CD5L/AIM Regulates Lipid Biosynthesis and Restrains Th17 Cell Pathogenicity

Highlights

- CD5L is preferentially expressed in non-pathogenic Th17 cells
- CD5L is a major switch of Th17 cell functional states in vivo
- CD5L regulates T cell lipidome in correlation with Th17 cell function
- CD5L alters T cell function through PUFA/SFA balance and Rorγt ligand availability

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In Brief

CD5L operates as a critical switch of Th17 cells functional states, regulating their ability to cause disease through changes in lipid metabolism and function of the master transcription factor Rorγt.

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CD5L/AIM Regulates Lipid Biosynthesis and Restrains Th17 Cell Pathogenicity

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SUMMARY

Th17 cells play a critical role in host defense against extracellular pathogens and tissue homeostasis but can induce autoimmune immunity. The mechanisms implicated in balancing “pathogenic” and “non-pathogenic” Th17 cell states remain largely unknown. We used single-cell RNA-seq to identify CD5L/AIM as a regulator expressed in non-pathogenic, but not in pathogenic Th17 cells. Although CD5L does not affect Th17 differentiation, it is a functional switch that regulates the pathogenicity of Th17 cells. Loss of CD5L converts non-pathogenic Th17 cells into pathogenic cells that induce autoimmunity. CD5L mediates this effect by modulating the intracellular lipidome, altering fatty acid composition and restricting cholesterol biosynthesis and, thus, ligand availability for Rorγt, the master transcription factor of Th17 cells. Our study identifies CD5L as a critical regulator of the Th17 cell functional state and highlights the importance of lipid metabolism in balancing immune protection and disease induced by T cells.

INTRODUCTION

IL-17-producing Th17 cells are present at tissue inflammation sites and contribute to the pathogenesis of human autoimmune diseases and relevant murine models (Kleineveldt and Hafler, 2013; Lee et al., 2014). However, not all Th17 cells induce tissue inflammation and disease (i.e., are “pathogenic”). Th17 cells that line the normal gut mucosa regulate tissue homeostasis by preventing invasion of gut microflora and promoting epithelial barrier functions (Gugliani and Khader, 2010). In addition, Th17 cells play a crucial role in host defense against pathogens such as fungi (Candida albicans) and extracellular bacteria (Staphylococcus aureus) (Gaffen et al., 2011; Romani, 2011). Therefore, Th17 cells exhibit great diversity in their function. The extracellular signals and intracellular mechanisms that control these distinct functions of Th17 cells in vivo have not been identified.

Th17 cells with distinct effector functions can also be generated in vitro by different cytokine combinations. We (Bettelli et al., 2006) and others (Mangan et al., 2006; Veldhoen et al., 2006) found that two cytokines, IL-6 and TGF-β1, can differentiate naive T cells into Th17 cells in vitro, although these cells are poor inducers of experimental autoimmune encephalomyelitis (EAE), an autoimmune disease model for human multiple sclerosis. Exposure to the pro-inflammatory cytokine IL-23 can transform these cells into disease-inducing pathogenic Th17 cells (Awasthi et al., 2009; Cua et al., 2003; Jäger et al., 2009; Langrish et al., 2005; McGeachy et al., 2007; McGeachy et al., 2009). Other cytokine combinations such as IL-1β+IL-6+IL-23 (Chung et al., 2009; Ghoreschi et al., 2010) or TGF-β3+IL-6+IL-23 (Lee et al., 2012) can also induce Th17 cells that elicit EAE with severe tissue inflammation. Comparing gene expression profiles of Th17 cells generated with distinct in vitro differentiation protocols led to the identification of a signature that distinguishes pathogenic from non-pathogenic Th17 cells (Lee et al., 2012), consisting of 16 pro-inflammatory genes expressed in pathogenic Th17 cells (e.g., T-bet, GMCSF and IL-23R) and 7 regulatory genes in non-pathogenic cells (e.g., IL-10). Exposure of non-pathogenic Th17 cells to IL-23 converts them into a pathogenic phenotype, with diminished expression of the regulatory signature and with acquisition of the pro-inflammatory signature.

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genes, suggesting that IL-23 is a master cytokine that dictates the functional phenotype of Th17 cells.

In humans, two subtypes of Th17 cells were described with specificity for different pathogens. Th17 cells that co-produce IL-17 with IFN-γ were generated in response to Candida albicans, whereas Th17 cells that co-produce IL-17 with IL-10 have specificity for Staphylococcus aureus infection (Zielinski et al., 2012). Both IL-1 and IL-23 can differentially affect the development of distinct Th17 subtypes in humans. Comparison of the human Th17 subsets with Th17 cells in mice suggests that the C. albicans-specific Th17 cells may mirror the pathogenic Th17 cells we and others have described in mouse models of autoimmunity with expression of proinflammatory signature genes, whereas S. aureus-specific Th17 cells are more similar to the non-pathogenic Th17 cells.

Identifying specific molecular switches that drive pathogenic versus non-pathogenic Th17 cells will allow selective inhibition of pathogenic Th17 cells while sparing non-pathogenic, potentially tissue-destructive Th17 cells. To date, the intracellular mechanisms by which IL-23 evokes the pathogenic phenotype in differentiating Th17 cells are not well understood. Genomic approaches provide a compelling unbiased method to find such mechanisms (Wu et al., 2013; Yosef et al., 2013), but it is likely that pathogenic and non-pathogenic cells co-exist in vivo and co-differentiate in vitro, limiting our power to detect subtler signals on a population level. Indeed, our previous study comparing populations of in-vitro-derived pathogenic and non-pathogenic cells (Lee et al., 2012) did not find strong candidate regulators but, rather, effector molecules. The advent of single-cell RNA-seq (Ramsköld et al., 2012) opens the way to identify such subtler yet physiologically important regulators.

Here, we used single-cell RNA-seq profiles of Th17 cells from in vivo autoimmune lesions and from in vitro differentiation (Gaublomme et al., 2015, this issue of Cell) and identified a novel regulator of Th17 pathogenicity, CD5L (CD5L). CD5L is predominantly expressed in non-pathogenic Th17 cells and is downregulated upon exposure to IL-23. Loss of CD5L converges non-pathogenic Th17 cells into disease-inducing Th17 cells by regulating the Th17-cell lipidome. CD5L decreases the level of polyunsaturated fatty acids (PUFA), affecting the expression of key cholesterol biosynthesis enzymes and, in turn, affecting the binding and activity of RORγt, the master transcription factor of Th17-cell differentiation. Thus, we discovered CD5L as a critical regulator that distinguishes Th17 functional states and identified T cell lipid metabolism as an integral component of the pathways regulating Th17 cell pathogenicity.

RESULTS

Single-Cell RNA-Seq Identifies CD5L as a Candidate Regulator of Pathogenicity

To identify regulators of Th17 cell function, we analyzed single-cell RNA-seq profiles of Th17 cells isolated from the central nervous system (CNS) during EAE in vivo or differentiated in vitro under non-pathogenic (TGF-β1+IL-6) and pathogenic (IL-1β+IL-6+IL-23) conditions (Gaublomme et al., 2015). We used three lines of evidence to rank genes for their potential association with pathogenicity: (1) transcript’s correlation with the first principal component (PC) of single Th17 cells differentiated in vitro (TGF-β1+IL-6), which showed the presence of two anti-correlated modules: a “pro-inflammatory module” (positively correlated with Il17a expression) and a “regulatory module” (positively correlated with Il10 expression); (2) co-variance of transcripts in vitro (TGF-β1+IL-6) with a cell pathogenicity score, defined as the difference in the average expression of genes from our previously defined pro-inflammatory and regulatory signatures (Lee et al., 2012); and (3) transcript’s correlation with the first two PCs of single Th17 cells isolated from the CNS and lymph node during EAE in vivo. These showed that Th17 cells span a functional spectrum along the first PC (from effector, memory to exhausted state) and the second PC (from a naive-like to terminally differentiated state).

Cd5l is one of the high-ranking genes by single-cell analysis of potential regulators, exhibiting two surprising features: although Cd5l is expressed in Th17 cells derived under non-pathogenic conditions (Figure 1A), in these non-pathogenic cells, Cd5l positively correlates with the first PC of in-vitro-derived cells and co-varies with other genes in the pro-inflammatory module (Figures S1A–S1C). In addition, Cd5l positively correlates with the cell pathogenicity score (Figures 1B and 1C). Comparing Cd5l expression at the single-cell level in Th17 cells (sorted IL-17.GFP+) derived in vitro showed that ~80% of Th17 cells derived with IL-1β+IL-6+IL-23 lacked Cd5l expression, whereas Th17 cells differentiated with TGF-β1+IL-6 predominantly expressed Cd5l (Figure 1A). Neither Th17 cells differentiated under an alternative pathogenic condition (TGF-β2+IL-6), nor did encephalitogenic Th17 cells sorted from the CNS of mice undergoing active EAE expressed Cd5l at the single-cell level.

Figure 1. CD5L Is a Candidate Regulator of Th17 Cell Functional States

(A–C) Single-cell RNA-seq analysis. (A) Cd5l expression of single cells from in-vitro-generated and in-vivo-sorted Th17 cells (IL-17.GFP+) from mice at the peak of EAE. (B) and (C) Correlation of Cd5l expression in non-pathogenic Th17 cells (TGF-β1+IL-6) with (B) the cell pathogenicity score (based on the pathogenic signature of Lee et al., 2012); p = 2.63 × 10⁻⁴ (Wilcoxon rank-sum test, comparing signature scores of Cd5l expressing versus non-expressing cells). (C) The founding signature genes of the single-cell-based proinflammatory (red) and regulatory (green) modules (solid bars, significant correlation [p < 0.05]; striked bars, non-significant correlation).

(D–F) Validation of CD5L expression in vitro. Naive T cells (CD4⁺CD62L⁺CD44⁻CD25⁻) were sorted and differentiated as indicated and analyzed by qPCR for CDS expression at 48 (D) and 72 hr (E) and by flow cytometry at 48 hr (F). (E) IL-23 or control was added at 48 hr in fresh media. Figures are summary of three independent experiments.

(G–I) Validation of Cd5l expression in vivo. (G and H) IL-17A.GFP reporter mice were immunized to induce EAE. Cells were sorted from spleen (G) and CNS (H) at the peak of disease. Cd5l and Il17a expression are measured by qPCR. Figure shown is representative data of three technical replicates from two independent experiments. (I) Cells were sorted from the gut of naive mice and the number of RNA transcripts measured by nanostring nCounter platform (Supplemental Experimental Procedures).

See also Figure S1.
(Figure 1A). However, Cd5l expressed in non-pathogenic Th17 cells (unsorted single-cell analysis, Figure S1A) correlates with the first PC and co-varies with the pro-inflammatory module (Figure S1B) that is indicative of the pathogenic signature (Figure S1C), as previously defined (Lee et al., 2012). Furthermore, Cd5l correlates with the defining signature of the pro-inflammatory module and negatively correlates with that of the regulatory module (Figure 1C). Finally, it is among the top eight genes in the single-cell-based pro-inflammatory module whose expression most strongly correlates with our previously defined pathogenic gene signature (Figure 1B, p = 2.63 × 10⁻⁵).

Cd5l is a member of the scavenger receptor cysteine-rich superfamily (Sarrias et al., 2004). It is expressed in macrophages and can bind cytosolic fatty acid synthase in adipocytes following endocytosis (Miyazaki et al., 1999). Cd5l is also a receptor for pathogen-associated molecular patterns (PAMPs) and may regulate innate immune responses (Martinez et al., 2014). However, its expression has not been reported in T cells, and its role in T cell function has not been identified.

Cd5l Expression Is Associated with Non-pathogenic Th17 Cells In Vitro and In Vivo

We hypothesized that the preferential expression of Cd5l in non-pathogenic Th17 cells, but in association with the pro-inflammatory module, may reflect a unique role for Cd5l in regulating the transition between a non-pathogenic and pathogenic state. While co-expression with the pro-inflammatory module (Figure 1C) and correlation with a pathogenicity signature (Figure 1B) per se could suggest a function as a positive regulator of pathogenicity, the apparent absence of Cd5l from Th17 cells differentiated in vitro under the pathogenic conditions or isolated from lesions in the CNS (Figure 1A) suggested a more nuanced role. We hypothesized that Cd5l is a negative regulator of pathogenicity, explaining its absence from truly pathogenic cells. In fact, mRNAs encoding negative regulators of cell states are often positively co-regulated with the modules they suppress (Figures 2 A and S2B). This suggests that increased severity of the disease was not due to changes in the number of Tregs in Cd5l⁻/⁻ mice (Figure S2A). In contrast, more CD4⁺ T cells produced IL-17, and fewer cells produced IFNγ in the CNS of Cd5l⁻/⁻ mice (Figures 2A and S2B). In response to MOG reactivation in vitro, cells from the draining lymph nodes of Cd5l⁻/⁻ mice showed higher proliferative responses and produced more IL-17 (Figures S2C and S2D). These observations are consistent with either a direct or indirect role for Cd5l in defining Th17 cell function.

We studied the impact of Cd5l on Th17 cells differentiated from naive WT and Cd5l⁻/⁻ T cells by analyzing signature gene expression. Cd5l deficiency did not affect Th17 differentiation as measured by IL-17 expression (Figures 2B and 2C), nor did it affect other Th17 signature genes, including Il17f, Il21, Il23r, Rorc, or Rorα (Figure 2D). Of note, under the non-pathogenic differentiation condition, Cd5l⁻/⁻ Th17 cells made less IL-10 (Figures 2C and 2D). These observations suggest that changes in differentiation alone cannot explain the increased susceptibility to EAE in Cd5l⁻/⁻ mice but that Cd5l may indeed affect the internal state of differentiated Th17 cells.

We determined whether Cd5l regulates effector/memory Th17 cells by differentiation of non-pathogenic Th17 cells from naive cells. Upon restimulation, more Cd5l⁻/⁻ Th17 cells produced IL-17 and expressed IL-23R without affecting viability (Figure 2E and data not shown), suggesting that Cd5l deficiency leads to more stable expansion of Th17 cells. Consistently, Cd5l⁻/⁻ Th17 cells expressed more Il17 and Il23r, less Il10, and similar levels of Rorc or Rorα (Figure 2F). Thus, Cd5l does not regulate Th17 cell differentiation but affects Th17 cell expansion and/or effector functions over time. Similarly, effector
Figure 2. CD5L Represses Effector Functions without Affecting Th17 Cell Differentiation

(A) EAE was induced by MOG/CFA (40 μg) immunization. Left panel is pooled results from three independent experiments. (Right) Cytokine profile of CD4 T cells isolated from CNS at day 15 post immunization with summary data in Figure S2B.

(B–D) Naive splenic T cells were sorted and differentiated with TGF-β1+IL-6 for 48 hr. Th17 cell signature genes were measured by flow cytometry (B), ELISA (C), and qPCR (D).

(E and F) Effector Th17 cells were differentiated as in (B) and resuspended in fresh media with no cytokines for 72 hr followed by restimulation. Gene profile was measured by flow cytometry (E) and qPCR (F).

(G and H) Effector memory T cells (CD4+CD62L–/CD44+) (G) or Effector memory Th17 cells (CD4+CD62L–CD44+RorγtGFP+) (H) were sorted from spleen of naive mice and activated with TCR stimulation. All figures are summary of three independent experiments unless otherwise specified. See also Figure S2.
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A TGFβ1+IL-6

B

C

D

E

F

CD5L<sup>-/-</sup> / WT (non-pathogenic)

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memory cells (CD4+CD62L−CD44+) isolated ex vivo from CD5L−/− mice have higher frequencies of IL-17 and lower frequencies of IL-10 cells (Figures 2G and S2E), possibly reflecting the greater stability of Th17 cells that persist in the repertoire of CD5L−/− mice. To address whether Th17 cells isolated in vivo also produced more IL-17 per cell, we sorted RORγt+ (GFP+) effector/memory T cells from WT and CD5L−/− mice and found more IL-17+ and fewer IL-10+ cells in CD5L−/− cells, suggesting that RORγt+ cells are better IL-17 producers in the absence of CD5L (Figure 2H and S2F).

**CD5L Is a Major Switch that Regulates Th17 Cells Pathogenicity**

To determine whether loss of CD5L can convert non-pathogenic Th17 cells into disease-inducing Th17 cells, we crossed CD5L−/− mice to 2D2 transgenic mice expressing a T cell receptor specific for MOG35-55/IAb (Bettelli et al., 2003). Naive CD5L−/− 2D2 T cells were differentiated with the non-pathogenic (TGF-β1+IL-6) Th17 condition and transferred into WT recipients. A similar frequency of IL-17+ T cells was generated from WT and CD5L−/− 2D2 naive cells (Figure 3A), consistent with the observation that CD5L does not affect Th17 differentiation.

We compared clinical and histological disease progression in the recipients of WT and CD5L−/− 2D2 cells. As expected, recipients (6 out of 13) of WT 2D2 Th17 cells showed little sign of EAE. Strikingly, all (12 out of 12) CD5L−/− 2D2 recipients developed severe EAE with optic neuritis, had significant weight loss, and developed more ectopic lymphoid follicle-like structures in the CNS (Figures 3B and 3C), a hallmark of disease induced by pathogenic IL-23-treated Th17 cells (Peters et al., 2011). Thus, T-cell-intrinsic expression of CD5L plays a pivotal role in restraining Th17 cell pathogenicity. We analyzed the phenotype of T cells from the CNS of mice undergoing EAE. The 2D2 CD5L−/− Th17 cells retained more IL-17+ and fewer IL-10+ cells (Figures 3D and S3A). A considerable proportion of endogenous T cells produced IL-10 compared to transferred 2D2 T cells (Figure S3A), suggesting that extracellular IL-10 is not sufficient to restrain the pathogenicity of CD5L−/− Th17 cells. WT 2D2 T cells also acquired IFNγ expression in vivo, whereas CD5L−/− 2D2 T cells produced little IFNγ, suggesting that CD5L may also regulate Th17 cell stability. Consistently, naive CD5L−/− 2D2 T cells transferred into WT hosts immunized with MOG35-55/CFA without inducing EAE made more IL-17 and little IL-10 in contrast to WT 2D2 T cells (Figures 3E and S3B).

As IL-23 suppresses CD5L (Figure 1E) and CD5L restrains Th17 cell pathogenicity, we reasoned that sustained CD5L expression should antagonize IL-23-driven pathogenicity. To test this hypothesis, we generated a retroviral vector for ectopic expression of CD5L. Naive 2D2 T cells were differentiated with IL-1+IL-6+IL-23, transduced with CD5L, transferred into WT recipients, and followed for weight loss and the development of clinical EAE (Experimental Procedures). 2D2 T cells transduced with CD5L (CD5L-RV 2D2) had a small reduction in IL-17 and higher IL-10 levels (Figure S3C). Ectopic expression of CD5L in pathogenic Th17 cells reduced their pathogenicity, as CD5L-RV 2D2 recipient mice had reduced weight loss and a significant decrease in the incidence and peak severity of EAE (Figures S3D and S3E). Furthermore, CD5L-RV 2D2 Th17 cells transferred in vivo lost IL-17 production and began producing IFNγ (Figure S3F). Therefore, sustained expression of CD5L in pathogenic Th17 cells converts them to a less pathogenic and less stable phenotype in that these cells lose the expression of IL-17 and acquire an IFNγ-producing phenotype in vivo. This observation, combined with the observation that the loss of CD5L converts non-pathogenic Th17 cells into pathogenic Th17 cells in vivo, unequivocally supports the role of CD5L as a negative regulator of the functional pathogenic state of Th17 cells.

Consistent with our functional findings, CD5L regulates expression of several pathogenicity signature genes. We differentiated naive WT and CD5L−/− T cells with TGF-β1+IL-6. CD5L−/− Th17 cells upregulated several effector molecules of the pathogenic signature, including Il23r, Il13, Ccl4, GzmB, Lrmp, Lag3, and Sgk1 and downregulated several non-pathogenic signature genes, including Il10, Il9, and Maf as compared to WT Th17 cells (Figure 3F). Several other signature genes were not affected by the loss of CD5L, suggesting a more nuanced mechanism.

**CD5L Shifts the Fatty Acid Composition in Th17 Cell Lipidome and Restricts Cholesterol Synthesis, an Endogenous Source of Rorγt Ligand**

As CD5L regulates lipid metabolism by binding to fatty acid synthase in the cytoplasm of adipocytes (Kurokawa et al., 2010), we hypothesized that CD5L could regulate Th17 cell function by regulating fatty acid (FA) profiles in T cells. We asked whether lipid metabolites are regulated by CD5L and whether any such changes are associated with the increased pathogenicity of CD5L−/− Th17 cells. We profiled the lipidome of WT and CD5L−/− Th17 cells differentiated under the non-pathogenic (TGF-β1+IL-6) and pathogenic (TGF-β1+IL-6+IL-23) conditions using a non-targeted approach. We detected 178 lipid metabolites from Th17 cells, 39 of which showed differences among various Th17-polarizing conditions (Figures 4A and S4A; p < 0.05, fold change > 1.5; Table S1). Strikingly, non-pathogenic
WT Th17 cells had a unique lipidome profile that was distinct from those of CD5L⁻/⁻ Th17 cells and WT Th17 cells differentiated with TGF-β1+IL-6+IL-23 (Figures 4A and S4A).

We analyzed the FA profile and lipid class in the Th17 cell lipidome. As we did not detect free FA except myristic acid, we analyzed the FA content (side chain) of the lipids in Figure 4A. WT non-pathogenic Th17 cells (compared to CD5L⁻/⁻ Th17 cells of the same conditions) have increased polyunsaturated fatty acid (PUFA), accompanied by a decrease in lipids containing saturated (SFA) and monounsaturated fatty acids (MUFA) (Figure 4B). We then extended our analysis to the 178 lipids detected. Not all PUFA are different in WT versus CD5L⁻/⁻ Th17 cells: linoleic acid (C18:2) and linolenic acid (C18:3) are equally distributed in the lipidome, whereas downstream PUFA—in particular, arachidonic acid (C20:4)—are elevated in WT non-pathogenic Th17 cells (Figure S4C). In contrast, MUFA is equivalently distributed, and the corresponding SFA is decreased in WT non-pathogenic Th17 cells (Figure S4C). The PUFA increase in WT non-pathogenic Th17 is equivalently distributed among the phospholipid and neutral lipid compartments (Figure 4C), whereas the relative decrease of SFA is only significant in phospholipid (Figure 4C). Finally, comparing the difference in specific lipid species (Figure S4D), we found a higher level of cholesterol ester (CE), lyso phosphatidylcholine (LPC), and phosphatidylcholine (PC), as well as decreased triacylglyceride (TAG) in both the CD5L⁻/⁻ and more pathogenic cells (Figure S4D). Taken together, these findings suggest that CD5L predominantly regulates FA composition in Th17 cells, resulting in elevation of PUFA and changes in specific lipid species, including cholesterol metabolites. Similar changes are also observed in WT Th17 cells differentiated under the pathogenic condition.

Cholesterol metabolites, such as oxysterols, can function as agonists of Rorγt (Jin et al., 2010; Soroosh et al., 2014), and found the cholesterol synthesis pathway has been linked to the production of endogenous Rorγt ligand. While we did not detect any oxysterols or intermediates of cholesterol synthesis, the higher level of cholesterol esters (Figure S4D) prompted us to further investigate the cholesterol pathway.

We confirmed the higher intensity of free cholesterol in CD5L⁻/⁻ Th17 cells using microscopy (Figure S4E). Next, we analyzed the expression of cyp51 and sc4mol, two enzymes of the cholesterol synthesis pathway responsible for generating endogenous Rorγt ligands (Santori et al., 2015), and found both increased in CD5L⁻/⁻ Th17 cells or in pathogenic WT Th17 cells (Figure 4D), suggesting that this may be a common mechanism by which Th17 cells regulate their function.
We asked if the change in FA profile in CD5L−/− Th17 cells is responsible for the regulation of cyp51 and sc4mol. Indeed, while SFA had a modest effect, PUFA abolished the increased expression of the enzymes in CD5L−/− Th17 cells (Figure 4D). Thus, CD5L can regulate fatty acid composition in Th17 cells and alter the cholesterol synthesis pathway, a source of Rorγt ligand.

**CD5L and PUFA/SFA Profile Regulate Rorγt Function in a Ligand-Dependent Manner**

We analyzed whether CD5L and the PUFA/SFA profile can alter Rorγt binding and function. Our previous chromatin immunoprecipitation (ChiP)-Seq analysis (Xiao et al., 2014) suggested that Rorγt binds at several sites in the promoter and intronic regions of Il23r and Il17 and near CNS-9 of Il10 (Figure S5A), where other Il10-regulating transcription factors, such as cMaf, also bind (Xiao et al., 2014). As CD5L restraints IL-17 and promotes IL-10 in Rorγt+ Th17 cells (Figure 2H) and CD5L−/− Th17 cells have more cholesterol metabolites and lower PUFA (Figures 4A, 4B, S4D, S4E), we hypothesized that CD5L regulates the expression of IL-23R, IL-17, IL-10, and, in turn, pathogenicity by affecting the binding of Rorγt to these targets by changing the SFA/PUFA profile and cholesterol biosynthesis.

We assessed whether CD5L regulates Rorγt binding and transcription using ChiP-PCR and luciferase reporter assays. ChiP of Rorγt showed higher binding in the Il17 and Il23r region and reduced binding to the Il10 region in CD5L−/− Th17 cells despite similar Rorγt expression compared to WT (Figures 5A, 5B, and SSB). Further, CD5L overexpression was sufficient to suppress Rorγt-dependent transcription of Il17 and Il23r luciferase reporters (Figures 5C and S5C) and to enhance the transcription of the Il10 reporter (Figure S5D). This effect of CD5L is not observed with PPARγ, another regulator of Il10, further supporting the hypothesis that the effect of CD5L depends on Rorγt (Figure S5E).

We then examined whether changing the lipidome of WT Th17 cells with exogenous SFA or PUFA can regulate Rorγt binding to genomic regions (Figures 5A, 5B, and S5C). SFA enriched binding of Rorγt at Il17 and Il23r loci, and PUFA decreased such binding (Figures 5A and S5B). Instead, PUFA increased Rorγt binding to the Il10 CNS-9 locus (Figure 5B), suggesting that manipulation of the lipid content of Th17 cells can indeed modulate Rorγt binding to DNA.

We reasoned that, if CD5L regulates Rorγt transcriptional activity by limiting Rorγt ligand, adding exogenous agonists of Rorγt would rescue CD5L-induced suppression. Indeed, 7β, 27-dihydroxycholesterol, previously shown as an endogenous ligand of Rorγt (Soroosh et al., 2014), rescued the CD5L-driven suppression of Il17 reporter transcription, suggesting that ligand availability partly contributes to the regulation of Rorγt function by CD5L (Figure 5D). Consistently, CD5L inhibited IL-17 expression in unpolarized Th0 cells with ectopic Rorγt expression, and this inhibition could be partially rescued by the addition of a Rorγt ligand (Figure 5E). Addition of Rorγt ligand also increased IL-17 production from non-pathogenic Th17 cells (Figure 5F), suggesting that ligand restriction may be one of the mechanisms...
Figure 6. PUFA/SFA Regulate Th17 Cell Function and Contribute to CD5L-Dependent Regulation of Th17 Cells

(A and B) Naive T cells were sorted from WT or IL-23RGFP reporter mice (A) or WT or Rorc<sup>-/-</sup> mice (B) and were differentiated with TGF-β1+IL-6 followed by addition of IL-23 at 48 hr in fresh media with either PUFA (arachidonic acid, 10 μM) or SFA (palmitic acid, 20 μM). Cells were analyzed by flow cytometry (A) or qPCR (B) at 96 hr. The concentration of free fatty acids was predetermined in titration experiments (data not shown).

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by which CD5L regulates Th17 cell pathogenicity. We then determined whether SFA/PUFA regulate Rorγt activity through Rorγt ligand. While Rorγt strongly transactivates the Il23r enhancer in the presence of an agonistic ligand, the addition of PUFA to the agonist ligand inhibited Rorγt-mediated Il23r transactivation and enhanced Il10 transactivation (Figures SSF and SSG). Similarly, adding SFA alone had little impact on Rorγt-dependent transcription, but it modified the transcriptional effect of oxysterol (Figures SSF and SSG). Thus, PUFA/SFA can modulate Rorγt transcriptional activity via a Rorγt-ligand dependent mechanism, although the precise mechanism of exogenous PUFA and SFA require further studies. Taken together, these observations suggest that CD5L shifts the FA composition in the lipids, changes Rorγt ligand availability and Rorγt genomic binding, and regulates Il23r and Il10, members of the proinflammatory versus regulatory modules.

**PUFA/SFA Regulate Th17 Cells and Contribute to CD5L Function**

As CD5L+/− Th17 cells have an altered balance in lipid saturation and PUFA/SFA modulate Rorγt binding and function, we analyzed the relevance of FA moieties to Th17 cell function and their contribution to CD5L-driven Th17 cell pathogenicity. We first tested the effect of PUFA/SFA on the generation of Th17 cells. WT Th17 cells were differentiated with TGF-β1+IL-6 and expanded using IL-23 in fresh media with either PUFA or SFA. PUFA suppressed IL-17 and IL-23R expression, consistent with reduced transcription in WT, but not in Rorγt+/− Th17 cells, suggesting that PUFA can limit pathogenic Th17 cell function in a Rorγt-dependent manner (Figures 6A and 6B). CD5L+/− Th17 cells differentiated with TGF-β1+IL-6 were also sensitive to PUFA treatment, resulting in reduced percentage of IL-17+/CD4+ T cells (Figure 6C). In contrast, addition of SFA only slightly increased the expression of both IL-17 and IL-23R expression, and this effect was not significant, possibly because pathogenic Th17 cells had already very high levels of SFA.

We studied the contribution of lipid saturation to Th17 cell pathogenicity. We speculated that, if the balance of lipid saturation distinguishes non-pathogenic WT Th17 cells and pathogenic CD5L+/− Th17 cells, the addition of SFA to WT and PUFA to CD5L+/− Th17 cells can result in reciprocal changes in the transcriptional signature relevant to Th17 cell pathogenicity. We analyzed the expression of a 312-gene signature of Th17 cell differentiation and function (Yosef et al., 2013) in SFA- or control-treated WT Th17 cells and in PUFA- or control-treated CD5L+/− Th17 cells differentiated with TGF-β1+IL-6. Of those genes that are differentially expressed (Table S2, > 1.5 fold), PUFA-treated CD5L+/− Th17 cells resemble WT non-pathogenic Th17 cells, and SFA-treated WT non-pathogenic Th17 cells are more similar to CD5L+/− Th17 cells (Figure 6D and Table S2). qPCR analysis confirmed that PUFA and SFA reciprocally regulated effector molecule expression of the pathogenicity signature (Lee et al., 2012), including Il10, Il23r, Ccl5, Csf2, and Lag3 (Figure 6E). Notably, in some cases, PUFA and SFA have the same effects; for example, Il22 expression is increased following either FA treatment. Taken together, these observations suggest that the balance of lipid saturation contributes to CD5L-dependent regulation of Th17 cells by regulating the Th17-cell transcriptome.

**DISCUSSION**

Th17 cells are a helper cell lineage capable of diverse functions ranging from maintaining gut homeostasis and mounting host defense against pathogens to inducing autoimmune diseases. How Th17 cells can mediate such diverse and opposing functions remains a critical open question. Addressing this is especially important since anti-IL-17- and Th17-based therapies have been highly efficacious in some autoimmune diseases but had no impact on others (Baeten and Kuchroo, 2013; Genovese et al., 2010; Hueber et al., 2012; Leonard et al., 2012; Papp et al., 2012; Patel et al., 2013), even when Th17 cells have been genetically linked to the disease process (Cho, 2008; Lees et al., 2011). Using single-cell genomics we have addressed this issue and have identified novel functional regulators of pathogenicity in Th17 cells.

Here, we highlight and investigate CD5L as one of the novel regulators that affect the pathogenicity of Th17 cells. We show that: (1) among CD4 T cells, CD5L is highly expressed only in non-pathogenic Th17 cells but, in them, positively co-varies with a pro-inflammatory module, a pattern consistent with being a negative regulator of pathogenicity; (2) CD5L does not affect Th17 differentiation but affects their long-term expansion and function; (3) CD5L deficiency converts non-pathogenic Th17 cells into pathogenic Th17 cells; (4) CD5L regulates lipid metabolism in Th17 cells and alters their fatty acid composition; and (5) change in the lipidome in CD5L+/− Th17 cells affects the ligand availability and binding of Rorγt to its target genes.

In a seemingly paradoxical way, CD5L is expressed only in non-pathogenic Th17 cells but in co-variance with the pro-inflammatory module. This observation led us to hypothesize that CD5L is a negative regulator of a non-pathogenic-to-pathogenic transition, since negative regulators are often known to co-vary in regulatory networks with the targets that they repress in organisms from yeast (Segal et al., 2003) to mammals (Amit et al., 2007; Amit et al., 2009). Our functional analysis bears out this hypothesis, suggesting that CD5L might indeed be expressed to restrain the pro-inflammatory module in the non-pathogenic Th17 cells. Similarly, other genes with this specific pattern, i.e., exclusive expression in non-pathogenic cells but in co-variance with the pro-inflammatory module, may also be repressors that quench pro-inflammatory effector functions.
and make Th17 cells non-pathogenic. Thus, depending on the environmental context or trigger, non-pathogenic Th17 cells can be readily converted into pathogenic Th17 cells by inhibiting a single gene like CD5L. This is supported by our data showing that IL-23R signaling can suppress CD5L and that persistent CD5L expression inhibits the pro-inflammatory function of Th17 cells. In addition to suppressing the pro-inflammatory module, CD5L also promotes the regulatory module, acting as a switch to allow rapid responses to environmental triggers such that Th17 cells can change their functional phenotype without intermediary pathways.

Both pathogenic and non-pathogenic Th17 cells are present in peripheral lymphoid organs, but pathogenic Th17 cells appear at sites of tissue inflammation (CNS) and non-pathogenic Th17 cells appear in the gut or other mucosal surfaces. This is mirrored in the expression of CD5L. IL-23, which is present in the CNS during EAE, can suppress CD5L and convert non-pathogenic Th17 cells into pathogenic Th17 cells. At steady state, it is unknown what promotes CD5L expression and non-pathogenicity in the gut. TGF-β could be a candidate, given its abundance in the intestine and its role in both differentiation of IL-10-producing CD4 T cells in vivo (Konkel and Chen, 2011; Maynard et al., 2007) and Th17 cell differentiation (Bettelli et al., 2006; Veldhoen et al., 2006). Specific commensal bacteria (Ivanov et al., 2009; Yang et al., 2014) and metabolites from microbiota (Arpaia et al., 2013) can also regulate T cell differentiation. Notably, CD5L is reported as a secreted protein and can recognize PAMPs (Martinez et al., 2014). It is possible that CD5L expressed by non-pathogenic Th17 cells in the gut can interact with the immune cells interacting with gut microbiota and can maintain gut tolerance and a non-pathogenic Th17 phenotype. Other CD5L-expressing cells in the intestine may also contribute to such a function. Therefore, the two functional states of Th17 cells may be highly plastic, in that either pathogenic or non-pathogenic Th17 cells can be generated by sensing changes in the tissue microenvironment. CD5L is critical for maintaining the non-pathogenic functional state of Th17 cells, and IL-23 rapidly suppresses CD5L rendering the cells pathogenic. This hypothesis also predicts that non-pathogenic Th17 cells can be easily converted into pathogenic Th17 cells by production of IL-23 locally in the gut during inflammatory bowel disease.

How does CD5L regulate Th17 cell pathogenicity? We provide evidence that CD5L can regulate Th17 cell function by regulating intracellular lipid metabolism and limiting Rorγ ligand. CD5L inhibits the de novo synthesis of fatty acid through direct binding to fatty acid synthase. We discovered that, in Th17 cells, CD5L is more than a general inhibitor, as it regulates the fatty acid composition of PUFA versus SFA and MUFA. We showed that CD5L suppresses the cholesterol synthesis pathway by regulating critical enzymes sc4mol and cyp51, and the addition of PUFA could reverse this phenotype. Importantly, exogenous Rorγ ligand can rescue the suppressive effect of CD5L on IL-17 expression. PUFA metabolites can function as ligands of several transcription factors, and the exact mode of function for PUFA requires further investigation. We showed that PUFA limits ligand-dependent function for Rorγ, such that, in the presence of CD5L or PUFA, Rorγ binding to the Il17a and Il23r loci is decreased, along with reduced transactivation of both genes, whereas binding at ligand expression from the Il10 locus is enhanced. Notably, Rorγ’s ability to regulate Il10 expression was not reported previously. As CD5L does not impact overall Th17 cell differentiation, this suggests a nuanced effect of CD5L and lipid balance on Rorγ function, enhancing its binding to and transactivation at some loci while reducing it in others. In Th17 cells, Stat3 and C-Maf can promote Il10 (Stumhofer et al., 2007; Xu et al., 2009). As Stat3, C-Maf, and Rorγ can all bind to the same Il10 enhancer element, it is possible that, depending on the quality and quantity of the available ligands, Rorγ may interact with other transcription factors and regulate Il10 transcription. This supports a hypothesis in which the spectrum of Rorγ ligands depends, at least in part, on the CD5L-regulated PUFA versus SFA lipid balance in the cell, and these resulting ligands can impact the specificity of Rorγ, allowing it to assume a spectrum of functional states.

Several metabolic pathways are associated with Th17 cell differentiation. HIF1α regulates Th17 cells through direct transactivation of Rorγ (Dang et al., 2011; Shi et al., 2011), and acetyl-coA carboxylase influences the Th17/Treg balance through the glycolytic and lipogenic pathways (Berod et al., 2014). Mice harboring mutations in genes that regulate Th17 cell differentiation and function acquire an obese phenotype, associating Th17 cell development with obesity (Ahmed and Gaffen, 2010; Jhun et al., 2012; Mathews et al., 2014; Winer et al., 2009). A hallmark of obesity is the accumulation of saturated fat and cholesterol, and mice fed with a diet rich in PUFA were reported to have reduced severity of chronic colitis and Th17 cell polarization (Monk et al., 2012, 2013). In this study, we provided evidence that, at the cellular level, lipidome saturation can promote Th17 cell function by regulating Rorγ function.

In conclusion, by using single-cell genomics and computational analysis, we identified CD5L as a novel repressor of Th17 cell pathogenicity, highlighting the power of single-cell genomics to identify molecular switches that are otherwise obscured by population-level genomic profiles. CD5L appears to be a molecular switch that does not affect Th17 differentiation per se but one that impacts the function (pathogenic versus non-pathogenic phenotype) of Th17 cells, potentially by regulating the quality and/or quantity of available Rorγ ligands, allowing a single master regulator to possibly assume multiple functional states. Our results connect the lipidome to essential functions of immune cells, opening new avenues for sensitive and specific therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

For additional procedures, please refer to the Supplemental Experimental Procedures.

**Single-Cell RNA-Seq Data Acquisition and Analysis**

We profiled the transcriptome of 806 Th17 cells either harvested in vivo or differentiated in vitro. For in vivo experiments, CD3+CD4+IL-17A.GFP+ cells were isolated from draining LNs and CNS of mice at peak of EAE. For in vitro experiments, cells were sorted at 48 hr post induction of differentiation of naive CD4+ T cells under different conditions. We had at least two independent biological replicates for each in vivo and in vitro condition (except for TGF-β3+IL-6 for which we only had one replicate), as well as two technical replicates for two in vivo conditions. For analysis, refer to the Supplemental Experimental Procedures.
Lipidomics
Th17 cells were differentiated from naïve WT and CD3L−/− T cells. Culture media were snap frozen. Cells were harvested at 96 hr. 10 × 10^6 cells per sample were snap frozen and extracted in either 80% methanol (for fatty acids and oxylipids) or isopropanol (for polar and nonpolar lipids). Two liquid chromatography tandem mass spectrometry (LC-MS) methods were used to measure fatty acids and lipids in cell extracts. Further details are provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, figures, and tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.10.068.

AUTHOR CONTRIBUTIONS
C. Wang and V.K.K. conceived the project. C. Wang designed and performed most biological experiments. J.G., N.Y., L.H., A.R., and V.K.K. conceived and performed the original single-cell analysis and ranking to identify putative pathogenicity regulators, N.Y., and A.R. devised the computational analyses. C. Wang, J.G., L.H., and C.B.C. performed the lipidomics experiments, and C. Wang, J.K., and N.Y. analyzed the data. C. Wu contributed to experiments and design. I.W. processed RNA-seq samples, and A.M. analyzed RNA-seq data. S.X., G.M.Z.H., M.P., K.K., M.O.-M., and C.Z. contributed to vector constructs used for experiments. Y.K. and N.J. contributed to setting up the project and acquisition of the CD3L−/− mice. R.S. performed histology analysis for EAE. T.M. provided CD3L−/− mice. C. Wang, N.Y., A.R., and V.K.K. wrote the paper with input from all of the authors.

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