et al., 2014), appear to be preserved in *HDAC7-ΔP* mice (data not shown). Thus, as HDAC7 mainly functions by targeting and binding transcription factors in the nucleus, we then proceeded with the hypothesis that it may target a signature transcription factor expressed in iNKTs.

Figure 1

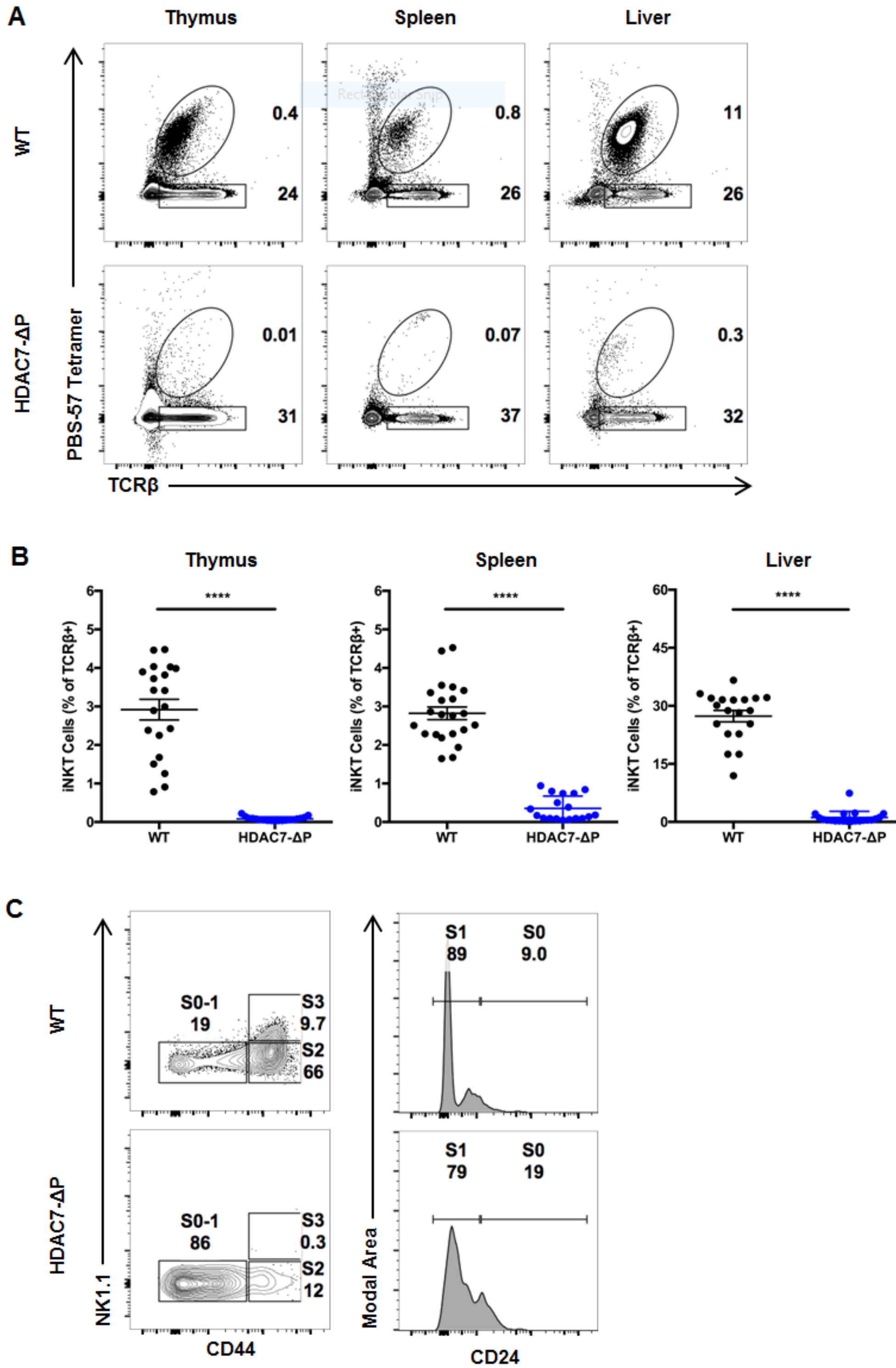


Figure 1. A Gain-of-Function HDAC7 Mutant, *HDAC7-ΔP*, Lacks Most iNKT Cells

(A, B) Flow cytometric plots (A) and total quantification (B) of iNKT cells and conventional αβ T-cells identified by staining with TCRβ and PBS-57-loaded tetramer ("Tet") in thymus, spleen and liver. iNKT cells are Tet^{+} $TCR\beta^{+}$, while conventional T-cells are Tet^{-} $TCR\beta^{+}$.

(C) Conventional staging of iNKT development based on CD24, CD44 and NK1.1 expression in Tet^{+} $TCR\beta^{+}$ thymic iNKT cells.

Bars on graphs indicate mean ± SEM (error bars); symbols represent individual mice. Data in (B) are combined from 8 independent experiments; data in (C) are representative of 3 experiments with 3-5 mice per group. Statistical significance was determined using unpaired two-tailed t tests; ***$p \leq 0.001$, ****$p \leq 0.0001$.

Figure 2

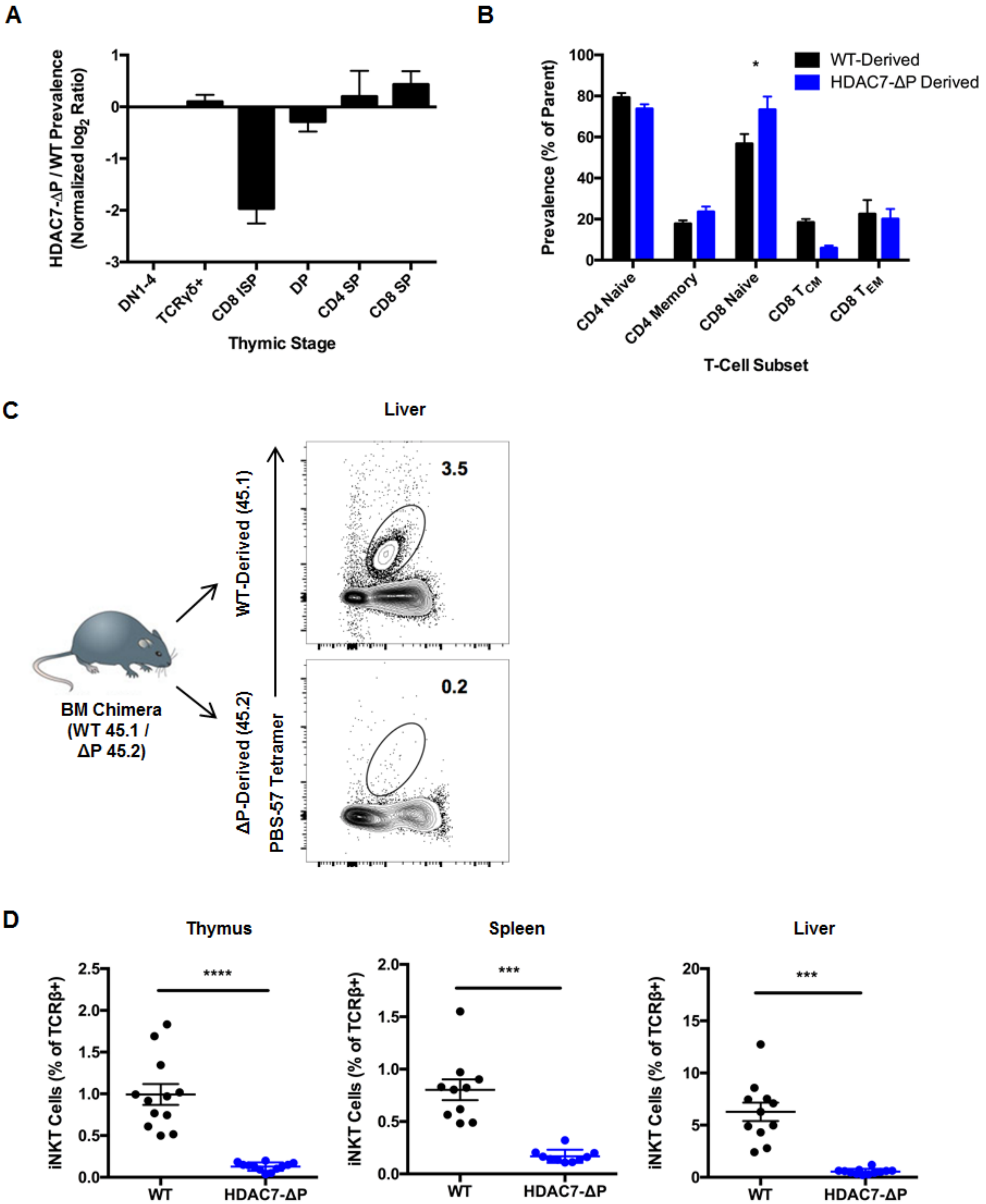


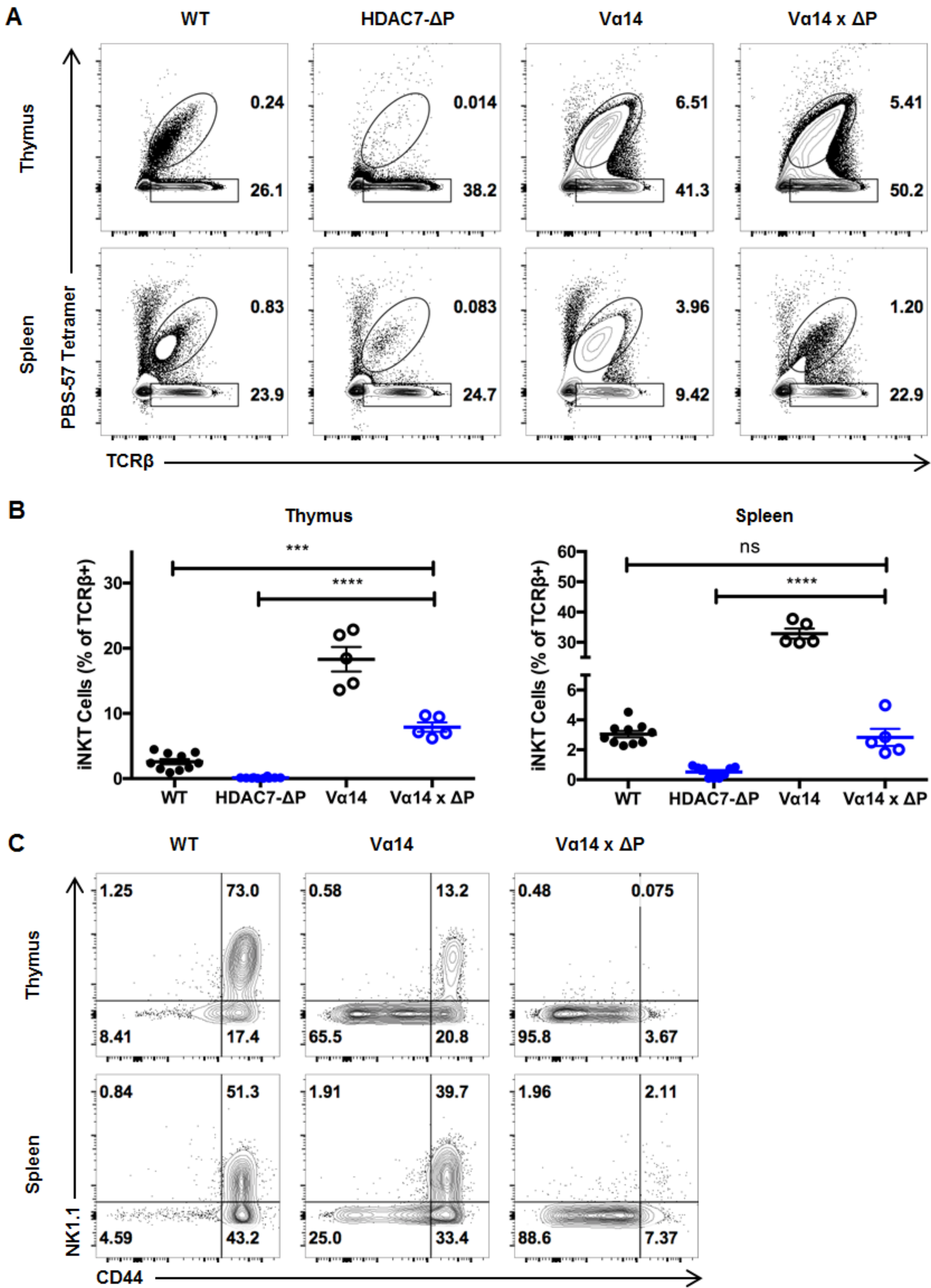
Figure 2. *HDAC7-ΔP* Cell-Autonomously Arrests iNKT Cells at Stage 1

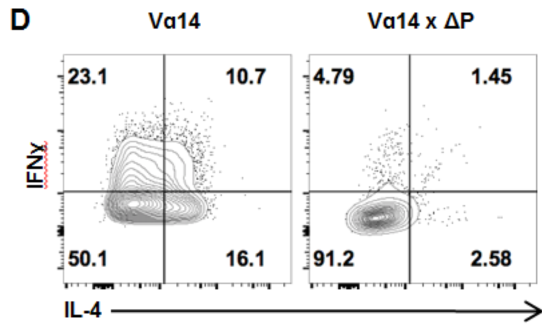
(A) Fold-enrichment of HDAC7-ΔP derived (CD45.2) to WT-derived (CD45.1) thymocyte stages in mixed bone marrow chimeras. A composite DN1-4 engraftment ratio ($Lin^-CD4^-CD8^-$) was calculated per mouse to normalize the ratio at each successive stage.

(B) Proportion of T-cell subsets plotted as percentage of parent from HDAC7-ΔP derived (CD45.2) or WT-derived (CD45.1) bone marrow in irradiated chimeras. CD4 naïve are defined as $CD44^{lo}CD4^+$,CD4 memory as $CD44^{hi}CD4^+$, CD8 naïve as $CD44^{lo}CD62L^{hi}CD8^+$, CD8 central memory (T_{CM}) as $CD44^{hi}CD62L^{hi}CD8^+$, and CD8 effector memory (TEM) as $CD44^{hi}CD62L^{lo}CD8^+$.

(C, D) Representative flow cytometric plots (D) and total quantification (E) of iNKT cells in irradiated mixed WT(CD45.1) / ∆P(CD45.2) bone-marrow chimeras from different organs. Data are compiled from 3 experiments with 4-6 mice per group. Statistical significance was determined using unpaired two-tailed t tests; *$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Figure 3

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10.7
50.1
16.1
4.79
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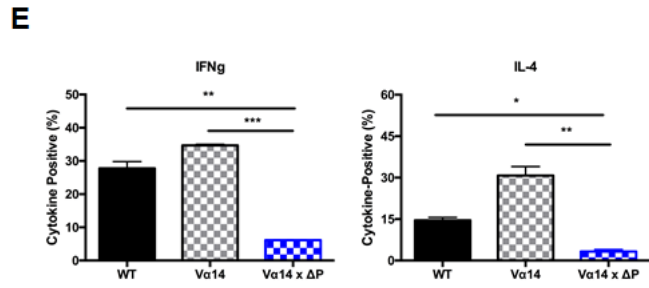
91.2
2.58
IFNγ
IL-4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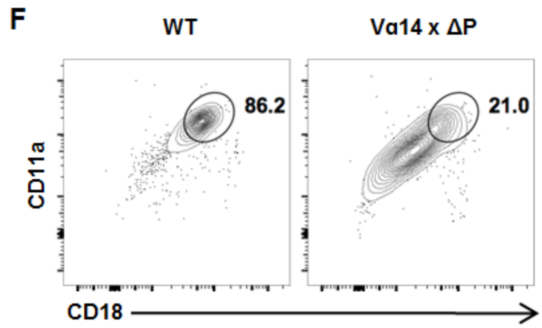
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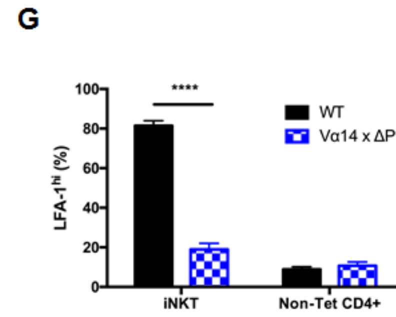
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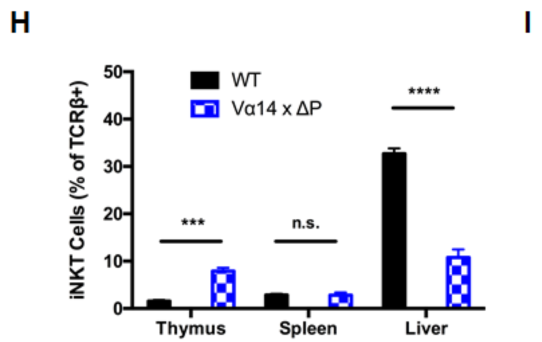
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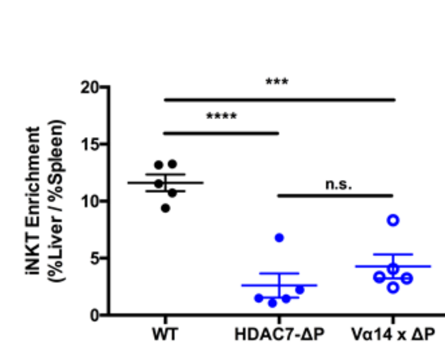
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n.s.
iNKT Enrichment
(%Liver / %Spleen)
WT
HDAC7-ΔP
Vα14 x ΔP

Figure 3. HDAC7-ΔP Blocks Innate Effector Development in iNKT Cells and Converts Them to Naive-Like T-cells

(A, B) Flow cytometric plots (A) and total quantification (B) of iNKT cells (Tet^{+} $TCR\beta^{+}$) from thymus and spleen of age-matched littermate mice.

(C) Representative surface expression of CD44 and NK1.1 in iNKT cells from thymus and spleen in age-matched littermate mice, based on mice from (B).

(D,E) Representative staining (D) and total quantification (E) of IFNγ and IL-4 secretion in ex vivo stimulated iNKT cells. Total splenocytes were harvested and stimulated for 3 hrs with PMA/Ionomycin, followed by 1 hour of concurrent brefeldin-A treatment.

(F) Surface expression of LFA-1 in iNKT cells from spleen based on CD11a and CD18 staining. Bars on graphs indicate mean ± SEM (error bars); symbols represent individual mice. Data in (B) are combined from 4 independent experiments; data in (E, G) are combined from 3 independent experiments. Statistical significance was determined using one-way (B, E) or two-way (G) ANOVA; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Tukey (B, E) or Bonferroni post-tests (G) were used for pairwise comparisons.

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CHAPTER 2

RATIONALE

The thymus-specific *HDAC7-ΔP* transgene represents a gain-of-function setting where HDAC7 has stronger than normal activity, and in that instance, innate effector development becomes blocked in iNKT cells. Another way to modulate HDAC7 is to weaken it *in* vivo through the use of standard *Cre/loxP* reverse genetics tools in mice to delete the *HDAC7* gene in a specific cell type. Therefore, to next examine if the converse, loss-of-function case with weakened HDAC7 also influences innate effector versus naïve T-cell development, we crossed the lymphocyte specific *Lck-Cre* transgene to a floxed *HDAC7* allele to generate lymphocyte-specific conditional knockout (*HDAC7-KO*), where HDAC7 is efficiently deleted from nearly all thymocytes (Kasler et al., 2011). The lab has also previously generated T-cell specific knockouts using a *CD4-Cre* driver, but found deletion to be more efficient with the *Lck-Cre* driver, in addition to benefiting from the recombinase turning on at an earlier stage to include the ɣδ T-lineage. Previous studies from our lab have shown that loss of HDAC7 seriously impairs positive selection of naïve lymphocytes, mainly due to modulation of MAP kinase signaling activity and attenuated lifespan, leading to the premature death of developing DP thymocytes. As the specific Vα14 TCR rearrangement in iNKT cells is a rather distal rearrangement, one might expect there to be fewer overall iNKT cells in *HDAC7-KO* mice as well given this sensitized apoptosis.

However, since stronger-than-normal HDAC7 activity impedes innate effector development, one might logically surmise that weaker-than-normal HDAC7 activity might promote innate effector development, leading to the expansion of iNKT or other innate effector lymphocyte subsets in the periphery that complete thymic development with a memory phenotype. Even if traditional invariant NKT cell numbers are reduced due to restricted ability to recombine for the correct TCR chain, other “innate-like effectors” such as the aberrant PLZF-expressing CD4 variant found in the Itk knockout could be expanded upon loss of HDAC7

(Prince et al., 2014). In general, most mouse mutants that either expand NKT numbers or promote innate effector lymphocytes typically exhibit a notable expansion of so-called innate memory CD8 T-cells (Jameson et al., 2015) that are $CD44^{hi}$, $T\text{-}bet^{-}$, $Eomes^{+}$ with concomitant CXCR3 and CD122 surface expression. Intriguingly, most published reports of genetic mouse mutants with this expanded innate memory CD8 population, which are normally quite rare in the standard C57Bl/6J strain of mice, have shown that these cells are generated in *trans* during thymic development by CD8 SP sensitization from IL-4 released from thymic-resident iNKT cells (Weinreich et al., 2010).

RESULTS AND DISCUSSION

Loss-of-Function HDAC7 Reduces iNKT Numbers

Regarding innate effector abundance, we immediately noticed that *HDAC7-KO* mice had fewer iNKT cells; for example, *HDAC7-KO* liver contained 80-90% fewer tetramer-binding T-cells as a proportion of TCRβ$^+$ T-cells (Figures 4A). This roughly five-fold reduction, though not as pronounced as the categorical block seen in *HDAC7-ΔP*, was seen throughout thymus, spleen and liver (Figures 4B-D). First, we attempted to establish whether this was due to similar aberrations in innate effector development, or if it may be due to a general effect of loss of HDAC7 on thymocyte lifespan.

The observation that both gain- and loss-of-function of *HDAC7* results in fewer iNKT cells initially appeared paradoxical; however, it has long been appreciated that the Vα14-Jα18 TCRα chain used by iNKT cells is a distal, secondary rearrangement, so any genetic alteration that sensitizes thymocytes to apoptosis reduces iNKT abundance (Engel and Kronenberg, 2012). As *HDAC7-KO* DP thymocytes have shortened lifespan (Kasler et al., 2011), this predicts HDAC7 loss would result in fewer iNKT cells (Hu et al., 2011). To verify the lower abundance of iNKT cells in *HDAC7-KO* resulted secondarily from changes in thymocyte lifespan, we crossed in the *Vα14* TCRα transgene and completely rescued iNKT abundance in the thymus and periphery (Figures 4A-D). In fact, as a general rule of thumb iNKT prevalence was greater even in *Vα14 x HDAC7-KO* mice than in *Vα14*–only mice, which contrasts with the case of *Vα14 x HDAC7-ΔP* that could only have tetramer-binding iNKT levels restored back to WT levels in the periphery. Additionally, and again in contrast to the case of *HDAC7-ΔP*, peripheral *HDAC7-KO* iNKT cells had a surface profile indistinguishable from WT iNKT cells, with a CD44^{hi} memory phenotype and equivalent upregulation of NK1.1 (Figure 4E). Thus, while *HDAC7-ΔP* blocks innate effector development, our data suggests *HDAC7-KO* leaves innate effector development intact and indirectly reduces iNKT abundance through shortening thymocyte lifespan.

Loss-of-Function HDAC7 Expands an Innate Memory CD8$^+$ Population

Genetic settings that promote innate effector development often result in the expansion of an innate memory $CD8^+$ population characterized by expression of the transcription factor Eomesodermin (Jameson et al., 2015). Though *HDAC7-KO* mice had fewer thymic iNKT cells, we nonetheless hypothesized that loss-of-function HDAC7 might promote innate effector development and generate innate memory, as the logical converse to the gain-of-function *HDAC7-ΔP* setting. Indeed, we uncovered more $CD44^{hi}$ $Eomes^+$ $CD8^+$ SP thymocytes in *HDAC7-KO* mice compared to WT counterparts (Figures 5A and 5B).

The $Eomes^+$ innate memory $CD8^+$ population is typically generated in *trans* from thymic-resident iNKT cells that sensitize $CD8^+$ SP thymocytes with secreted IL-4 (Lee et al., 2013; Weinreich et al., 2010). However, as noted earlier, *HDAC7-KO* mice actually have fewer thymic iNKT cells than normal, and we did not detect increased IL-4 secretion from cultured *HDAC7-KO* thymocytes stimulated *ex vivo* (data not shown). To resolve how innate memory $CD8^+$ was generated in the HDAC7 loss-of-function setting, we established irradiated mixed BM chimeras reconstituted with WT and *HDAC7-KO* bone marrow. Peripheral $CD8^+$ T-cells derived from *HDAC7-KO* had an even more pronounced $CD44^{hi}$ memory bias compared to WT-derived $CD8^+$ T-cells in the same chimeric hosts (Figures 5C and 5D). When splenocytes from chimeras were *ex vivo* restimulated, *HDAC7-KO*-derived CD8+ T-cells elaborated much more IFNγ than WT-derived $CD8^+$ T-cells (Figure 5E), assessed both as percent cytokine-positive (Figure 5F) and by median fluorescence intensity (MFI) of cytokine staining (Figure 5G). $CD8^+$ T-cells from *HDAC7-KO* mice also had increased expression of the Eomes-associated chemokine receptor CXCR3 and the trafficking receptor Ly6C (Figure 5H), and we noted increased IL-4 secretion but no memory or IFNγ enrichment in *HDAC7-KO* derived $CD4^+$ T-cells (data not shown).

In this chimeric setting, we again observed strongly impaired positive selection upon loss of HDAC7 (Kasler et al., 2011), resulting in severe underrepresentation of *HDAC7-KO* thymocytes compared to WT (Figure 5I). Because of this, $Eomes^+$ $CD8^+$ SP thymocytes were not reliably identified in chimeras because the vast majority of *HDAC7-KO* derived $CD8^+$ SP

cells were immature ISPs devoid of TCRβ expression (Figure 5J), which accords with previous observations that loss of HDAC7 severely reduces thymocyte lifespan and prevents CD8 ISPs from reaching a point where they recombine for distal TCR rearrangements – in fact, in the bone marrow chimeric setting, the vast majority of *HDAC7-KO* derived thymocytes are unable to compete with WT derived DP thymocytes. Nonetheless, as we uncovered expanded Eomes^{+} CD8^{+} SP thymocytes in individual *HDAC7-KO* mice and recapitulated their peripheral expansion into innate memory-like CD8^{+} T-cells in chimeras, we conclude that thymic loss of HDAC7 cell-autonomously promotes innate effector development, particularly in the CD8^{+} compartment.

This expansion of innate memory CD8, though somewhat expected given previous work on other genetic models that promote innate effector development, is also notable for being one of the rare, perhaps one of the first to our knowledge, reported examples of cell-autonomous generation that is not driven by IL-4 secretion in the thymus. We have attempted to examine for either mature IL-4 secreting thymocytes via primary restimulation *ex vivo* and could not find any population that actively produces this cytokine upon restimulation, nor have we been able to find aberrant PLZF-expressing CD4 lymphocytes that might be responsible for producing innate memory CD8 *in trans* (data not shown), and combined with the chimeric data, our data suggests that loss of HDAC7 intrinsically predisposes CD8 SP thymocytes to only be able to complete thymic develop by taking on in this innate memory developmental program.

Figure 4

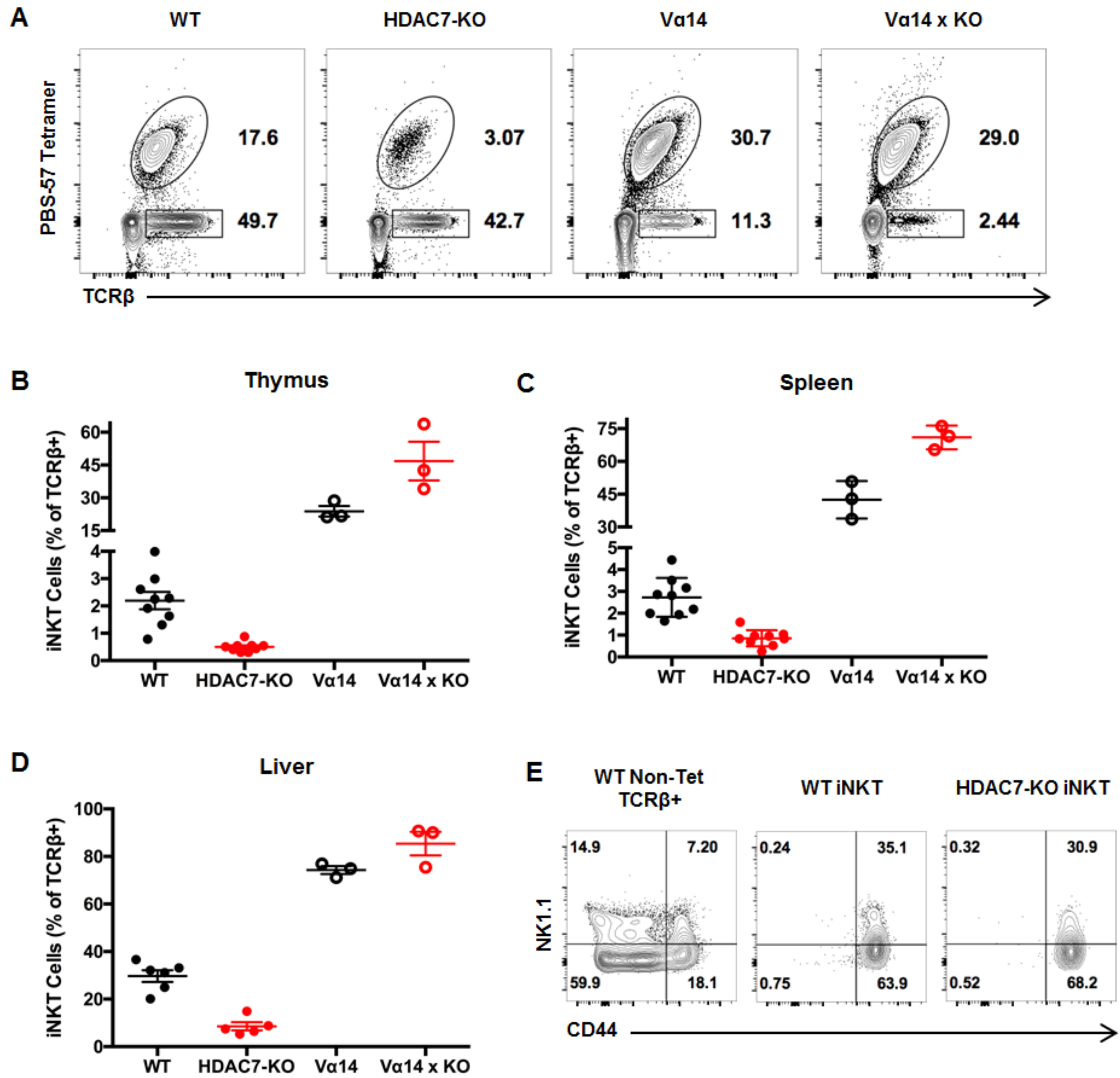


Figure 4. Loss-of-Function HDAC7-KO Reduces iNKTs Numbers But Does Not Impede Innate Effector Development

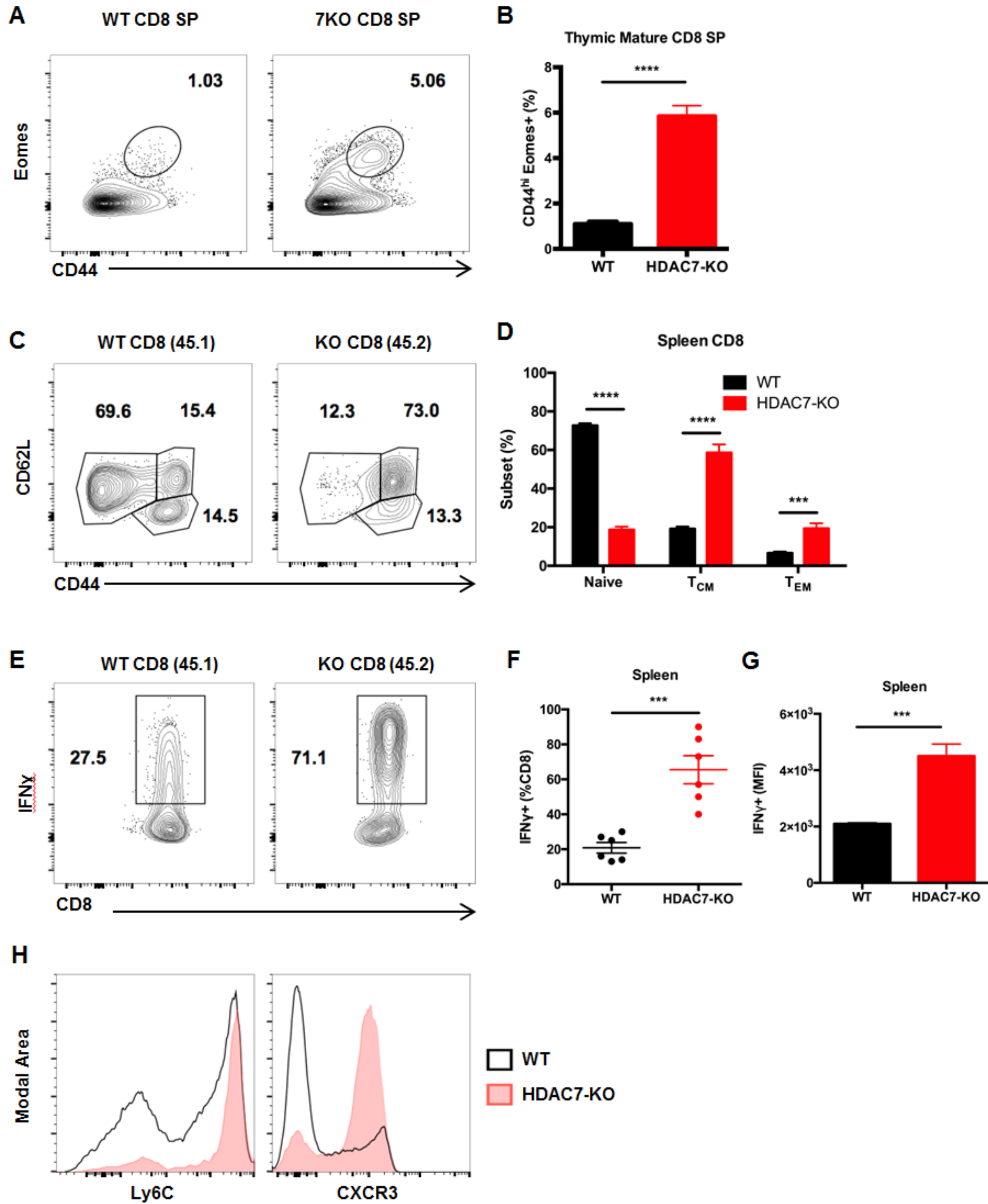
(A) Representative flow cytometric plots demonstrating restoration of iNKT cells (Tet$^+$ TCRβ^+) in HDAC7-KO using the Vα14-Jα18 TCRα transgene. Representative plots shown in (A) with total quantification shown in (B) for age-matched littermate mice.

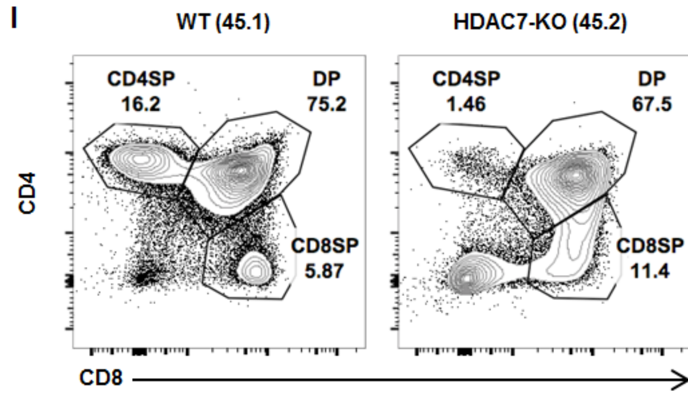
(B-D) Quantification of iNKT cells as a percentage of total TCRβ^+ T-cells in thymus (B), spleen (C), and liver (D). Data are combined from 3 independent experiments.

(E) Representative flow cytometric staining of CD44 and NK1.1 from peripheral iNKT (Tet$^+$ TCRβ^+) T-cells compared with non-tetramer binding (“Non-tet”) TCRβ^+ T-cells. Note that HDAC7-KO iNKT cells appear indistinguishable from WT.

Bars on graphs indicate mean ± SEM (error bars); symbols represent individual mice. Data in (B-D) are combined from 2 independent experiments with at least 2 mice per group. Plots in (E) are representative of at least 3 independent experiments with 2 mice per group.

Figure 5




I
WT (45.1)
HDAC7-KO (45.2)
CD4SP
16.2
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75.2
CD8SP
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CD4SP
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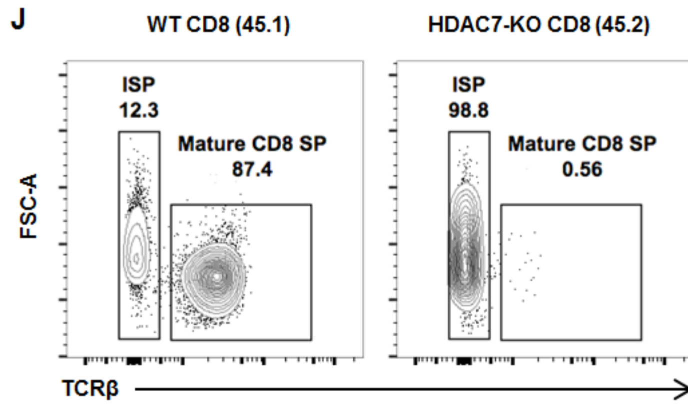
CD8SP
11.4
CD4
CD8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ISP
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Mature CD8 SP
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FSC-A
TCRβ

Figure 5. Loss-of-Function HDAC7-KO Cell Autonomously Generates an Innate Memory CD8 Population

(A, B) Identification of $CD44^{hi}$ $Eomes^{+}$ innate memory CD8 thymocytes expanded in HDAC7-KO mice. Representative plots shown in (C) with quantification shown in (D) for age-matched littermates. Mature CD8 SP T-cells are identified as $TCR\beta^{+}CD8^{+}CD4^{-}$ thymocytes.

(C, D) Representative flow cytometric plots (E) and total quantification (F) of peripheral naive, central memory (T_{CM}), and effector memory (T_{EM}) CD8 T-cell populations from WT (CD45.1) and 7KO (CD45.2) derived bone marrow in irradiated mixed BM chimeras. Naïve T-cells are $CD44^{lo}CD62L^{hi}$, T_{CM} are $CD44^{hi}CD62^{hi}$, and T_{EM} are $CD44^{hi}CD62^{lo}$, all among $TCR\beta^{+}CD8^{+}$ T-cells.

(E, F). Representative flow cytometric plots (E) and total quantification (F) of IFNγ secretion in ex vivo stimulated CD8 T-cells. Splenocytes were harvested from mixed WT (CD45.1) / HDAC7-KO (CD45.2) BM chimeras, stimulated ex vivo for 3 hrs with PMA/Ionomycin followed by 1 hr brefeldin A treatment.

(G) Median fluorescence intensity (MFI) of IFNγ+ secretion in ex vivo-stimulated CD8 T-cells from (F).

(H) Surface expression of Ly6C and CXCR3 from peripheral CD8 T-cells. Black unfilled corresponds to WT, red tinted to HDAC7-KO. Plots are representative of 3 independent experiments with 2-4 mice per group.

(I, J) Representative flow cytometric plots of total thymocytes (I) and CD8 SP thymocytes (J) from WT-derived (CD45.1) and HDAC7-KO derived (CD45.2) bone marrow in irradiated mixed bone-marrow chimeras. Note the near-complete absence of mature ($TCR\beta^{+}$) CD8 SP in HDAC7-KO derived bone marrow.

Bars on graphs indicate mean ± SEM (error bars); symbols represent individual mice. Data in (A, B) are combined from 3 independent experiments with at least 2 mice per group; data in (C-

J) are combined from 3 independent experiments with at least 3 mice per group. Statistical significance was determined using either unpaired two-tailed T-test (B), Two-way (D) or one-way (F, G) ANOVA; ***$p \leq 0.001$, ****$p \leq 0.0001$. Tukey (D) or Bonferroni post-tests (F, G) were used for pairwise comparisons.

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CHAPTER 3

RATIONALE

After discovering a crucial role for HDAC7 in balancing thymocyte development into either the naïve or innate effector lineage by modulating its activity *in vivo,* we next wanted to uncover some mechanistic understanding of how this broad-acting transcriptional corepressor could have such a pronounced effect on such a specific developmental decision. Upon reviewing additional reports from the literature, we were struck by how closely our results mirrored findings reported in similar gain- and loss-of-function studies of the gene *ZBTB16,* encoding for the transcription factor PLZF. Notably, the prominent Stage 1 iNKT block found in gain-of-function *HDAC7-ΔP* resembles the iNKT defect observed in loss-of-function PLZF knockouts (Kovalovsky et al., 2008; Savage et al., 2008). Additionally, the consequences of loss-of-function HDAC7 – notably expansion of $Eomes^{+}$ innate memory $CD8^{+}$ coupled with the appearance of IL-4 secreting $CD4^{+}$ T-cells (Figure 6A) – mirror results reported in gain-of-function PLZF transgenic strains (Kovalovsky et al., 2010; Savage et al., 2011), with the only major difference being a difference in iNKT cell quantity. The PLZF-transgenic strain leaves iNKT development largely intact with peripheral frequency comparable to WT numbers, while our *HDAC7-KO* strain actually has around five-fold fewer iNKT cells as noted previously, but this is due to a secondary effect of HDAC7 on sensitizing thymocyte apoptosis (See Chapter 2). Polyclonal (noninvariant) type II NKT cells are also thought to be PLZF-dependent (Zhao et al., 2014), and we similarly noted a near absence of tissue-resident type II NKTs in *HDAC7-ΔP* mice, defined by a $Tet^{-}TCR\beta^{+}CD8^{-}CD44^{hi}NK1.1^{+}$ profile (Figure 6B and 6C), again highlighting another area of overlap between HDAC7 and PLZF.

Several models could be invoked to explain this remarkable antiparallel phenotypic mirroring. One rather straightforward explanation is a simple hierarchical transcriptional model where nuclear HDAC7 represses the expression of *ZBTB16*, preventing *HDAC7-ΔP* thymocytes from initiating PLZF induction (Seiler et al., 2012). If this were the sole mechanism responsible,

one would predict that there would be greatly diminished, essentially absent PLZF expression in *HDAC7-ΔP* mice. Furthermore, one would also predict that downstream rescue of the missing protein, perhaps using a transgenic line that enforces PLZF expression in all developing thymocytes, should be sufficient to complete rescue the *HDAC7- ΔP* developmental block and restore the iNKT population.

Another possibility, not necessarily mutually exclusive with the previous model of upstream transcriptional repression, is that HDAC7 might act as a functional antagonist of PLZF activity through direct physical binding, converting it from a transcriptional activator to corepressor, or vice versa – most reports examining the mechanism of PLZF activity have suggested that it can act both as activator or repressor depending on cellular context and binding partners (Choi et al., 2014; Kang et al., 2003; Sadler et al., 2015). As HDAC7 has not intrinsic DNA-binding specificity, it requires binding to key molecular targets, often transcription factors, in order to induce any sort of transcriptional regulation while in the nucleus, so direct physical binding to and modulation of PLZF remains another possibility.

RESULTS AND DISCUSSION

PLZF Expression is Absent from T-Lymphocytes in *HDAC7-ΔP* Mice

First we wished to examine the hierarchical transcriptional model where nuclear HDAC7 represses the expression of *ZBTB16*, preventing *HDAC7-ΔP* thymocytes from initiating PLZF induction (Seiler et al., 2012). Indeed, there was a pronounced reduction in PLZF expression in TCRβ$^+$ T-cells from *HDAC7-ΔP* mice in all organs examined, including thymus, spleen and liver (Figures 6D and 6E), which lends some support to the idea that HDAC7 might in part regulate PLZF transcription from its gene locus.

To add a wrinkle to this simple model though, robust PLZF was still detected in CD4$^+$ SP thymocytes from *Vα14 x HDAC7-ΔP* mice; although expression was restricted compared to *Vα14*-only thymus (Figure 7A), PLZF expression was maintained in roughly half of splenic *Vα14 x HDAC7-ΔP* iNKT cells (Figure 7B, right panel). Not all *Vα14 x HDAC7-ΔP* iNKT cells expressed PLZF, and given the reduced (but not absent) expression of PLZF compared to only *Vα14*-only thymus, we suspect that there might be some degree of upstream regulation of HDAC7 on PLZF transcription. However, all *Vα14 x HDAC7-ΔP* iNKT cells were developmentally arrested at Stage 1 (see Chapter 2) despite a large proportion of them expressing PLZF. Overall, this suggests that simple transcriptional repression of *ZBTB16* by HDAC7 is insufficient to explain the iNKT block, even PLZF$^+$ *Vα14 x HDAC7-ΔP* iNKT cells remain naïve-like, Stage 1-arrested T-cells (Figure 3C).

Nuclear HDAC7 is Epistatically Dominant to PLZF Expression

To prove that the absence of iNKT cells did not result from HDAC7-mediated repression of PLZF transcription, we crossed *HDAC7-ΔP* with a *PLZF-Tg* line (Savage et al., 2011) that upregulates PLZF in all DP thymocytes and maintains PLZF in peripheral CD4$^+$ T-cells. Under the simple hierarchical model, this should be sufficient to rescue iNKT innate effector development, but if there are alternate modes of regulation, then the nuclear-localized HDAC7 should be able to antagonize the PLZF transgene and continue blocking iNKT cells,

demonstrating genetic epistasis. This would also support the idea that physical binding of HDAC7 to PLZF can inhibit or antagonize its activity. As PLZF is considered a signature transcription factor of innate effector development, something of a master regulator (Raberger et al., 2008; Savage et al., 2011), we first noted that all $CD4^+$ T-cells in this strain had a $CD44^{hi}$ $CD62L^{lo}$ surface phenotype (Figure 7E). However, in *PLZF-Tg x HDAC7-ΔP* double transgenics, thymic and peripheral iNKT cells remained notably absent while peripheral $CD4^+$ T-cells remained $CD44^{hi}$ $CD62L^{lo}$ (Figure 7E). Note that the *HDAC7-ΔP* transgene turns on at a similar timing pattern as the *Lck*-Cre driver used previously, but then has a CD2 silencing element that essentially turns off expression in the periphery, so it is essentially undetectable in peripheral CD4 T-cells. In contrast, the PLZF transgene is driven by a CD4 promoter with a CD8-specific silencing element, so it maintains peripheral expression only in CD4 T-cells to directly induce an innate effector phenotype. Overall, we showed that enforced PLZF expression was insufficient to overcome the *HDAC7-ΔP* mediated absence of iNKT cells, and we conclude nuclear HDAC7 is epistatically dominant to PLZF expression in blocking innate effector development.

HDAC7 Physically Binds to PLZF as a Negative Corepressor

HDAC7 is a class IIA histone deacetylase that lacks intrinsic DNA binding capacity and requires binding to target transcription factors to modulate transcription at specific loci (Yang and Seto, 2008). Intriguingly, PLZF belongs to the BTB-ZF family of transcription factors (Beaulieu and Sant'Angelo, 2011) previously reported to interact with class IIA HDACs (Chauchereau et al., 2004; Verdin et al., 2003); indeed, one group has even demonstrated *in vitro* and *in vivo* binding of HDAC7 to PLZF in a separate cell type (Lemercier et al., 2002). This suggested that HDAC7 might modulate PLZF activity through direct physical binding. Indeed, though the abundance of PLZF in crude WT thymocytes is fairly low, by taking advantage of the large numbers of PLZF-expressing thymocytes from the previously used *PLZF-Tg* mouse, we

were able to detect a physical interaction between HDAC7 and PLZF in *ex vivo* isolated primary thymocytes via endogenous co-immunoprecipitation and Western blotting (Figure 7F).

After direct binding to a target, class IIA HDACs often act as dominant corepressors to antagonize transcriptional activation, as best understood in the example of MEF2, which is converted from an activator to a repressor upon class IIA HDAC docking (McKinsey et al., 2000). Though the precise mechanism of PLZF transcriptional activity remains unclear, with different domains demonstrating activating and repressive capacities in varying contexts (Melnick et al., 2002; Puszyk et al., 2013; Sadler et al., 2015), we next wanted to examine if HDAC7 physical binding to PLZF could counteract or antagonize its transcriptional activity. Experiments are continuing with other members of the lab, but so far, at least using a GAL4-based transcriptional reporter format, we find that HDAC7 binding-competent mutants of PLZF induce transcriptional repression compared to basal activity in empty vector alone, while HDAC7-binding incompetent mutants induce transcriptional activation (personal communication, H. Lim and H. Kasler). Overall, these data strongly support the notion that physical binding of HDAC7 to PLZF antagonizes its function or inhibits its activity, basically acting as dominant negative binding partner.

The identification of a committed precursor to innate lymphoid cells that transiently expresses high amounts of PLZF (Constantinides et al., 2014) also represents an intriguing cell lineage whose development may be regulated by class IIA HDAC binding. Furthermore, the main mechanism of action we investigate here, HDAC7 antagonism of PLZF via direct interaction, may be generalizable to other members of the BTB -ZF family. Given the primacy of one closely related homolog and known binding target, Bcl6 (Lemercier et al., 2002), in regulating follicular helper T-cell development (Crotty, 2014), a class IIA HDAC/BTB-ZF axis may regulate lymphoid development at additional branch points even outside the thymus.

Additionally, in recent years a number of transcriptional regulators and epigenetic modifiers – including Jarid2, NKAP, HDAC3, and Ezh2 (Dobenecker et al., 2015; Pereira et al.,

2014; Thapa et al., 2013) – have been identified that regulate iNKT ontogeny. At least one member, HDAC3, physically associates with class IIA HDACs as part of a much larger corepressive complex (Fischle et al., 2002). We anticipate that leveraging new advances in ChiP-Seq and other genome-scale approaches will help uncover the complex interplay between these factors and how they coordinately regulate innate effector development, as we suspect that in addition to altered interaction partners, changes in HDAC7 localization will be associated with broad-scale epigenomic modifications and dynamic resetting of the accessible chromatin landscape.

Figure 6

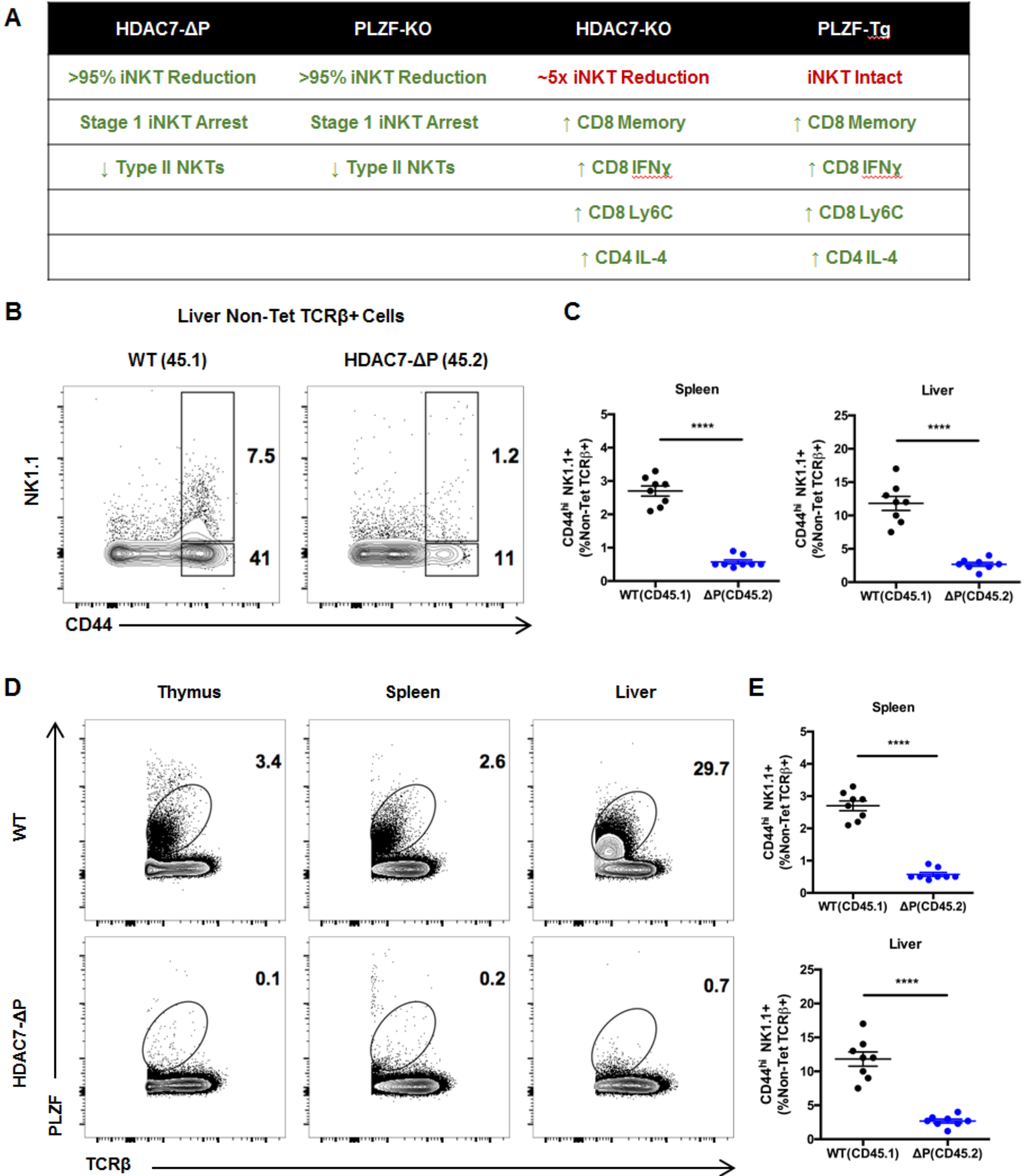

Figure 6. Mutations in *HDAC7* Mirror *ZBTB16* Phenotypes, and Gain-of-Function HDAC7 Results in Loss of PLZF Expression

(A) Summary table comparing phenotypes in HDAC7-ΔP, PLZF-KO, HDAC7-KO and PLZF-Tg mice with respect to iNKT and conventional T-cell development.

(B, C) Representative flow cytometric plots (B) from liver and total quantification (C) from liver and spleen of type II iNKT cells (Tet^{-} CD44^{hi} NK1.1^{+} TCRβ^{+}) in mixed bone-marrow chimeras depending on bone marrow of origin.

(D, E) Representative flow cytometric plots (D) and total quantification (E) of PLZF expression in TCRβ^{+} cells from thymus, spleen, and liver.

Bars on graphs indicate mean ± SEM (error bars); symbols represent individual mice. Data in (C) are combined from a subset of experiments described in Fig 1A-B, with N=8 per group; data in (E) are combined from 4 independent experiments with at least 2 mice per group. Statistical significance was determined using unpaired two-tailed T-tests (B); ***$p \leq 0.001$, ****$p \leq 0.0001$.

Figure 7

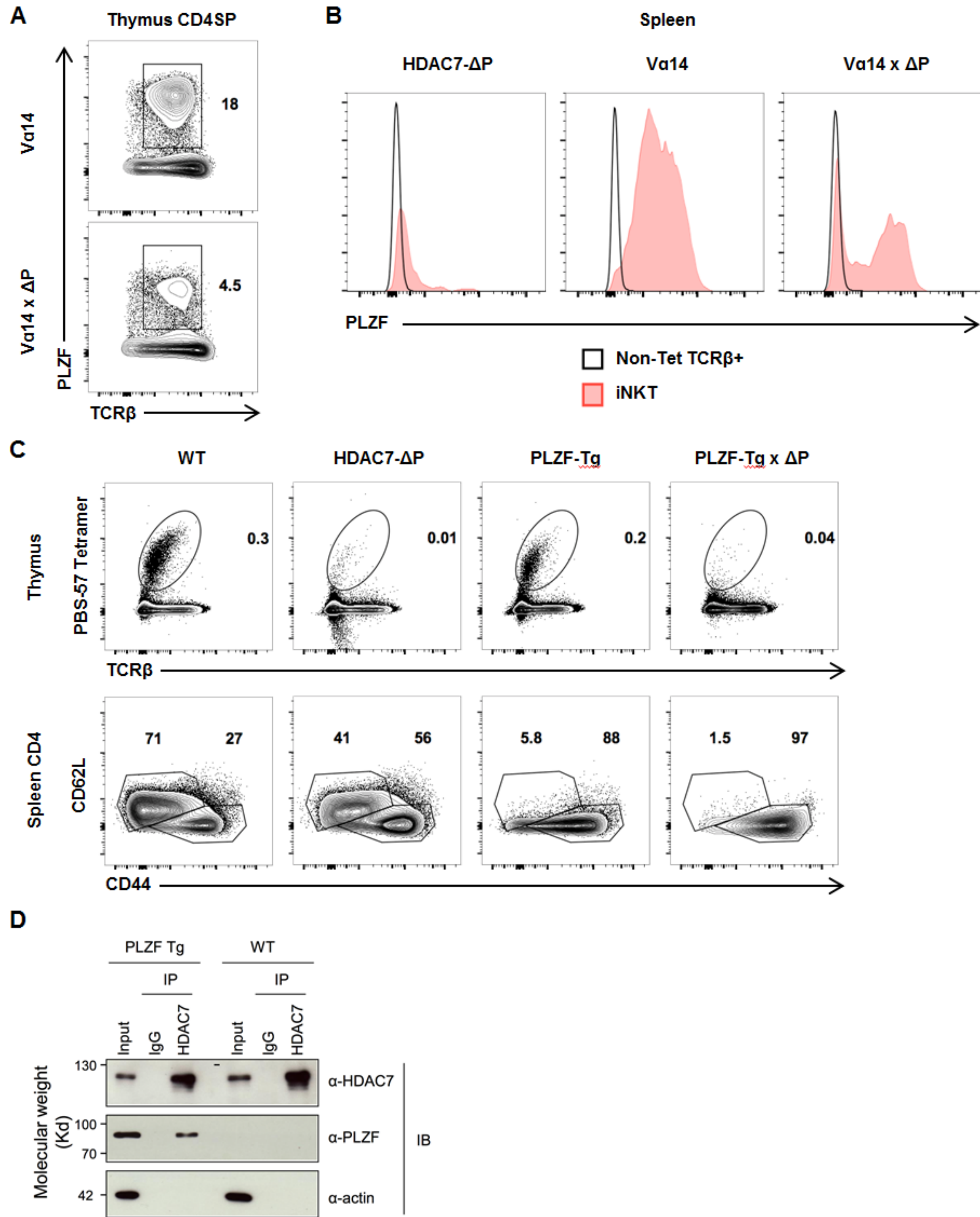

Figure 7. Nuclear HDAC7 Does Not Completely Block Expression of PLZF but is Epistatically Dominant in Arresting iNKT Development

(A) Representative flow cytometric plots of PLZF expression in mature CD4 SP ($CD4^+$ $CD8^-$ $TCR\beta^+$) thymocytes from indicated genotypes.

(B) Representative flow cytometric plots PLZF expression in peripheral iNKT (Tet^+ $TCR\beta^+$) cells from spleen of indicated genotypes. Black unfilled corresponds to conventional ($Tet^-TCR\beta^+$) T-cells, red tinted to iNKT ($Tet^+TCR\beta^+$) cells. Y-axis is normalized to modal area to account for differences in cell number.

(C) HDAC7-ΔP is genetically epistatic to the PLZF-Tg in blocking iNKT development. Thymocyte iNKT cells are Tet^+ $TCR\beta^+$ (top). Continued PLZF expression in the periphery still induces the innate effector phenotype ($CD44^{hi}$ $CD62L^{lo}$) in peripheral CD4 T-cells (bottom) as expected, yet iNKT cells were not rescued by thymic expression of PLZF in HDAC7-ΔP.

(D) Immunoblot demonstrating co-immunoprecipitation of PLZF from HDAC7-directed pulldown. Total WT or PLZF-Tg thymocytes (unisolated) were isolated, lysed in RIPA buffer and complexes probed with a polyclonal HDAC7 antibody.

Data in (A,B) are representative of 3 independent experiments with 2 mice per group; data in (C) are representative of 2 experiments with 1-2 mice per group. Immunoblot in (D) is representative of 3 independent experiments. Statistical significance was determined using unpaired two-tailed T-tests (B); ***$p \leq 0.001$, ****$p \leq 0.0001$.

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CHAPTER 4

RATIONALE

So far, we have demonstrated a critical cellular role for HDAC7 in mediating naïve versus innate effector development in developing thymocytes and begun to probe the molecular mechanism through which this corepressor might exert its function. At a broader level, we also wished to frame our findings from a physiological perspective and investigate whether disruptions in this balance might influence the development or time course of disease. On a very direct level, we think these studies have broad relevance to understanding autoimmune disease, because as we earlier reported, *HDAC7-ΔP* mice develop spontaneous tissue-specific autoimmunity, with about 80% developing obliterative exocrine pancreatitis and concomitant T-cell infiltration in stomach, liver and small intestine within eight months (Kasler et al., 2012). Although this had been previously attributed to a defect in negative selection of conventional thymocytes, the striking absence of iNKT cells in *HDAC7-ΔP* spurred us to consider whether disrupted innate effector development might also contribute to this autoimmune syndrome. Indeed, the very tissues vulnerable to T-cell infiltration in *HDAC7-ΔP*, notably the small intestine, liver and hepatobiliary mucosa, are typically replete with PLZF-dependent innate effectors such as iNKT and MAIT cells (Fan and Rudensky, 2016).

Thus, to modify the previous model slightly, we surmised that this specific autoimmune syndrome could result from a combination of two defects: a block in negative selection of conventional, naïve lymphocytes leading to the escape of autoreactive precursors, and a block in agonist selection of PLZF-dependent innate effectors leading to the loss of iNKTs and other related innate lymphocytes at vulnerable tissue sites, particularly in the gastrointestinal mucosa and hepatobiliary tissues where innate effectors are typically abundant. Depending on cellular context, iNKTs can either promote or inhibit inflammatory responses at the early phase of infection, and though most previous studies have focused on these proinflammatory roles, our model posits that missing iNKT cells in *HDAC7-ΔP* mice could normally have a homeostatic,

protective role, possibly by generating IL-22 or controlling vascular access into privileged tissue sites (Bandyopadhyay et al., 2016; Guidotti et al., 2015; Lu et al., 2016). We thus set out to determine if restoring iNKT cells could modify or palliate the course of autoimmune pathology either completely blocking the onset of disease or at least mollifying its progression and severity. Though we believe that many PLZF-dependent subtypes are probably absent in *HDAC7-ΔP* mice, experimental considerations – particularly given the immense scarcity of other innate effector types such as MAIT cells – restricted us to focus on the restoration of iNKT cells that could be effectively increased to large numbers with the use of the *Vα14* transgene.

RESULTS AND DISCUSSION

Restoring iNKT Cells Ameliorates Tissue-Specific Autoimmunity

As an early attempt, we first tried to restore iNKT cells directly by adoptively transfer large numbers of iNKT cells into *HDAC7-ΔP* recipient to see if the autoimmune outcomes could be improved. However, we quickly found that memory-phenotype innate effectors were extremely inefficient at engrafting into new hosts, and we could not effectively restore tissue resident iNKT populations with this approach (Figure 8A and 8B), as within 3 days post engraftment close to 98% of the transferred cells went missing, likely due to either apoptosis or trapping in the lung vasculature (A. Abbas, personal communication). However, as an alternative approach, we took advantage of our earlier demonstration that *HDAC7-ΔP*-mediated autoimmunity is dominantly transferable in mixed BM chimeras (Kasler et al., 2012). Thus, we generated two sets of chimeras to determine if restoring iNKT cells using *Vα14* bone marrow, which gives very good restoration of iNKT numbers in spleen and liver, could ameliorate disease (Figure 8C) compared to WT bone marrow, which in this highly damaging irradiated setting does not effectively reconstitute iNKT cells. Thus, when irradiated recipients were reconstituted with a 1:5 mixture of *Vα14:HDAC7-ΔP* bone marrow, peripheral iNKT cells were effectively rescued to normal levels, while recipients receiving a 1:5 *WT:HDAC7-ΔP* mixture were not (Figure 8D), giving us two different experimental groups of mice we could compare over time.

Monitoring these cohorts, we noted *Vα14:HDAC7-ΔP* chimeras had significantly lower peak plasma levels of ALT and AST, commonly used as an indication of liver damage, than *WT:HDAC7-ΔP* chimeras (Figure 9A). Both cohorts eventually perished and had equivalent pancreatic lipase levels in plasma (Figure 9B), though the lipase levels were not prominently upregulated compared to healthy WT controls, so the proximate cause of death may not be obliterative pancreatitis but disruptions in some other gastrointestinal or hepatobiliary tissue. At

any rate, *Vα14:HDAC7-ΔP* chimeras exhibited significantly improved body weight maintenance in the first two months post-engraftment (Figure 9C) and a reduced overall mortality rate (Figure 9D) compared to *WT:HDAC7-ΔP* chimeras. These results provide evidence that disruptions in innate effector development, particularly the loss of iNKT cells in the hepatobiliary tract, exacerbates tissue specific autoimmunity in the *HDAC7-ΔP* setting. Restoring this missing innate effector population resulted in significant palliation of the severity and time course of autoimmune pathology, especially in the typically iNKT-replete liver.

Physiological Implications of HDAC7 Control of Thymocyte Development

By restoring the missing iNKT population with the use of *Vα14* donor bone marrow, we significantly attenuated the severity and time course of HDAC7-ΔP mediated autoimmune disease, resulting in improved liver function, better body weight maintenance, and reduced overall mortality. Though iNKT rescue did not categorically block autoimmunity nor seem to provide protection in the pancreas – almost all *Vα14:HDAC7-ΔP* chimeras eventually developed the same autoimmune constellation as *WT:HDAC7-ΔP* chimeras, culminating in exocrine pancreatitis – our studies have nonetheless revealed an important physiological role for dysregulated innate effector development as an exacerbating factor in autoimmunity. Additionally, we note that the HDAC7-PLZF axis influences the development of several non-iNKT innate effector subtypes that were not restored with *Vα14* bone marrow, and most of the benefits in our *in vivo* setting accrued in liver, a major site of iNKT residence. It is tempting to speculate that restoring other subsets – reestablishing MAIT cells in the intestine, for example – might ameliorate tissue destruction and T-cell infiltration in an organ-specific manner.

Innate effector T-cells are often considered frontline first-responders to infection that amplify and orchestrate the early immune response to invading pathogens. Thus, it was somewhat surprising to uncover a protective or anti-inflammatory role for iNKT cells in attenuating tissue destruction, even given the recent discovery of IL-10 producing regulatory

iNKT cells whose development is largely PLZF- independent (Lynch et al., 2015). Additional studies will be required to uncover the mechanisms through which iNKT cells provide protection, but for now we favor a model in which innate effectors occupy homeostatic niches to limit access of other immune cells into specific tissue sites. In *HDAC-ΔP*, escape of autoreactive lymphocytes due to impaired negative selection coupled with the loss of PLZF-dependent innate effectors seems to result in hepatobiliary and gastrointestinal directed T-cell attack. This intriguing “two-hit” model to explain tissue-specific autoimmunity, namely (1) defective tolerance mechanisms resulting in self-reactive lymphocytes coupled with (2) dysregulated tissue access from loss of innate lymphocytes at specific sites, seems amenable to verification in other model systems.

Our findings likely hold immense relevance to understanding the etiology and mechanisms contributing to human autoimmunity. Indeed, common variant single nucleotide polymorphisms (SNPs) in the *HDAC7* gene are significantly associated with human autoimmune and autoinflammatory diseases, namely primary sclerosing cholangitis (Liu et al., 2013) and inflammatory bowel disease (Jostins et al., 2012). Additional common variant SNPs in kinases known to export Class IIA HDACs via phosphorylation, including *SIK2* and *PRKD2*, are also associated with PSC (Liu et al., 2013), suggesting that the broad HDAC7 regulatory network may be a crucial nexus that underlies susceptibility and severity of much of the global autoimmune disease burden. As primary sclerosing cholangitis is a tissue-specific autoimmune disease directed against the hepatobiliary tracts, our discovery that reestablishing missing iNKT cells ameliorates *HDAC7-ΔP* induced hepatic damage also holds important implications in the use of innate effector restoration as a potential therapeutic modality.

Figure 8

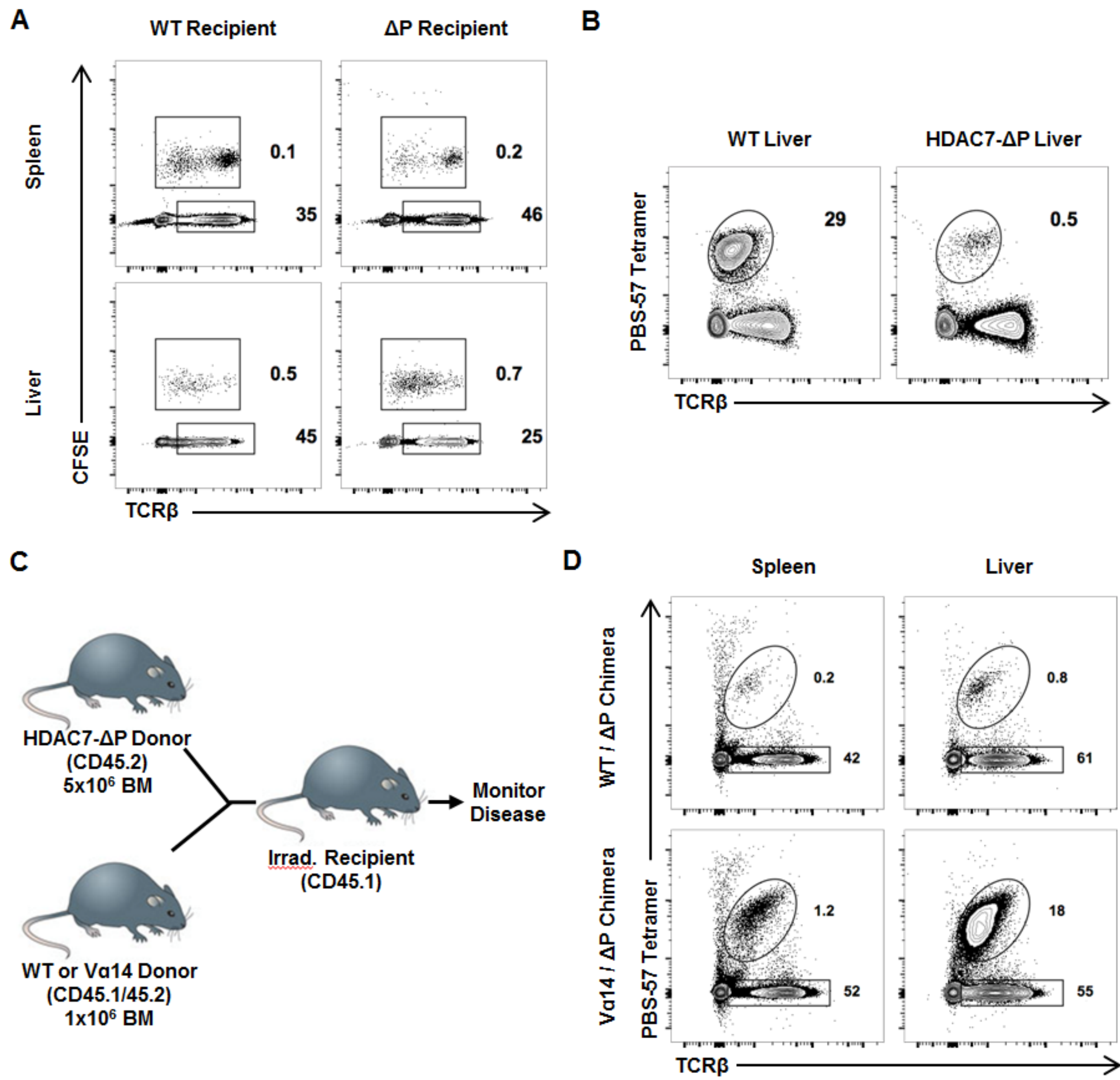

Figure 8. Using *Vα14* bone marrow can effectively restore peripheral iNKT populations in mixed irradiated chimeras.

(A) Identification of CFSE-labeled adoptively transferred T-cells in liver and spleen of WT and HDAC7-ΔP mice 3 days post-retroorbital injection of $5x10^6$ T-cells (iNKT-enriched to 85+%) isolated from spleens and livers of Vα14-Jα18 transgenic mice. Data are representative of two independent experiments, N=3 mice per group total.

(B) Quantification of total iNKT cells from livers of WT and HDAC7-ΔP mice 3 days post-adoptive transfer, described in S7A.

(C) Schematic of the generation of mixed BM chimeras used to monitor HDAC7-ΔP-mediated autoimmunity time course and severity. Lethally irradiated CD45.1 Boy/J recipients were retroorbitally reconstituted ($6x10^6$ cells) with a 1:5 mixture of either WT(CD45.1) / ΔP(CD45.2) or Vα14-Jα18(CD45.1) / ΔP(CD45.2) bone marrow cells.

(D) Vα14-Jα18 bone marrow (bottom) robustly restores peripheral iNKT cells (Tet^+ $TCR\beta^+$ in liver and spleen in mixed BM chimeras, while WT bone marrow does not. Plots are representative of two sets of independent chimera takedowns.

Figure 9

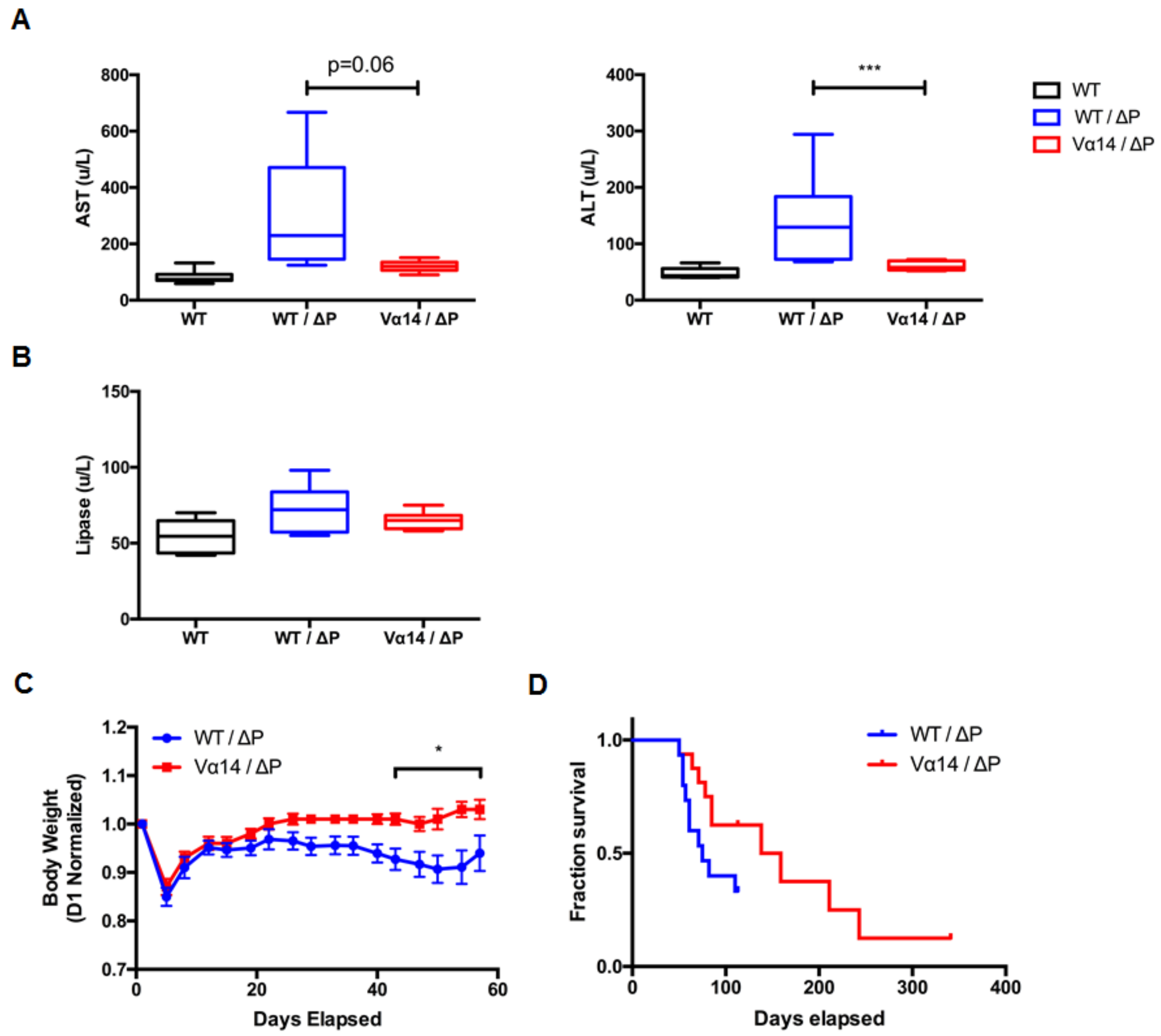

Figure 9. Loss of PLZF-Dependent Innate Effectors in HDAC7-ΔP Contributes to Tissue-Specific Autoimmunity

(A-C) Plasma concentrations of tissue damage markers, including liver (AST and ALT; A) and pancrease (lipase; B) over time measured in WT mice compared to Vα14-Jα18/ΔP and WT/ΔP BM chimeras. Plasma was collected and measured monthly over 4 months and the peak values reached per individual mouse are plotted.

(C, D) Body weight and survival of mixed BM chimeras over time post-irradiation. Weights in (C) were normalized to starting weight on Day 1 post-irradiation and measured twice a week thereafter. Survival (D) was assessed by monitoring for spontaneous death twice a week or by euthanization after reaching a clinical endpoint of at least 20% body weight loss compared to peak weight post-irradiation.

Bars on graphs indicate mean ± SEM (error bars); whiskers on box-and-whiskers plots represent min to max. Data in (C) were collected from N=6 mice per group; data in (D) and (E) were combined from three independent experiments with N=16 mice total per group. Statistical significance was determined using two-way ANOVA (C, D); $^*p \leq 0.05$. Bonferroni post-tests were used for pairwise comparisons. Using Kaplan-Meier analysis, $p = 0.0343$ by Log-rank and $p=0.0446$ by Gehan-Breslow-Wilcoxon tests in (D).

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