REGULATION OF ADAPTIVE AND INNATE IMMUNITY BY THE CIRCADIAN TRANSCRIPTION FACTOR NFIL3

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DEDICATION

Dedicated to Dad and Mom

for

their love and support

REGULATION OF ADAPTIVE AND INNATE IMMUNITY BY THE CIRCADIAN TRANSCRIPTION FACTOR NFIL3

by

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DISSERTATION

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The day-night cycle has a profound impact on animal physiology, which has been shown to be mediated by an intracellular timing system called the circadian clock. However, little is known about whether and how the day-night cycle and the circadian clock influence host immunity. Here, I show that the circadian transcription factor NFIL3 is critical for both adaptive and innate immunity by regulating T_H17 cells and innate lymphoid cells (ILCs), respectively.

First, NFIL3 transcriptionally represses RORγt, the master regulator of T_H17 cells, by directly binding to the Rorγt promoter and thereby suppresses T_H17 cell development from naïve T helper cells. Consistent with oscillation of *Nfil3* expression during the circadian cycle, Rorγt expression also oscillates in mice, with higher expression at noon and lower expression at midnight. Accordingly, naïve T helper cells show greater potential to develop into T_H17 cells at noon than at midnight. Furthermore, artificially disturbing the circadian cycles of mice by manipulating their light exposure results in circadian clock-mediated disruption of T_H17 homeostasis and increased susceptibility to experimentally-induced colitis. Therefore, NFIL3 regulates T_H17 cell development in a circadian manner.

Second, NFIL3 is essential for the development of all major types of innate lymphoid cells (ILCs) by regulating the generation of ILC progenitors in the bone marrow. One of the NFIL3-dependent progenitor populations, αLP, can differentiate into all major types of ILCs *in vivo*. NFIL3 controls progenitor development by activating a High Mobility Group (HMG) transcription factor *Tox* directly in common lymphoid progenitors (CLPs). Accordingly, restoring *Tox* expression in *Nfil3*-deficient progenitors rescues ILC development in mice. So NFIL3 regulates ILC development by activating *Tox* expression in bone marrow precursors.

Taken together, my work demonstrates that NFIL3 is a critical regulator of host immunity and that it modulates immune functions along the circadian cycle. This study is among the first to reveal the influence of the circadian clock on host immunity and provides novel insights into the regulatory mechanisms underlying variations of immune cell development and function during the circadian cycle.

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a.k.a.	–	Also known as
AMP	–	Adenosine Monophosphate
AMPK	–	Adenosine Monophosphate (AMP)-activated protein kinase
ANOVA	–	Analysis of variance
AP-1	–	Activator protein 1
APC	–	Antigen presenting cells
bHLH	–	Basic helix-loop-helix
BMAL1	–	Brain and muscle Arnt-like protein-1
BMDC	–	Bone marrow derived dendritic cells
bZIP	–	Basic leucine zipper
C/EBP	–	CCAAT-enhancer-binding protein
cAMP	–	Cyclic adenosine monophosphate
CCG	–	Clock-Controlled Genes
CCL	–	Chemokine (C-C motif) ligand
CD	–	Cluster of differentiation
cDNA	–	Complementary DNA
CDS	–	Coding DNA Sequence
CFU	–	Colony forming unit
CHILP	–	Common “helper-like” innate lymphoid progenitor
ChIP	–	chromatin immunoprecipitation

CILP	–	Common ILC progenitor
CLP	–	Common lymphoid progenitor
CRY	–	Cryptochrome
CTL	–	Cytotoxic T lymphocytes
CXCL	–	Chemokine (C-X-C motif) ligand
DAMP	–	Damage-associated molecular patterns
DBP	–	D site albumin promoter binding protein
DC	–	Dendritic cells
DEC1/2	–	Differentiated embryo chondrocyte 1/2
DSS	–	Dextran sulfate sodium
DTT	–	Dithiothreitol
EDTA	–	Ethylenediaminetetraacetic acid
EGFP	–	Enhanced green fluorescent protein
EMSA	–	Electrophoretic mobility shift assays
EOMES	–	Eomesodermin
FACS	–	Fluorescence-activated cell sorting
FBS	–	Fetal bovine serum
FGF1	–	Fibroblast growth factor 1
GATA3	–	GATA binding protein 3
G-CSF	–	Granulocyte colony-stimulating factor
GI	–	Gastrointestinal

GICD	–	Glucocorticoid-induced cell death
GSK-3β	–	Glycogen Synthase Kinase 3 beta
GWAS	–	Genome-wide association studies
Gy	–	The Gray ionizing radiation dose
H&E	–	Hematoxylin and eosin
HBSS	–	Hank's Balanced Salt Solution
HLF	–	Hepatic leukemia factor
HMG	–	High Mobility Group
hr	–	Hour
HSC	–	Hematopoietic stem cell
IBD	–	Inflammatory bowel disease
ID2	–	Inhibitor of DNA binding 2
IEC	–	Intestinal epithelial cells
IFN	–	Interferon
IL	–	Interleukin
ILC	–	Innate lymphoid cell
ILC1	–	Type 1 innate lymphoid cell
ILC2	–	Type 2 innate lymphoid cell
ILC2P	–	Type 2 innate lymphoid cell progenitor
ILC3	–	Type 3 innate lymphoid cell
ILF	–	isolated lymphoid follicles

ires	–	Internal ribosome entry site
LB	–	Luria-Bertani broth
LMPP	–	Lymphoid primed multipotent progenitor
LPS	–	Lipopolysaccharide
LSK	–	Lineage marker$^-$ Sca1$^+$ cKit$^+$ cells
LTi	–	lymphoid tissue inducer cell
LXR	–	Liver X receptor
MACS	–	Magnetic-activated cell sorting
MAMP	–	Microbial-associated molecular patterns
MHC	–	Major histocompatibility complex
min	–	Minute
MPP	–	Multipotent progenitor
mRNA	–	Messenger RNA
MyD88	–	Myeloid differentiation primary response gene (88)
NFIL3	–	Nuclear factor, IL-3 regulated
NFκB	–	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	–	Natural Killer cells
NKP	–	NK cell precursor
ODN	–	oligodeoxynucleotides
PACAP	–	Pituitary adenylate cyclase activating peptide
PAR-bZIP	–	Proline and acidic amino acid-rich basic leucine zipper

PBS	–	Phosphate buffered saline solution
PER	–	Period
PLZF	–	Promyelocytic leukemia zinc finger
PMA	–	Phorbol myristate acetate
PreNKP	–	Precursors to NKP
PRR	–	Pattern recognition receptor
PTH	–	Parathyroid hormone
REG3γ	–	Regenerating islet-derived 3 gamma
REV-ERB	–	The reverse strand of *c-erbA* encoded protein
RHT	–	Retinohypothalamic tract
rNKP	–	Refined NK cell precursor
RORα	–	Retinoic acid receptor-related orphan nuclear receptor alpha
RORγt	–	Retinoic acid receptor-related orphan nuclear receptor gamma, isoform t
RPMI	–	Roswell Park Memorial Institute medium
RRE	–	*Rev*-responsive element
RT-PCR	–	Reverse transcription polymerase chain reaction
SCF	–	Stem cell factor
SCN	–	Suprachiasmatic Nucleus
SFB	–	Segmented filamentous bacteria
SFEM	–	Serum-free expansion medium

shRNA	–	Short hairpin RNA
siRNA	–	Small interfering RNA
SPF	–	Specific pathogen-free
STAT3	–	Signal transducer and activator of transcription 3
SUMO	–	The small ubiquitin-like modifier
T-bet	–	T-cell-specific T-box transcription factor
TCF-1	–	Transcription factor 7, T cell specific
TEF	–	Thyrotroph embryonic factor
TGF	–	Transforming growth factor
Th	–	T helper cells
T_H1	–	Type 1 T helper cell
T_H17	–	T helper 17 cell
T_H2	–	Type 2 T helper cell
TLR	–	Toll-like receptor
TNF	–	Tumor necrosis factor
TOX	–	Thymocyte selection-associated high mobility group box protein
TPO	–	Thrombopoietin
T_{reg}	–	Regulatory T helper cell
TUNEL	–	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay
WT	–	Wild-type

ZT	–	Zeitgeber Time
α	–	anti
αLP	–	$\alpha_4\beta_7$ integrin-expressing common lymphoid progenitor
αMEM	–	Minimum essential medium eagle - alpha modification

CHAPTER ONE

INTRODUCTION

The day-night cycle is a fundamental feature of life on earth, which creates rhythmic changes in environmental factors such as light and temperature. During evolution, living organisms have developed sophisticated internal timing systems, known as circadian clocks, to anticipate such changes and coordinate physiological processes accordingly [1]. This can be observed macroscopically as rhythms in behaviors such as sleep-wake and feed-fast or microscopically as oscillation in cellular activities such as endocrine secretion. A growing number of studies have started to reveal the molecular mechanisms by which the circadian clock controls cell metabolism [2]. However, little is known about whether and how the circadian clock impacts immune functions in animals [3].

THE CIRCADIAN CLOCK

The circadian clock is an evolutionarily conserved mechanism that allows living organisms to adapt to the rhythmic day-night transition. So far, the circadian clock has been found in virtually all major life forms, including cyanobacteria [4], plants [5], insects [6] and mammals [7], though their organizations and components can be different. Based on its biochemical nature, the circadian clock can be roughly categorized into two forms: the transcriptional/translational autoregulatory network [1] and the reduction-oxidation (redox) loop [8-10]. They are also called the “genetic clock” and the “metabolic clock” [11], respectively. The genetic clock usually consists of a group of transcriptional regulators that

cross-regulate each other's expression, while the metabolic clock mainly functions through oxidation and reduction of peroxiredoxin enzymes. Because the metabolic clock has been recently identified and most well-studied clocks in various species fall into the genetic clock category, the term "circadian clock" will hereafter refer to the genetic clock only, unless stated otherwise.

The hierarchical organization of the mammalian circadian clock system

The circadian clock is believed to exist in virtually every cell in mammals. To coordinate the clock function in a large number of cells, the mammalian circadian clock is organized into two hierarchical levels: the central clock and the peripheral clock [1]. The central clock is located in the brain, which receives signals from the outside environment and sends out signals to modulate the peripheral clock. The peripheral clock refers to the clock in non-brain tissues such as muscle and liver, which governs local physiological activities.

The central clock

The central clock, a.k.a. the "master pacemaker", is comprised of ~20,000 neurons in the Suprachiasmatic Nucleus (SCN) of the hypothalamus. Because the period of the circadian clock is not exactly 24 hours, external environmental signals are necessary to entrain and synchronize the internal clock in order to align it with the day-night cycle. Arguably the most important entraining signal is light, which is sensed by the retina in the eyes and conveyed to the SCN through the retinohypothalamic tract (RHT) [12]. The RHT innervates the SCN and releases glutamate and the neuropeptide pituitary adenylate cyclase activating peptide

(PACAP), which leads to activation of SCN neurons [13]. After activation, SCN neurons send out synchronization signals to other tissues, either directly through neural projections or indirectly through body temperature (via the hypothalamus) and hormones (such as glucocorticoids from the adrenal gland), to modulate the peripheral clock [1]. In addition, the central clock can also relay day-night temporal information to the peripheral clock through food signals by controlling feeding behaviors of animals.

The peripheral clock

The peripheral clock functions autonomously in individual cells in peripheral tissues such as, but not limited to, the liver, intestine, heart, kidney, skeletal muscles and adipose tissues. There are key differences between the peripheral clock and the central clock. First, the peripheral clock does not process environmental signals such as light directly, but instead receives circadian information through synchronizing signals originating from the SCN, such as hormones and body temperature. Second, the peripheral clock is more sensitive to body temperature and food signals. For example, the peripheral clock can be reset and synchronized by body temperature, while the SCN is resistant to temperature variation [14], which makes body temperature the universal resetting cue of the peripheral clock. Food intake can also greatly influence the peripheral clock in certain tissues, such as the liver, as limiting food intake in mice during the day can readily dissociate the activity of the peripheral clock from that of the SCN. This has been shown to involve Sirtuin 1 (SIRT1) and Poly(ADP-Ribose) Polymerase 1 (PARP1) [15-17]. Third, the peripheral clock is more involved in regulating the physiological activities of local tissues, such as metabolism [2, 18].

The molecular basis of the mammalian circadian clock

Though circadian oscillation was first observed in the early 1700s, understanding the molecular basis of the circadian clock only began to be established in the late 1900s [7, 19]. The first identified gene involved in the circadian clock was *Period* in *Drosophila melanogaster* [20], which was discovered by screening mutants that had shown an altered circadian pattern of eclosion, as wild-type *Drosophila* flies preferentially emerge from the pupal case in the morning. The first gene in the mammalian circadian clock, named *Clock*, was identified in 1994 by genetic screening too [21] and subsequently cloned in 1997 [22]. Soon after, the mammalian homologs of the fruit fly *Period* gene were also identified [23]. Since then, many other circadian clock components have been discovered, which have collectively led to our current understanding of the molecular clock (FIGURE 1) [1].

The molecular clock

The core of the clock consists of two transcription activators, *Bmal1* and *Clock*, which are basic helix-loop-helix (bHLH)-PAS transcription factors. Upon expression, BMAL1 and CLOCK dimerize and activate downstream genes by binding directly to the E-box motif (CACGTG) in the promoters [24]. One group of the downstream genes are *Period*s and *Cryptochrome*s, which form dimers and interact with BMAL1:CLOCK to suppress the latter's function [25]. Another group of BMAL1:CLOCK target genes include nuclear hormone receptors *Rev-erbα* and *Rev-erbβ*, which bind to the RRE motif (GGTCA) in the *Bmal1* promoter to repress *Bmal1* expression [26]. Thus PER:CRY and REV-ERBs form two

negative feedback loops to inhibit BMAL1:CLOCK post-translationally and transcriptionally, respectively. Once BMAL1:CLOCK activity drops, expression of these negative regulators also decreases and eventually leads to de-repression of BMAL1:CLOCK. Therefore, expression of the circadian clock components oscillates during the day-night cycle with a period of ~24 hours (FIGURE 1).

Additional clock regulatory mechanisms

The function of the core clock is fine-tuned by additional mechanisms, particularly post-translational modifications. First, SUMOylation of BMAL1 increases BMAL1 turnover, which is critical for maintaining rhythmicity of BMAL1 *in vivo* [27]. Second, PER1 and PER2 are phosphorylated by Casein Kinase I delta and epsilon (CKIδ/ε), which leads to nuclear translocation and ubiquitin-mediated degradation [28, 29]. Third, the Cryptochrome CRY1 can be phosphorylated by the Adenosine Monophosphate (AMP)-activated protein kinase (AMPK) at Serine-71, which targets CRY1 for ubiquitination and degradation [30]. Fourth, the Glycogen Synthase Kinase 3 beta (GSK-3β) can phosphorylate and stabilize REV-ERBα, leading to persistent repression of *Bmal1* [31]. Fifth, the activity of REV-ERBs can be inhibited by nuclear hormone receptors Retinoid-Related Orphan Receptors (RORs), which are also transcriptionally activated by BMAL1:CLOCK, but functionally compete with REV-ERBs by binding to the same DNA motif (GGTCA) and activating *Bmal1* [32].

Other clock-related genes

In addition to the circadian clock genes mentioned above, there are many other circadian clock-related genes in mammals, which can be categorized into two groups. The first group consists of homologs of the circadian clock genes, which may exhibit tissue-specific expression patterns but can functionally replace the circadian clock genes. For example, NPAS2 is a paralog of CLOCK that forms a dimer with BMAL1 to participate in the clock network in the mouse forebrain [33]. Similarly, BMAL2 is a homolog of BMAL1 and can dimerize and function with CLOCK [34]. The second group involves genes regulated by the circadian clock, also known as Clock-Controlled Genes (CCGs). The circadian clock components not only regulate each other within the circadian clock network, but also regulate genes outside the network [35], thereby converting rhythms of the circadian clock activity into oscillations of cellular processes. Functions of many CCGs have been well studied, such as the PAR-bZip transcription factors DBP, HLF, and TEF; the bZip transcription factor NFIL3; and bHLH transcription factors DEC1 and DEC2. For example, NFIL3, the focus of my thesis work, is directly activated by BMAL1:CLOCK and repressed by REV-ERBα [35, 36].

CIRCADIAN REGULATION OF IMMUNITY

Mammals, including humans, are densely colonized by microbes on both exterior surfaces, such as skin, and interior surfaces such as the gastrointestinal tract (GI tract), upper respiratory tract as well as the urogenital tract. Meanwhile, pathogens including bacteria,

fungi and viruses from the outside environment pose great threats to the body. In order to defend themselves, mammals have developed a sophisticated immune system during evolution to prevent and resolve infections, which eventually helps establish a symbiotic relationship between the host and indigenous microbes [37].

The immune system consists of two branches: the innate immune system and the adaptive immune system. The innate branch includes cells that can directly sense microbes and respond immediately. Macrophages, dendritic cells (DCs), granulocytes, intestinal epithelial cells and innate lymphoid cells fall into this category. These cells sense infection using germline-encoded pattern recognition receptors (PRR) to recognize microbe-associated molecular patterns (MAMP) and damage-associated molecular patterns (DAMP). These molecular patterns usually associate with a group of microbes or host cells and therefore can initiate a broad, rather than specific, immune defense program. The adaptive branch involves cells that require priming to mount optimal responses against infections, such as T cells and B cells. They utilize somatically rearranged receptors to sense specific antigens. Once an antigen receptor-bearing B or T cell clone recognizes antigens presented by the antigen presenting cells (APCs), this clone will undergo extensive proliferation (clonal expansion) and differentiation so as to mount immune protection specifically against this particular antigen. In addition to defending against current infection, a subset of adaptive immune cells generated during the primary immune response can live from months to years in the body, which allows for faster and stronger immune responses against the same pathogens during reinfection in the future (immunological memory). Despite such differences, both innate and adaptive immune cells have been shown to harbor a functional circadian clock [38].

Daily oscillation of immune factors

Early evidence that immune functions may be regulated by the circadian clock stemmed from the observation that some immune factors show daily oscillations in the body [3]. First, it has been reported that levels of many immune cytokines oscillate in the blood [39, 40]. These cytokines include, but are not limited to, interleukin (IL)-1β, IL-6, IL-10, IL-12, Tumor Necrosis Factor (TNF)-α and Interferon (IFN)-α. Second, the cellularity of immune cells also oscillates in the blood and other immune tissues [40-44]. For example, numbers of eosinophils, B cells, macrophages, monocytes and neutrophils show daily oscillation in blood, spleen and other tissues, which may indicate circadian regulation of cell differentiation and/or recruitment. Eosinophils are of particular interest as they have a very short life span in circulation, with a half-life of 3~18 hours. The half-life is short enough to cause cell number variation during the circadian cycle, with 200~300 eosinophils per microliter blood in the morning (10:00) and ~100 eosinophils in the same amount of blood at night (22:00) [45]. However, the exact role of the circadian clock in the oscillation of immune factors requires further examination.

Circadian oscillations of immune cells may also serve as a homeostatic signal in the body. For example, the number of neutrophils oscillates in the blood during the circadian cycle, partly due to homing of "aged" neutrophils back to the bone marrow at the end of the resting period (late afternoon for mice) [44]. Macrophages in the bone marrow take up these "aged" neutrophils and activate their LXR pathway, which leads to downregulation of the

hematopoietic stem cell (HSC)-retaining chemokine CXCL12 in the bone marrow and thus release of HSCs into blood circulation [44].

Circadian regulation of immune functions

It has been known for more than 50 years that responses to endotoxin (lipopolysaccharide, LPS) in mice vary during the circadian cycle [46]. In 1960, Halberg and colleagues challenged mice by injecting LPS at different times of the day and discovered that mice challenged during the day exhibited greater mortality than those treated at night, with peak mortality seen in mice treated in the late afternoon and the nadir at midnight. This is probably the first observation demonstrating that the immune responses are impacted by the circadian cycle. However, not much was known about either the circadian clock or the immune receptor to LPS (i.e. TLR4) when this observation was initially published. With advances in understanding of both the circadian clock and immunity, several recent studies have established molecular pathways by which the circadian clock regulates immune responses.

Circadian regulation of sepsis responses

Injection of LPS into mice leads to systemic immune responses characterized by acute production of large quantities of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, which mimics immune responses during systemic bacterial infection. This leads to a disease condition called sepsis, with a resulting mortality due to immunopathology [46].

Early studies revealed that macrophage production of pro-inflammatory cytokines responding to LPS stimulation differs during the circadian cycle, with higher responses in the late afternoon and lower in early morning [47], indicating that the sepsis response is

regulated by the circadian clock. This variation is dependent on the circadian clock components REV-ERBα and BMAL1 [47]. With a setting similar to Halberg *et al* [46], a recent study shows that the core circadian clock component, CLOCK, directly interacts with the p65 subunit of NFκB [48], a critical transcription factor that acts downstream of Toll-like Receptors (TLR) as well as cytokine (such as TNF-α and IL-1β) receptors. By interacting with p65, CLOCK enhances NFκB-activated gene expression in the nucleus and therefore modulates NFκB-mediated immune responses along the circadian cycle.

Circadian regulation of T cell responses

T cells are an important group of adaptive immune cells that function by either secreting cytokines to coordinate the function of other immune cells ($CD4^+$ T cells, a.k.a T helper cells) or by directly killing infected cells ($CD8^+$ T cells, a.k.a cytotoxic T cells or CTL). With the evidence of a functional circadian clock in T cells [49], it is intriguing to test whether T cell responses can be regulated by the circadian clock. By stimulating T cells *in vitro*, Fortier *et al* demonstrated that mouse T cells respond to stimulation in a circadian manner, with higher responses in the late afternoon and early evening [50]. This circadian pattern of responses is dependent on the circadian clock, as deficiency in the *clock* gene abolishes such circadian variation. Interestingly, delivery of ovalbumin as an antigen by bone marrow derived dendritic cells (BMDCs) at noon results in more ovalbumin-specific ($CD8^+$) T cells in mice than delivery at midnight. The discrepancy between *in vitro* stimulation and *in vivo* activation may reflect the time it takes for the BMDCs to migrate to lymphoid tissues and interact with T cells *in vivo*.

Variation in T cell responses also correlates with circadian expression of the key tyrosine kinase ZAP70 downstream of the T cell receptor (TCR) [50]. However, there remain questions as to i) whether and how the circadian clock regulates ZAP70 expression and ii) if circadian variation in ZAP70 protein levels is responsible for overall T cell response variation during the circadian cycle. Also using bulk cells from the spleen and lymph nodes without purifying and fractionating T cell subtypes in this study could complicate interpretation of the data.

Circadian regulation of B cell responses

B cells are the adaptive immune cells that produce antibodies against invading pathogens. Silver *et al* have shown that TLR9 expression in mouse B cells oscillates during the circadian cycle, with higher expression in the early evening [51]. Expression of TLR9 is dependent on the circadian clock, as mutation in PER2 or knockdown CLOCK by siRNA decreases TLR9 expression. Accordingly, immunizing mice at midnight leads to better immune responses in mice as measured by cell proliferation and cytokine production, when the TLR9 ligand, CpG oligodeoxynucleotides (CpG ODN), is used as adjuvant. However, the author did not assess how the antibody production by B cells was impacted by differing the time of immunization. In addition, because immunization was performed by intraperitoneal injection of ovalbumin and CpG, potential circadian variation in migration of antigen presenting cells (including B cells) and antigen processing could play a role in the final immune responses. Therefore, the optimal time of immunization described in this study does not necessarily agree with the

optimal immunization time described in the T cell study [50], in which antigen was delivered by intravenous injection of antigen-loaded BMDCs.

Circadian regulation of monocyte recruitment

Monocytes are a group of myeloid cells in the blood that are capable of phagocytosis as well as secretion of cytokines, such as IL-1, IL-6, and TNF-α, upon activation. Monocytes develop in the bone marrow and retain proliferative capacity when released into blood. Monocytes can further differentiate into tissue-resident macrophages and dendritic cells once they migrate into peripheral tissues such as liver and skeletal muscle. Two recent studies demonstrated that recruitment of monocytes is regulated by the circadian clock [42, 43]. In mice, numbers of monocytes in the blood and spleen are greater during the day and lower at night, while the numbers in the bone marrow and skeletal muscle show an opposite oscillation pattern. This circadian recruitment pattern is abolished by genetic deficiency of the circadian clock component BMAL1 or surgical removal of local adrenergic nerves. Further studies revealed that BMAL1:CLOCK regulates expression of the monocyte-migration signal CCL2, which is responsible for circadian recruitment of monocytes into peripheral tissues.

NFIL3

NFIL3 denotes “nuclear factor, interleukin 3 regulated” and is also known as E4BP4. It was first identified as a transcription factor that can bind the adenovirus E4 promoter in a

sequence-specific manner (and hence the name E4BP4) [52]. It was later independently discovered as a factor that binds the IL-3 promoter in human T cells and activates IL-3 expression, hence it was named as NF-IL3A [53]. This and a later study showed that it could also be induced by IL-3 in T cells and B cells, respectively, which led to its current name, NFIL3 [54].

The biochemical nature of NFIL3

NFIL3 is well conserved in mouse and other species at the both protein and DNA levels (FIGURE 2A). It contains a DNA binding domain at the N-terminus and a regulatory domain at the C-terminus (FIGURE 2B).

The DNA-binding domain

A basic leucine zipper (bZIP) domain at the N-terminus is responsible for the DNA-binding activity of NFIL3. Accordingly, NFIL3 binds DNA as a dimer [52], as do most other bZIP transcription factors. The basic region directly interacts with DNA and the leucine zipper mediates dimerization. This is also consistent with the palindromic sequences of the NFIL3 binding site RTTAYRTAA [52]. However, NFIL3 can also bind to motifs that do not align perfectly with this consensus sequence. For example, NFIL3 binds to the D-box motif (ATTATGCAAC) to compete with D-box-binding proteins such as DBP [55]. The binding site of NFIL3 in human IL-3 promoter is ATAATTAC, which deviates even more from the consensus [53].

The regulatory domain

NFIL3 can function as either a transcriptional activator or a repressor, depending on the context of the promoter it occupies. For example, NFIL3 activates IL-3 expression but represses the adenovirus E4 promoter activity [52, 53]. While the activation domain has not been identified, the repression domain was found at the C-terminus of the NFIL3 protein [56]. The minimum repression domain lies between I299 and F363 of the human NFIL3 protein, and positively-charged K330 and K332 are critical for the suppression activity, as mutating these two lysine residuals to glutamates (K →E) abolishes the suppressive effect [56].

Regulation of NFIL3

Transcriptional regulation of Nfil3

Expression of *Nfil3* is regulated by multiple signaling pathways, such as those activated by cytokines and hormones. First, it has been shown that IL-3 can induce *Nfil3* in T and B cells [53, 54], which is mediated by transcription factors GATA-1 and GATA-2 [57]. Second, *Nfil3* expression can be induced in macrophages by exposing to LPS [58]. However, the LPS-sensing TLR4-MyD88 signaling pathway does not induce *Nfil3* directly. Instead, LPS engagement of TLR4 leads to release of IL-10, which then acts back on macrophages to induce *Nfil3* [58]. When macrophages are deficient in IL-10 or an IL-10-neutralizing antibody is present, LPS fails to induce *Nfil3* expression. Indeed, an earlier study reveals that IL-10 induces *Nfil3* in macrophages through the transcription factor STAT3 [59]. Third, IL-4 activates *Nfil3* expression in T and B cells through STAT6 [60, 61]. Fourth, *Nfil3* expression

can be induced by IL-15 in human T cells [62], though the detailed mechanism is not clear. Fifth, p53 suppresses *Nfil3* expression in the murine myeloid leukemia cells M1p53ts$_{val}$, which can be overridden by IL-6 [63]. This is not surprising as IL-6 also signals through STAT3. Sixth, Parathyroid hormone (PTH) can also act as an *Nfil3* inducer through the cAMP pathway in osteoblasts [64, 65]. Seventh, upregulation of *Nfil3* can also occur when adipocytes and fibroblasts receive insulin signals, which is mediated by AKT activation [66, 67]. The complexity of signals that regulate *Nfil3* expression in a wide range of tissues may reflect the broad role of *Nfil3* in various physiological processes in the body.

The basal expression of *Nfil3* is regulated by the circadian clock. As mentioned before, BMAL1:CLOCK directly activates *Nfil3* expression while REV-ERBα directly represses its expression [35, 36]. Interestingly, in *Clock*-deficient mice, *Nfil3* expression is higher than in wild-type mice, consistent with lower expression of both *Rev-erbα* and *Rev-erbβ*, suggesting that repression of *Nfil3* by REV-ERBs is a dominant pathway in circadian regulation of *Nfil3* [68].

Post-Translational regulation of NFIL3

It has been reported that NFIL3 can be phosphorylated in B cells, but the physiological role of this phosphorylation is not clear [69]. However, it has been shown that phosphorylation of NFIL3 at Ser-182 by CKIε mediates proteasomal degradation of NFIL3 [70].

The function of NFIL3 in immune cells

The function of NFIL3 has been widely studied in multiple physiological contexts such as circadian clock, metabolism, apoptosis, and neuron development [55, 67, 71, 72]. Due to the scope of my thesis research, I will focus on its role in immune cells and immune responses in this section.

Role of Nfil3 in macrophages and dendritic cells

In macrophages, NFIL3 represses *Il12b*, encoding IL-12p40, downstream of IL-10 signaling [58, 73]. After induction by IL-10, NFIL3 binds directly to the ATGATGTAAG motif in the *Il12b* promoter, thereby suppressing production of IL-12. Though IL-12p40 is a shared subunit of IL-12 and IL-23, IL-23 production by *Nfil3*$^{-/-}$ DCs stimulated with LPS or LPS together with IL-10 is comparable to wild-type DCs, indicating differential regulation of IL-12 and IL-23 in DCs [73].

In addition to regulating *Il12b*, NFIL3 is critical for *in vivo* development of CD8α^+ DCs, a class of DCs that predominantly produce IL-12 after activation [74]. Furthermore, residual DCs in *Nfil3*$^{-/-}$ mice fail to produce IL-12 in response to poly (I:C), the TLR3 ligand. However, their responses to LPS (TLR4 ligand) and CpG (TLR9 ligand) are partially retained, suggesting that NFIL3 impacts TLR signaling in a receptor-specific manner [74].

Role of Nfil3 in T cells

Immature T cells in the thymus, especially CD4$^+$ CD8$^+$ (DP) T cells, are susceptible to apoptosis induced by glucocorticoid, a process termed glucocorticoid-induced cell death (GICD) [75, 76]. It has shown that GICD is mediated by NFIL3 downstream of

glucocorticoid signaling. The glucocorticoid receptor directly activates *Nfil3* by binding to the *Nfil3* promoter and, conversely, knockdown of NFIL3 confers cell resistance to GICD in a cell culture model (CTLL-2) [75].

In mature T cells, NFIL3 has been shown to regulate type 2 cytokine production, such as IL-5, IL-10 and IL-13 [77, 78]. In T_H2 cells, the T helper cell subtype that predominately produces type 2 cytokines, loss of *Nfil3* leads to impaired production of IL-4 and elevated production of IL-5 and IL-13, which can be partially explained by binding of NFIL3 to the *Il13* promoter [78]. Interestingly, T_H1 cells that usually produce type 1 cytokines such as IFNγ can also produce IL-10 and IL-13 under chronic infection or repetitive stimulation, which is mediated by NFIL3 through binding to their promoters [77]. Indeed, overexpression of *Nfil3* in T_H1 cells can force T_H1 cells to produce IL-10 and IL-13. Therefore, NFIL3 functions differently in different T helper lineages.

Role of Nfil3 in B cells

It has been shown that NFIL3 is critical for IL-3-mediated survival of pro-B cells [54]. However, it is not clear whether NFIL3 promotes survival by inhibiting FOXO-induced apoptosis as it does in cancer cells [71].

In mature B cells, NFIL3 promotes class switch recombination to IgE by binding to the Iε promoter and activating Ig GLε transcription [69]. Accordingly, *Nfil3*$^{-/-}$ mice have much lower levels of IgE in the serum than in wild-type mice, while overexpression of *Nfil3* in wild-type B cells elevates IgE production. Interestingly, NFIL3 seems to regulate IgE specifically as neither *Nfil3* deficiency nor *Nfil3* overexpression impacts IgG production.

Role of Nfil3 in Natural Killer (NK) cells

The role of *Nfil3* in NK cells has been intensively studied. Early studies showed that *Nfil3*$^{-/-}$ mice are deficient in NK cell development compared to wild-type mice [69, 79, 80]. Further, NK cell deficiency was attributed to impaired differentiation of immature NK cells from committed NK cell precursors (NKP) as the frequencies of NKP in the bone marrow of wild-type and *Nfil3*$^{-/-}$ mice are comparable but immature NK cells in *Nfil3*$^{-/-}$ mice are markedly reduced [79, 80]. Though NKP cells are committed to develop into NK cells with no T, B, myeloid or erythroid differentiation potential, the NKP population is heterogeneous, as only a small fraction of NKP cells (estimated 10%~30%) can differentiate into NK cells [81, 82]. So deficiency of NK progenitors upstream of immature NK can go unnoticed when examined this way.

Recently, two studies further defined early NK precursors in the mouse bone marrow, namely PreNKP and rNKP [83, 84]. Re-examination of *Nfil3*$^{-/-}$ mice revealed that both PreNKP and rNKP cells are reduced in *Nfil3*$^{-/-}$ mice compared to wild-type mice while upstream common lymphoid progenitors (CLPs) are largely intact [85, 86], indicating the NK cell deficiency occurs during differentiation from CLP to PreNKP. Despite conflicting data on the role of NFIL3 in *Id2* expression during NK development, NFIL3 directly binds to the promoter of *Eomes,* another transcription factor involved in NK cell development. Accordingly, restoring *Eomes* expression in multipotent progenitors rescues NK cell development *in vivo* and *in vitro* [85, 86]. For the residual NK cells in *Nfil3*$^{-/-}$ mice, their

maintenance and function appear to be normal compared to wild-type NK cells during viral infection, which may be due to strong immunological stimulation during that process [87].

Because of the heterogeneity of NK cells, NFIL3 is not required for the development of all NK cells as several groups of NK cells are largely unaffected in *Nfil3*$^{-/-}$ mice [88, 89].

Taken together, NFIL3 has emerged as a critical regulator of the immune system in the past few years [90]. Given that it is regulated by the circadian clock, NFIL3 may serve as a molecular link that bridges the circadian clock and immune functions.

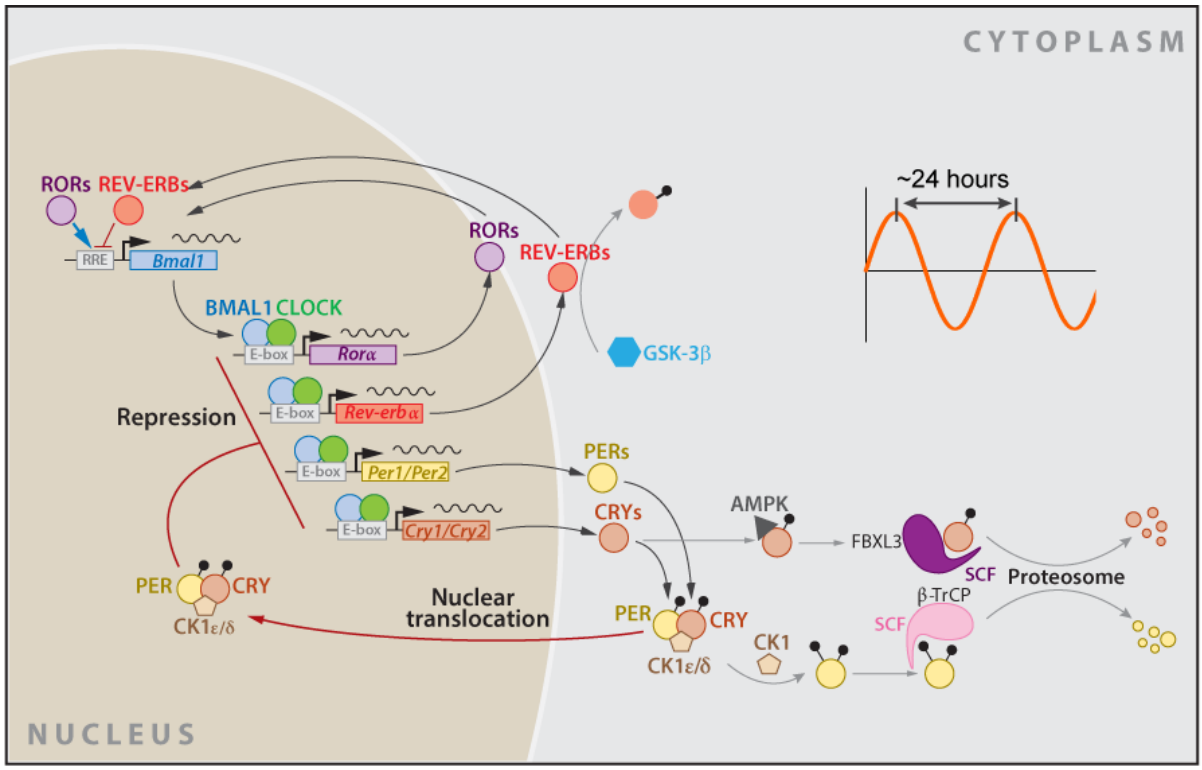

FIGURE 1: The mammalian circadian clock.

Schematic illustrating the molecular clock in mammalian cells (adapted from [1]). Expression of BMAL1 and CLOCK leads to activation of PER/CRY and REB-ERBα/β, which repress BMAL1:CLOCK post-translationally and transcriptionally, respectively. These two negative feedback loops are fine-tuned by CKIε/δ and GSK-3β.

A.

M. musculus	Protein Identity (%)	DNA Identity (%)
vs. H.sapiens	84.6	79.5
vs. P.troglodytes	84.6	79.5
vs. M.mulatta	85.0	79.5
vs. C.lupus	80.3	78.6
vs. B.taurus	83.9	78.7
vs. R.norvegicus	96.1	93.9
vs. G.gallus	76.6	73.5
vs. X.tropicalis	67.4	65.9
vs. D.rerio	53.8	58.4

B.

FIGURE 2: The biochemical nature of NFIL3.

(A) Sequence similarities between mouse NFIL3 and its homologs in other species at the protein and DNA levels. **(B)** Conserved protein domains in mouse NFIL3. The coordinates indicate the starting and ending amino acids of the given domain.

CHAPTER TWO

MATERIALS AND METHODS

Mice

Wild-type C57BL/6J mice were bred in the specified pathogen-free (SPF) barrier facility at the University of Texas Southwestern Medical Center. *Nfil3*$^{-/-}$ mice were imported from the University of Iowa [69] and maintained in the SPF barrier facility. *Rag1*$^{-/-}$ mice (B6.129S7-Rag1^{tm1Mom}/J) and CD45.1^{+} mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were purchased from the Jackson Laboratory, Bar Harbor, Maine. *Nfil3*$^{-/-}$ mice were intercrossed with *Rag1*$^{-/-}$ mice to create *Nfil3*$^{-/-}$;*Rag1*$^{-/-}$ double knockout mice. *Rag2*$^{-/-}$;*Il2rg*$^{-/-}$ mice (B10;B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl}) were acquired from the Taconic Farms, New York. *Rev-erbα*$^{-/-}$ mice (B6.129S2-Nr1d1^{tm1Schb}/Cnrm) were obtained from the European Mutant Mouse Archive [26]. *Rev-erbβ*$^{-/-}$ (B6;129P2-Nr1d2^{tm1Dgen}) mice were purchased from Deltagen, San Mateo, California. *Rev-erbα*$^{+/-}$ and *Rev-erbβ*$^{+/-}$ mice were crossed to generate *Rev-erbα/β*$^{-/-}$ double knockout mice. *Clock*$^{\Delta 19/\Delta 19}$ mice were created as described [21, 22]. Germ-free C57BL/6J mice were raised in gnotobiotic isolators as described previously [91]. All mouse strains, if not specified otherwise, were maintained under 12-h (7:00-19:00) light and 12-h (19:00-7:00) dark conditions. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Texas Southwestern Medical Center at Dallas.

Lamina propria lymphocyte isolation and analysis

Lamina propria lymphocytes were isolated as previously described [92]. Briefly, intestines were dissected from mice and Peyer's patches were removed. Intestines were cut into small pieces and thoroughly washed with ice-cold phosphate buffered saline solution (PBS). Epithelial cells were removed by incubating intestine tissues in Hank's Balanced Salt Solution (HBSS) supplemented with 1mM EDTA and 1mM DTT at 37ºC for 30min with gentle shaking, followed by extensive washing with PBS. Residual tissues were then digested by Collagenase IV (Sigma), DNase I (Sigma) and Dispase (BDbiosciences) at 37ºC for 60 min with gentle shaking. Cells were filtered through 100 μm cell strainers and applied onto a 40%:80% Percoll gradient (GEhealthcare), in which LPLs were found at the interface between the 40% and 80% Percoll fractions.

LPLs were washed with PBS containing 2mM EDTA and 3% Fetal Bovine Serum (FBS). For detection of T_H17 and T_H1 cells by cytokine production, 1~2 x 10^6 cells were stimulated with 50 ng/mL phorbol myristate acetate (PMA), 1 μM ionomycin and 1 μg/mL brefeldin A (Sigma) at 37ºC for 4 hr. Cells were then fixed and permeabilized with BD Cytofix/Cytoperm solution for 30 min, followed by staining with α-CD3ε (500A2), α-IFNγ (XMG1.2), α-CD4 (RM4-5) and α-IL-17A (TC11-18H10.1). For detection T_H17 and T_{reg} by transcription factor expression, freshly-isolated LPL cells were fixed and permeabilized with eBiosciences Mouse Regulatory T Cell Staining Kit #3 per the manufacturer's instruction and stained with α-CD4 (RM4-5), α-Foxp3 (FJK-16s) and α-RORγ (AFKJS-9). To examine ILCs, freshly-isolated LPL cells were first blocked with α-CD16/32 (2.4G2) and then stained with antibodies against cell surface markers such as α-CD3ε (500A2), α-CD19 (ebio1D3),

α-CD5 (53-7.3), α-TCRβ (H57-597), α-TCRγδ (GL3), α-NK1.1 (PK136), α-Sca1 (D7), α-KLRG1 (2F1), α-NKp46 (29A1.4), α-CD45 (30-F11), α-CD45.1 (A20), α-CD45.2 (104), α-hCD2 (RPA-2.10), and α-CD127 (A7R34). Cells were then fixed/permeabilized with eBiosciences Mouse Regulatory T Cell Staining Kit #3 and subjected to nuclear staining with α-RORγ (AFKJS-9) and α-GATA3 (TWAJ). Analysis was performed with a FACSCalibur (BD Biosciences), LSRII (BD Biosciences) or CyAn ADP (Beckman Coulter) flow cytometer and data were processed with the FlowJo software (Tree Star).

Microbiota adoptive transfer

The small intestine, cecum and colon were dissected out of conventionally raised wild-type and *Nfil3*$^{-/-}$ mice and homogenized in ice-cold sterile PBS. The homogenate was spread in the cages as well as on the fur of germ-free wild-type mice [93]. Ex-germ-free mice that received microbiota from wild-type and *Nfil3*$^{-/-}$ mice were maintained in separate gnotobiotic isolators for 6 weeks. Lamina propria lymphocytes were isolated and analyzed as mentioned above.

T cell adoptive transfer

Adoptive transfer of T cells was performed as previously published [94, 95]. Briefly, total CD4^{+} T cells were isolated from the spleens of wild-type and *Nfil3*$^{-/-}$ mice by negative selection with the Dynal® Mouse CD4 Negative Isolation Kit (Invitrogen). Cells were stained with α-CD4 (RM4-5) and CD45RB (C363.16A) and CD4^{+} CD45RB^{hi} cells were sorted out with a MoFlo cell sorter (Beckman Coulter). 4 x 10^{5} purified CD4^{+} CD45RB^{hi} cells were transferred into *Rag1*$^{-/-}$ mice through tail vein injection. Disease conditions were

monitored by weight loss. Lamina propria lymphocytes were isolated and analyzed 4 weeks post transfer.

In vitro differentiation of T_H17 cells

Naïve $CD4^+$ T cells were purified from mouse spleen using the $CD4^+CD62L^+$ T Cell Isolation Kit II (Miltenyi Biotec), as described above and 95% purities were routinely obtained. To culture cells under T_H17 polarizing conditions, 96-well plates were coated with 5μg/mL α-hamster antibody (MP Bio) overnight at 4ºC. Afterwards, plates were washed three times with sterile PBS, and 2 x 10^5 cells were added to each well in the presence of 0.25 μg/mL α-CD3ε (145-2C11), 1 μg/mL α-CD28 (37.51), 5 μg/mL α-IFNγ (XMG1.2), 5 μg/mL α-IL-4 (11B11), 20 ng/mL recombinant murine IL-6 and 1 ng/mL recombinant human TGF-β1. Cells were analyzed 72hr later by intracellular staining of IL-17A after PMA/ionomycin/brefeldin A treatment.

Lentiviral transduction of naïve T_H cells

The *egfp* sequence was amplified from pEGFP-C1 (Clontech) by PCR and cloned into pCDH-CMV-MCS-EF1-Puro (SBI) to generate vector pXY81. *Nfil3* cDNA was cloned from total intestinal cDNA by PCR and inserted downstream of *egfp* in pXY81 to create vector pXY82. HEK293T cells were transfected with either pXY81 or pXY82 in combination with packaging plasmids pVSV-G and pCMVDR9 [96]. Lentiviral particles were purified from cell culture supernatant by poly(ethylene glycol)-8000 precipitation (Sigma), which was subsequently removed by dialysis.

Naïve $CD4^+$ T cells were purified from the spleen of wild-type mice using the $CD4^+CD62L^+$ T Cell Isolation Kit II (Miltenyi Biotec), following the manufacturer's instructions. 2 x 10^5 purified naïve T cells were cultured under T_H17-polarizing conditions in one well of a 96-well plate with plate-bound α-CD3ε (145-2C11) and α-CD28 (37.51), 10 μg/mL α-IFNγ (XMG1.2), 10 μg/mL α-IL-4 (11B11), 20 ng/mL recombinant murine IL-6 and 5 ng/mL recombinant human TGF-β1 (Peprotech) as described [97]. Lentiviral transduction was performed by spinoculation at 1200 *g* for 1 hr at 32°C on the day of polarization. T_H17 cells were analyzed by intracellular cytokine staining 4 days later.

Isolation and analysis of bone marrow progenitors

The femur and tibia were dissected from adult mice and bone marrow cells were released in PBS buffer containing 2mM EDTA and 3% FBS by a pestle and a mortar. Cells were filtered through 70 μm cell strainers and blocked with α-CD16/32 (2.4G2), followed by incubation with biotinylated lineage marker (Lin) antibodies including α-CD3ε (145-2C11), α-B220 (RA3-6B2), α-CD11b (M1/70), α-Gr1 (RB6-8C5), α-Erythroid Cells (TER119), α-CD5 (53-7.3), α-TCRγδ (GL3), and α-NK1.1 (PK136). Cells were then washed and incubated with α-biotin magnetic microbeads (Miltenyi Biotec). Lineage-negative cells were enriched by an autoMACS sorter with the "Depletes" setting. Surface staining were performed with antibodies including α-biotin (Bio3-18E7), α-CD45 (30-F11), α-cKit (2B8), α-CD127 (A7R34), α-Sca1 (D7), α-Flt3 (A2F10) and $\alpha\text{-}\alpha_4\beta_7$ (DATK32). FACS sorting was performed with a FACSAria cell sorter (BD Biosciences) while flow cytometry analysis was carried out with an LSRII (BD Biosciences). Under either situation, LSK cells were

identified as Lin$^-$ Sca1$^+$ cKit$^+$, CLP as Lin$^-$ cKit^{low} CD127$^+$ Sca1^{low} Flt3$^+$ and αLP as Lin$^-$ cKit^{low} CD127$^+$ Sca1^{low} Flt3$^-$ α4β7$^+$. Data were processed with the FlowJo software (Tree Star).

Adoptive transfer of CLPs and αLPs

CLPs and αLPs were purified from CD45.1$^+$ mice by FACS sorting as described above. *Rag2*$^{-/-}$*;Il2rg*$^{-/-}$ recipient mice were sublethally irradiated with a dose of 4.2 Gy on the same day with an XRAD320 irradiator (Precision X-ray, Inc, North Branford, CT). Cells were transplanted into recipient mice by retro-orbital injection. ILCs in recipient mice were examined 5-6 weeks later.

For CLP co-transfer experiments, CLPs were purified from wild-type and *Nfil3*$^{-/-}$ mice and mixed at the 1:1 ratio before transfer.

Retroviral transduction of LSK cells

The TOX coding sequences (CDS) were cloned by PCR from total mouse thymus cDNA into a bicistronic retroviral vector MSCV-ires-hCD2 (generous gift from Dr. Chandrashekhar Pasare at UT Southwestern) to generate a TOX-encoding plasmid, MSCV-*Tox*-ires-hCD2 (pXY130). The MSCV-ires-hCD2 and MSCV-*Tox*-ires-hCD2 plasmids were transfected into the Plat-E packaging cell line [98] with FugeneHD (Promega) to produce retroviral particles. Cell culture supernatant was harvested 48 and 72 hr post transfection. Cell debris was first cleared by spinning at 400 g for 10 min, followed by passing through 0.2 μm sterile filters.

LSK cells were purified from *Nfil3*$^{-/-}$ mice by FACS sorting as described above and seeded into 96-well round-bottom plates at the cell density of 10,000 cells/well in STEMSPAN Serum-Free Expansion Medium (SFEM) (Stemcell Technologies) [99]. During retroviral transduction, cells were mixed with equal volume of cleared retrovirus-containing cell culture supernatant, supplemented with 2 U/mL Heparin (Sigma), 10 ng/mL mouse SCF (Peprotech), 20 ng/mL mouse TPO (Peprotech), 10 ng/mL mouse FGF-1 (Life Technologies) and 4 μg/mL polybrene. Spinoculation was carried out at 1200 g for 90 min at 32ºC to enhance retroviral transduction. Three hours later, cell media was replaced with fresh STEMSPAN SFEM media supplemented with above cytokines but without polybrene. Transduction was performed on two consecutive days, using retroviral supernatant harvest 48 and 72 hr post transfection, respectively.

On day 3, CD45.1^{+} wild-type recipient mice were lethally irradiated at a dose of 9.2 Gy. LSK cells were collected from the 96-well plate with Cell Dissociation Buffer (Life Technologies) and washed with sterile PBS. 2000-4000 cells were transferred into recipient mice in 200uL sterile PBS by retro-orbital injection. ILCs in recipient mice were examined 5-6 weeks later.

NFIL3 knockdown by shRNA in EL4 cells

Five independent shRNA constructs (sh38-sh42) targeting mouse NFIL3 and a control construct containing scramble sequences (sh-scramble) were purchased from Sigma (St. Louis, Missouri). To screen shRNA constructs that can effectively knockdown NFIL3, 1 μg of shRNA plasmid and 1 μg of NFIL3-encoding plasmid (pXY52) were co-transfected into

HEK293T in a 6-well plate with FugeneHD (Promega). Cells were harvested 36 hr later, lysed and used for western blotting with an α-NFIL3 antibody.

shRNA constructs that can effective knockdown NFIL3, namely sh39 and sh40, were then co-transfected with packaging plasmids pCMVDR9 and pVSV-G into HEK293T cells. Cell culture supernatant was harvest 48 and 72 hr post transfection and cleared by spinning and filtering as described above. Lentiviral particles were concentrated by ultracentrifugation at 75,000 g for 2 hr at 4°C and resuspended in RPMI media.

EL4 cells were then mixed with lentiviral particles in the presence of 4 μg/mL polybrene, and spinoculated at 1,200 g for 90 min at 32°C. Two days later, EL4 were selected with 8 μg/mL puromycin for 2 weeks. Live cells were sorted with a FACSAria cell sorter based on propidium iodide exclusion.

NFIL3 overexpression in EL4 cells

NFIL3 coding sequences were subcloned into MSCV-ires-hCD2 to generate MSCV-*Nfil3*-ires-hCD2 (pXY92). MSCV-ires-hCD2 and MSCV-*Nfil3*-ires-hCD2 were then transfected into Plat-E cells to produce retroviral particles as described before, which were used to transduce EL4 cells. Three days after transduction, EL4 cells were stained with α-hCD2 and $hCD2^+$ EL4 cells were purified with a FACSAria cell sorter. Sorted cells were maintained in RPMI media for another 3-4 days, followed by staining and sorting again and resultant cells were expanded. Expression of *Nfil3* and *Tox* was examined by SYBR green-based real-time PCR with specific primers (TABLE 1).

Chromatin immunoprecipitation (ChIP) assays

To detect binding of NFIL3 to the *Rorγt* promoter, total $CD4^+$ T cells were purified from the spleen and lymph nodes of wild-type mice by negative selection with the Mouse CD4 Negative Isolation Kit (Invitrogen). Cells were cross-linked in PBS with 1% formaldehyde for 10 min at room temperature. Crosslinking was terminated by adding 1/5 volume of 1 M glycine and incubating for another 10 min.

Fixed cells were used for chromatin immunoprecipitation (ChIP) with the Magna ChIP assay kit (Millipore) according to the manufacturer's instructions, except that Magna protein A beads were saturated with BSA and salmon sperm DNA before use. Chromatin from approximately 5 x 10^6 cells was used for each immunoprecipitation reaction in combination with 5 μg of goat α-NFIL3 or total goat IgG antibody and 20 μl of Magna protein A beads. The *Rorγt* promoter was detected using SYBR green-based real-time PCR with specific primers: RORγt-ChIPF1: 5'-AAGTGCCAGGAGACGGGCCA-3' and RORγt-ChIPR1: 5'-ACAAGACTGCTAGTCTGGGACACA-3'. Abundance of the *Rorγt* promoter in precipitated DNA was determined as the percentage of total input DNA. Enrichment of the DNA fragment was calculated as the ratio of its abundance in α-NFIL3-immunoprecipitated DNA to that in total goat IgG- immunoprecipitated DNA.

To test binding of NFIL3 to the *Tox* promoter, EL4 cells or *Nfil3*-overexpressing EL4 cells were cultured in RPMI media to 1~0.8 $X10^6$ cells/mL and fixed as described above. Nuclei were isolated with a dounce tissue grinder (Wheaton) in Nuclear Isolation Solution containing 10 mM Tris pH 7.4, 5 mM $MgCl_2$, 25 mM KCl and 250 mM Sucrose, and purified by spinning at 1,000g for 10 min over the Hypertonic Solution containing 10 mM

Tris pH 7.4, 5 mM $MgCl_2$, 25 mM KCl and 30% (wt/v) Sucrose. Purified nuclei were then used for ChIP with the Megna ChIP assay kit (Millipore) as described before. The *Tox* promoter was detected by real-time PCR with specific primers: Tox-ChIPF6: 5'-GACACTGACAGCAAGGACCA-3' and Tox-ChIPR6: 5'-CAGGGCTTCATAGCACCGAT-3'. Enrichment of the *Tox* promoter was determined as before.

Electrophoretic mobility shift assays (EMSA)

To prepare protein for EMSA, HEK293T cells were transfected with either an empty vector or an NFIL3-encoding plasmid. Cells were harvested 2 days after transfection and nuclear extract was prepared with nuclear extraction buffer containing 20 mM Tris pH 8.0, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol and 1X protease inhibitor cocktail (Roche). Protein concentration of nuclear extracts was determined by the Bradford assay and adjusted to 1 mg/mL with nuclear extraction buffer.

EMSA assays were performed as previously described [100], with modifications. DNA oligos were synthesized according to the *Rorγt* promoter sequences encompassing the putative NFIL3-binding site (WT: 5'-GATCTTGAGCAGGTTACTTAATCTCTCTGT-3'; Mutant: 5'-GATCTTGAGCAGGCGGCGGCGTCTCTCTGT-3'). Probes were terminally labeled with ^{32}P by T4 polynucleotide kinase (PNK) and α-^{32}P-ATP (PerkinElmer). 8000 cpm of labeled probes were mixed with 4 μg of nuclear extract in EMSA buffer containing 20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 2 mM DTT, 2 mM $MgCl_2$, 0.25 mg/mL BSA, 5% glycerol, 50 μg/mL poly(dI-dC). For competition with cold probes, 3 pmole of cold

probe was added to the reaction. For supershifting, 100 ng of α–NFIL3 antibody was used. Reactions were incubated at 30ºC for 45 min and loaded onto a 3.5% polyacrylamide gel. Autoradiography was carried out with a storage phosphor screen and images were scanned with a Storm scanner (GE healthcare).

***Luciferase* reporter assays**

To test whether NFIL3 binds to the *Rorγt* promoter, a 1018 bp (from -1013 to +5) fragment of the *Rorγt* promoter was amplified from C57BL/6J genomic DNA by PCR and cloned into pGL3-Basic (Promega) to generate the WT reporter. The putative NFIL3-binding site in the WT reporter was mutated from GTTACTTAA to GTTACTTTA to generate the mutant reporter. Jurkat T cells were transfected with 300 ng of reporter plasmid (WT or mutant), 400 ng of an empty vector (pXY47) or an NFIL3-encoding plasmid (pXY52), 300 ng of P1P2N-HIF1α (a kind gift of Dr. Kevin Gardner at UT Southwestern Medical Center) and 50 ng of pCMV-Renilla-Luciferase, followed by stimulation with 12.5 ng/mL PMA and 0.25 mM ionomycin for 16 hr. Luciferase activities were determined by the Dual-Glo Luciferase Assay kit (Promega) and measured with a SpectraMax M5e plate reader (Molecular Devices). Firefly luciferase activities were first normalized against Renilla luciferase activities and then referenced to the values obtained from cells transfected with pXY47.

To examine regulation of *Tox* by NFIL3, a 2.3kb fragment (-2133 to 232) of the *Tox* promoter were cloned into the pGL3-Basic vector to create the *Tox-luciferase* reporter. HEK293T cells were cultured in a 96-well plate overnight and were co-transfected with the *Tox-luciferase* reporter and an empty (pXY47) or NFIL3-encoding (pXY52) vector. A

pCMV-Renilla-Luciferase reporter was co-transfected into HEK293T to serve as an internal control. Luciferase activities were analyzed as above.

Light cycle perturbation experiments

Four-to-six week old C57BL/6J male mice were housed in ventilated, light-tight cabinets on a 12 hr-light, 12 hr-dark cycle (Phenome Technologies). After acclimation for 3 days, light cycles were changed for mice in treatment groups while remained the same for mice in the control group. On day 1, lights were turned on 6 hr earlier than they were previously (6 hr phase advance). The light cycles were maintained for 4 days and changed in the same way again on day 5. After changing light cycles four times, the mice were maintained on a normal light cycle for 4 days. Mouse intestines were then harvested and lamina propria lymphocytes were isolated and analyzed.

DSS treatment

Age- and sex-matched C57BL/6J mice were maintained under normal light cycles or subjected to perturbed light cycles as described above. After the treatment period, mice were provided 3% DSS (wt/vol) in drinking water. 200 μg mouse IL-17A neutralizing antibody (BioXCell, clone 17F3) or isotype control antibody (BioXCell, clone MOPC-21) in 100 μL PBS was delivered by intraperitoneal injection on day 0, 2 and 4. Mouse weight was monitored daily. All mice were sacrificed on day 5 and colon length was measured.

Citrobacter rodentium infection

The *C. rodentium* (DBS100) strain was originally obtained from ATCC. To infect mice, *C. rodentium* (DBS100) was first inoculated in Luria-Bertani **(**LB**)** broth overnight at 37ºC with shaking in the presence of 50 μg/mL nalidixic acid, and was subcultured into fresh LB media the next morning until OD600 =0.8~1.0. Bacteria were then harvest by centrifugation and resuspended in sterile PBS. *Rag1*$^{-/-}$ and *Nfil3*$^{-/-}$;*Rag1*$^{-/-}$ mice were deprived of food the night before infection and were orally gavaged with 5 x 10^9 CFU in 200 uL sterile PBS. The number of viable *C. rodentium* (DBS100) in the inoculum was confirmed by retrospective plating on nalidixic acid-containing LB-agar plates. Mouse disease conditions were monitored by weight loss.

Statistical analysis

Comparisons between groups were performed with two-tailed Student's t-test, two-tailed Mann-Whitney test, one-way ANOVA or two-way ANOVA as specified in each experiment.

TABLE 1: Primers used in Q-PCR

Primer	Sequences	Description
RORγt-ChIPF1	5'-AAGTGCCAGGAGACGGGCCA-3'	Detection of the mouse *Rorγt* promoter by ChIP-PCR
RORγt-ChIPR1	5'-ACAAGACTGCTAGTCTGGGACACA-3'	
Nfil3-ChIPF1	5'-GCGATGAAACGTGGGCACCG-3'	Detection of the mouse *Nfil3* gene locus by ChIP-PCR
Nfil3-ChIPR1	5'-CGTCACAATGGCTCGTCCGGG-3'	
Nfil3-RTF	5'-CTTTCAGGACTACCAGACATCCAA-3'	Detection of mouse *Nfil3* expression by RT-PCR
Nfil3-RTR	5'-GATGCAACTTCCGGCTACCA-3'	
Rorγt-RTF	5'-TTCACCCCACCTCCACTG-3'	Detection of mouse *Rorγt* expression by RT-PCR
Rorγt-RTR	5'-GTGCAGGAGTAGGCCACATT-3'	
Gapdh-F	5'-TGGCAAAGTGGAGATTGTTGCC-3'	Detection of mouse *Gapdh* expression by RT-PCR
Gapdh-R	5'-AAGATGGTGATGGGCTTCCCG-3'	
Tox-ChIPF6	5'-GACACTGACAGCAAGGACCA-3'	Detection of the mouse *Tox* promoter by ChIP-PCR
Tox-ChIPR6	5'-CAGGGCTTCATAGCACCGAT-3'	
Tox-RTF	5'-CACAAGTTGTCACCCAAGCG-3'	Detection of mouse *Tox* expression by RT-PCR
Tox-RTR	5'-TACAGCGCTTTGTCCCTCTG-3'	

CHAPTER THREE

NFIL3 SUPPRESSES RORγt TRANSCRIPTION AND LINKS T_H17 CELL DIFFERENTIATION TO THE CIRCADIAN CLOCK

The data presented in this chapter has been published in *Science*, volume 342, pages 727-730. This work is reproduced with the permission of the American Association for the Advancement of Science (AAAS).

INTRODUCTION TO T_H17 CELLS IN INTESTINAL IMMUNITY

Intestinal immunity

The mammalian intestine is home to trillions of microbes, which help the host digest food and metabolize nutrients but also pose great challenges to the host's health [37]. Multiple immune mechanisms are in place to defend the host so as to maintain the largely mutually-beneficial relationship between the host and microbes (FIGURE 3A). For example, the intestinal epithelial cells (IEC) form a single cell layer to separate microbes from underlying tissues and thus serve as the first line of defense against invading microbes. IECs can sense microbial signals through TLRs, triggering secretion of antimicrobial peptides like REG3γ and defensins that directly kill invading bacteria [91, 101-103], secretion of mucin glycoproteins to form a viscous mucus layer that physically separates microbes from epithelial cells [104, 105], and mobilization of autophagy machinery to kill intracellular

pathogens by fusing with lysosomes [106]. Other intestinal defense mechanisms include, but are not limited to, IgA production by intestinal B cells and cytokine secretion and microbe-engulfment by phagocytes in the intestinal lamina propria [37]. The functions of these immune cells are partly coordinated by T_H17 cells, which may help achieve optimal immune defenses [107, 108].

The role of T_H17 cells in intestinal immunity

T_H17 cells are a distinct subset of $CD4^+$ T helper (Th) cells that produce the immunoregulatory cytokines IL-17A, IL-17F, IL-21 and IL-22 [109, 110] and are crucial for host defense against extracellular bacteria and fungi. Within the gut, T_H17 cells reside in the lamina propria compartment, directly beneath the intestinal epithelial cells (FIGURE 3B). This location enables them to mount prompt responses against microbial infection.

T_H17 cells regulate the functions of multiple immune compartments through cytokine secretion (FIGURE 3A). IL-17A and IL-17F can function as homodimers or heterodimers and engage a heterotrimeric receptor consisting of two IL-17RA subunits and one IL-17RC subunit, which leads to activation of transcription factors NFκB, AP-1 and C/EBP [111]. IL-22 signals through a heterodimeric receptor comprised of IL-22R1 and IL-10R2 to activate STAT3, STAT1 and STAT5 [112]. Because IL-22R1 expression is mostly restricted to epithelial cells, the biological function of IL-22 is largely limited to the epithelial compartment. T_H17-derived IL-17 and IL-22 activate epithelial cells to produce antimicrobial proteins such as REG3γ, β-Defensins and S100 proteins, cytokines such as IL-6 and granulocyte colony-stimulating factor (G-CSF), and chemokines including chemokine CXC

ligand 8 (CXCL8) [113, 114]. G-CSF is essential in proliferation, survival, and differentiation of granulocyte precursors in the bone marrow, and CXCL8 is critical for neutrophil migration. Thus, T_H17 cells regulate neutrophil development and recruitment to intestinal tissue in order to clear microbial infection [113]. In addition, IL-21 exhibits pleiotropic effects on T cells, B cells and NK cells. Because both IL-17 and IL-21 are important for formation of lymphoid follicles in the intestine, T_H17 cells therefore play an important role in production of microbe-specific antibodies [115]. Additionally, a recent study has shown that T_H17 cells are essential for B cell class-switch to IgA in mice [116].

T_H17 cells not only protect the host from bacterial and fungal infection at mucosal surfaces but also contribute to autoimmune and inflammatory disease if dysregulated [107, 117]. T_H17 and IL-17/22 have been found to be elevated in a wide variety of human diseases, such as psoriasis, rheumatoid arthritis and inflammatory bowel disease (IBD). IL23R, the receptor for T_H17-activating cytokine IL-23, has been identified as an IBD- and psoriasis-risk gene in genome-wide association studies (GWAS) [118, 119]. High T_H17 cytokine expression is also predictive of prognosis in human colorectal cancer patients [120].

Development of T_H17 cells

T helper (Th) cells, including T_H17 cells, differentiate from naïve $CD4^+$ T cells. Upon encountering antigens presented by the antigen presenting cells (APCs), naïve $CD4^+$ T cells can differentiate into several lineages, depending on the ambient cytokine environment (FIGURE 4) [121]. In the presence of IL-12, naïve $CD4^+$ T cells up-regulate the transcription factor T-bet (encoded by *Tbx21*) and become capable of producing the cytokine IFN-γ,

thereby differentiating into T_H1 cells. Similarly, IL-4 guides naïve T helper cells to express the transcription factor GATA-3 and become IL-4/5/13-producing T_H2 cells. TGF-β activates Foxp3 expression and converts naive T helper cells into IL-10/TGF-β-producing T_{reg} cells.

Lineage-specification of T_H17 cells requires cytokines IL-6 and TGF-β, followed by expression of the retinoic acid receptor-related orphan nuclear receptor RORγt [92]. This process involves a complex transcriptional regulatory network, consisting of transcription factors IRF4, BATF, STAT3, c-Maf, and RORγt [122]. IRF4 and BATF bind to DNA in close proximity and pre-pattern chromatin for T_H17 specification. IRF4, BATF and STAT3 together initiate the specification program, reinforce each other's expression, and activate RORγt expression. In the meantime, c-Maf represses BATF expression to serve as a negative regulator, which can be suppressed by RORγt. Interestingly, the major role of RORγt is not to participate in the initiation program but to regulate key loci involving T_H17 cell effector functions such as *Il17a*, *Il17f* and *Il23r*.

The development of intestinal immunity is profoundly affected by environmental factors such as microorganisms [91, 123], dietary nutrients [124], and light cues [125]. In the case of T_H17 cells, it has been shown that their development is influenced by the intestinal microbiota composition, particularly, segmented filamentous bacteria (SFB) [93, 123]. However, little is known about other environmental cues that influence intestinal T_H17 cell development.

RESULTS

NFIL3 suppress T_H17 development in a cell-intrinsic manner.

NFIL3 has been shown to regulate a number of immune processes including natural killer (NK) cell development, pro-B cell survival, and cytokine production by various immune cells [126]. Because *Nfil3* polymorphisms are associated with human inflammatory bowel disease (IBD) [127], we sought to characterize the function of NFIL3 in intestinal immunity by studying *Nfil3*$^{-/-}$ mice. Approximately 10% of *Nfil3*$^{-/-}$ mice housed in our SPF barrier facility, but none of the wild-type (WT) mice housed in the same facility, exhibited rectal prolapse at 6-9 months of age (FIGURE 5A). This was consistent with histopathological evidence of immune cell infiltration into the intestinal mucosa (FIGURE 5B), which was confirmed by a later study [128]. This observation agrees with genome-wide association studies that identified *Nfil3* polymorphisms as a human IBD risk factor.

The spontaneous colitis phenotype prompted us to examine $CD4^+$ T helper subtypes, which are critical for maintaining intestinal homeostasis and often dysregulated in pathological states and inflammatory disease [37]. Intracellular cytokine staining revealed that *Nfil3*$^{-/-}$ mice had significantly higher IL-$17A^+$ T_H17 frequencies than WT mice in both small intestine (FIGURE 6A,B) and colon (FIGURE 6C,D). This was confirmed by nuclear staining of RORγt, the key transcription factor regulating T_H17 development (FIGURE 6E,F). In contrast, there were no differences in IFNγ^+ T_H1 cell frequencies (FIGURE 6A,B). $Foxp3^+$ regulatory T (T_{reg}) frequencies also did not differ between WT and *Nfil3*$^{-/-}$ mice (FIGURE 6E,F), in agreement with a previous report [77]. These findings indicate that NFIL3 deficiency impacts intestinal T_H17 but not T_H1 or T_{reg} cell frequencies.

Because intestinal T_H17 cell development is sensitive to microflora composition [93, 123], we considered whether the differences in T_H17 frequencies between WT and *Nfil3*$^{-/-}$ mice arose from differences in microflora composition between the two genotypes. To test this idea, we adoptively transferred microbiota from conventionally raised WT and *Nfil3*$^{-/-}$ mice into germ-free WT mice. Six weeks after transfer there was no significant difference in T_H17 cell frequencies between mice that had received the WT and *Nfil3*$^{-/-}$ microbiotas (FIGURE 7A,B), indicating that intestinal T_H17 cell expansion in *Nfil3*$^{-/-}$ mice is unlikely to arise from altered microbiota composition. This is further supported by the fact that T_H17 cell frequencies, but not T_H1 frequencies, also increased in the spleens of *Nfil3*$^{-/-}$ mice compared to WT littermates (FIGURE 7C), suggesting that loss of NFIL3 leads to a systemic defect in T_H17 cell development rather than trafficking. However, because T_H17 cell frequencies can be markedly influenced by intestinal microbiota composition as well as age [93, 123], all experiments involving comparisons of different genotypes used age- and sex-matched littermates that were co-housed in order to minimize microbiota composition differences among mice in a given experiment.

T_H17 cell specification is influenced by other immune cells, such as dendritic cells, via cytokine production [108]. We therefore tested whether the increased T_H17 cell frequencies in *Nfil3*$^{-/-}$ mice were due to a T cell-intrinsic NFIL3 deficiency by adoptively transferring naïve WT and *Nfil3*$^{-/-}$ $CD4^+$ T cells into lymphopenic *Rag1*$^{-/-}$ mice [94]. We examined T_H17 and T_H1 cell frequencies in the small intestines of recipient mice 4 weeks after transfer. Mice receiving *Nfil3*$^{-/-}$ T cells had higher T_H17 cell frequencies than those receiving WT T cells, suggesting that NFIL3 suppresses T_H17 cell development in a T cell-

intrinsic manner (FIGURE 7E,F). No significant difference in IFNγ^+ T_H1 cell frequencies was observed between the two groups of mice, confirming that NFIL3 preferentially impacts T_H17 cell development (FIGURE 7E,F).

To assess directly whether NFIL3 suppresses T_H17 development, we overexpressed EGFP-tagged NFIL3 in naïve $CD4^+$ T cells by lentiviral transduction and grew cells under T_H17-polarizing conditions. Since only a fraction of the T cells became transduced, we were able to analyze both transduced ($EGFP^+$) and non-transduced ($EGFP^-$) cells in each experimental sample. $CD4^+$ T cells transduced with lentiviral particles encoding NFIL3 yielded lower T_H17 cell frequencies than non-transduced T cells (FIGURE 7D). In contrast, transduced and non-transduced T cells yielded similar T_H17 cell frequencies when lentiviral particles that did not encode NFIL3 were used. Together, these data indicate that NFIL3 suppresses T_H17 cell development in a T cell-intrinsic manner *in vitro*. Although a prior study found that retroviral transduction of *Nfil3* did not significantly impact T_H17 cell development [122], key differences in the transduction protocol (e.g., lentiviral versus retroviral transduction) likely account for the different experimental outcomes.

NFIL3 represses *Rorγt* expression by binding directly to the *Rorγt* promoter.

We next sought to delineate the mechanism by which NFIL3 suppresses T_H17 development. Analysis of the *Rorγt* promoter sequence revealed a putative NFIL3 binding site (GTTACTTAA) that was conserved in both human and mouse (FIGURE 8A). This suggested that *Rorγt* transcription might be regulated by NFIL3. Accordingly, we found that

expression of *Rorγt* was higher in *Nfil3*$^{-/-}$ T_H17 cells than in WT cells (FIGURE 8B), indicating NFIL3 functions as a repressor on the *Rorγt* promoter.

To test this hypothesis, we performed a chromatin immunoprecipitation (ChIP) assay with an NFIL3-specific antibody [69] and found that NFIL3 directly bound to the *Rorγt* promoter in mouse $CD4^+$ T cells (FIGURE 8C). Electrophoretic mobility-shift assay (EMSA) demonstrated that NFIL3 binding is mediated by the GTTACTTAA motif identified in Fig. 7A (FIGURE 8D). While NFIL3 bound to wild-type probes, mutation of the consensus motif abolished binding. Binding specificity of NFIL3 was further established by competition with unlabeled probe and supershift with an α–NFIL3 antibody.

To assess the biological effects of NFIL3 binding to the *Rorγt* promoter, we cloned the proximal 1 kb promoter fragment upstream of the firefly *luciferase* gene. Luciferase activity was lower in Jurkat T cells when NFIL3 was overexpressed, supporting the idea that NFIL3 represses *Rorγt* transcription (FIGURE 8E). Repression was dependent on the GTTACTTAA motif in the *Rorγt* promoter, as introduction of a point mutation (GTTACTTTA) abolished the repressive effect. Together, our data demonstrate that NFIL3 binds to the GTTACTTAA motif in the *Rorγt* promoter and represses promoter activity.

The circadian clock regulates *Nfil3* expression and thus impacts T_H17 development.

NFIL3 coordinates inputs from multiple regulatory pathways, including the circadian clock [35, 67, 126, 129]. The circadian clock is an autoregulatory transcriptional network driven by the primary activators BMAL1 and CLOCK. It is negatively regulated by two feedback arms, one of which comprises the nuclear receptors REV-ERBα and its close homolog REV-ERBβ

[1], which also have a role in transmitting output pathways [26, 130, 131]. This circadian clock circuitry has been shown to function in $CD4^+$ T cells [49].

Because REV-ERBα directly represses *Nfil3* transcription by binding to the *Nfil3* locus in non-immune cells [36, 131], we asked whether REV-ERBα influences *Nfil3* expression in T cells. We examined activated $CD4^+$ T cells, as *Nfil3* mRNAs are more abundant in activated than in naïve $CD4^+$ T cells (FIGURE 9A), consistent with the global increase in transcriptional activity that occurs in activated lymphocytes [132]. By a ChIP assay with a REV-ERBα-specific antibody, we found that REV-ERBα directly bound to *Nfil3* locus (FIGURE 9B-D). Consistent with the repressive activity of REV-ERBα [36], *Nfil3* expression was higher in *Rev-erbα*$^{-/-}$ $CD4^+$ T cells than in WT cells (FIGURE 10A). Accordingly, naïve *Rev-erbα*$^{-/-}$ $CD4^+$ T cells showed a decreased capacity to differentiate into T_H17 cells after *in vitro* polarization (FIGURE 10B). Consistent with *in vitro* differentiation deficiency of naïve *Rev-erbα*$^{-/-}$ $CD4^+$ T cells, T_H17 cell frequencies were reduced in the intestines of *Rev-erbα*$^{-/-}$ mice (FIGURE 10C,D). In contrast, no differences were observed in T_H1 cell frequencies (FIGURE 10C,D). Together, these data demonstrate that T_H17 cell lineage specification is linked to the clock regulatory network through NFIL3 and REV-ERBα.

To determine whether targeted disruption of other clock components also affects T_H17 cells, we assessed *Clock* $^{\Delta 19/\Delta 19}$ mice, which produce a dominant-negative CLOCK lacking sequences encoded by Exon 19. The mutant CLOCK protein inhibits the function of the BMAL1/CLOCK complex, which is required for REV-ERBα expression (FIGURE 11A) [22, 133]. Consistent with this function of CLOCK, *Clock*$^{\Delta 19/\Delta 19}$ mice exhibited higher *Nfil3*

expression in activated CD4$^+$ T cells (FIGURE 11A). Accordingly, *Clock*$^{\Delta 19/\Delta 19}$ naïve CD4$^+$ T cells shown lowered capacity for T_H17 cell differentiation *in vitro* (FIGURE 11B). This was confirmed by lowered intestinal T_H17 cell frequencies in *Clock*$^{\Delta 19/\Delta 19}$ mice compared to WT mice (FIGURE 11C,D). Interestingly, unlike *Rev-erbα*$^{-/-}$ mice, *Clock* $^{\Delta 19/\Delta 19}$ mice also exhibited lower T_H1 cell frequencies in the intestine (FIGURE 11C,D). This suggests that the circadian clock regulatory network also impacts other intestinal CD4$^+$ T cell subsets such as T_H1 cells. However, since *Rev-erbα*$^{-/-}$ mice do not show altered intestinal T_H1 cell frequencies (FIGURE 11C,D), this must involve regulatory pathways that are distinct from the REV-ERBα-NFIL3 pathway and offers opportunities for future investigation.

T_H17 cell differentiation is circadianly regulated.

Our finding that T_H17 cell development is linked to the clock transcriptional network suggested that T_H17 lineage specification might be influenced by the day-night (light-dark) cycles that entrain the circadian clock. To examine T_H17 development during the circadian cycle, we housed age- and sex- matched mice under either normal light cycles (LD, 12hr light: 12hr dark) or reversed light cycles (DL, 12hr dark: 12hr light) and processed samples from both groups in parallel (FIGURE 12A). We first examined *Nfil3* and *Rorγt* expression in CD4$^+$ T cells at different times during the circadian cycle. *Nfil3* expression was lower during the day and higher at night, while *Rorγt* expression was higher during the day and lower at night (FIGURE 12B,C). There were no significant differences in CD4$^+$ T cell composition at these time points (FIGURE 12D), suggesting that diurnal variation of *Nfil3* and *Rorγt* expression is not due to T helper cell composition difference at these time points.

The expression of *Nfil3* and *Rorγt* in opposite phases of the circadian cycle was consistent with diurnal variation in binding of NFIL3 to the *Rorγt* promoter (FIGURE 12E) and supports our finding that NFIL3 represses *Rorγt* transcription.

Since NFIL3 regulates *Rorγt* in a diurnal manner, we asked whether T_H17 lineage specification varies during the circadian cycle. We found that naïve $CD4^+$ T cells isolated during the day were more likely to differentiate into T_H17 cells after *in vitro* polarization than those isolated at night (FIGURE 12F), in agreement with the diurnal expression patterns of *Nfil3* and *Rorγt*. This difference was abolished in cells lacking *Nfil3* (FIGURE 12F), showing that the diurnal variation in T_H17 lineage specification is *Nfil3*-dependent. Together, these findings show that traversal of the developmental checkpoint specifying the T_H17 lineage is regulated in a diurnal manner and is synchronized across the T cell population by the circadian clock.

Although T cell lineage specification varied in a diurnal manner, we found that intestinal T_H17 cell frequencies did not change significantly during a single 24 hour cycle (FIGURE 12G). This is consistent with the relatively long half-life of these cells [134], and the fact that the entire process of differentiation and proliferation of T_H17 cells requires time periods longer than a single day-night cycle [110, 123]. We therefore tested whether chronic light cycle perturbations, which desynchronize the body's circadian clocks [135], altered T_H17 cell frequencies. We subjected mice to perturbed light cycles by shortening the dark period by 6 hours every 4 days (6 hr phase advance). The whole treatment encompasses 4 phase advances and comprises 16 days, which allows the jet-lag effects on T cells to accumulate (FIGURE 13A). We observed higher T_H17 cell frequencies in the intestines and

spleens of mice maintained under such altered light cycles as compared to mice maintained under a normal light cycle (12h light: 12h dark) (FIGURE 13B). The increase was suppressed in *Rev-erbα*$^{-/-}$ and *Rev-erbα/β*$^{-/-}$ double knockout mice (FIGURE 13C), indicating that the increase in T_H17 cell frequencies requires REV-ERBα/β and is unlikely to arise from non-specific effects of light cycle perturbation. Note that circadian disruption didn't impact T helper cell proliferation (FIGURE 14A-C) and survival (FIGURE 14D,E), suggesting that elevated T_H17 frequencies was due to altered lineage specification during light cycle perturbation. Together, these results show that chronic light cycle perturbation leads to elevated T_H17 cell frequencies in the intestine, and suggest that normal diurnal regulation of T_H17 cell differentiation is important for limiting over-accumulation of T_H17 cells in tissues.

We next tested whether mice subjected to chronic light cycle perturbation exhibit increased susceptibility to intestinal inflammatory disease due to the elevated T_H17 cell frequencies. Administration of dextran sulfate sodium (DSS) to mice induces intestinal injury and leads to intestinal pathology and disease. Mice maintained under a perturbed light cycle were more susceptible to DSS, as measured by weight loss and colon shortening (FIGURE 15A-C). The enhanced DSS-induced pathology was ameliorated by neutralizing IL17A (FIGURE 15A-C), supporting the idea that enhanced disease susceptibility was due to the increased T_H17 cell frequencies.

DISCUSSION

Together, our results demonstrate that NFIL3 suppresses T_H17 cell development by directly repressing *Rorγt* transcription, and links T_H17 cell development to the circadian clock

transcriptional network (FIGURE 15D). This ensures that T_H17 lineage specification occurs during a specific time period during the day-night cycle and is synchronized across the entire T cell population by the circadian clock. Chronic light cycle perturbation results in elevated T_H17 cell frequencies in tissues and enhanced susceptibility to inflammatory disease. This suggests that synchronization of T_H17 lineage specification to the day-night cycle is important for maintaining homeostatic T_H17 cell frequencies and restraining inflammation. We suggest that overaccumulation of T_H17 cells may be limited by ensuring that all T cells within a population traverse this critical developmental checkpoint in synchrony rather than at random times during the day-night cycle.

Modern life often involves chronic circadian disruptions such as those that arise from night shift work or jet lag due to international travel. These light cycle disruptions can contribute to a wide range of human diseases including intestinal inflammatory diseases [136, 137]. Our findings suggest that the pathologic consequences of severe light cycle disturbances may be due in part to direct interactions between the circadian clock transcriptional network and the transcriptional pathways that regulate the development of pro-inflammatory intestinal immune cells such as T_H17 cells.

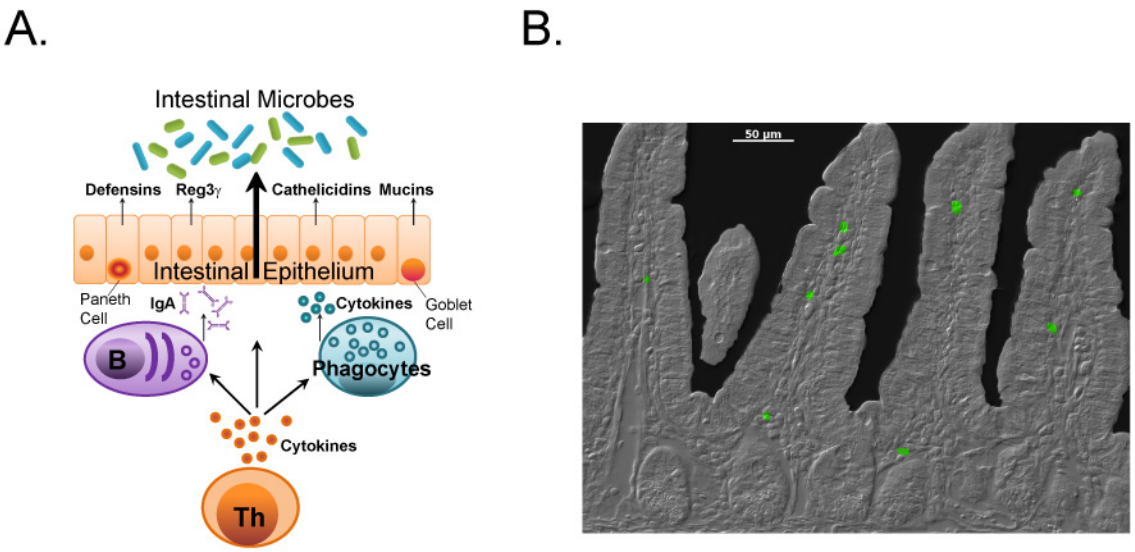

FIGURE 3: T_H17 cells in intestinal immunity.

(A) A simplified model of intestinal immunity. Intestinal epithelial cells secret anti-microbial proteins such as Defensins (mainly by a special type of epithelial cell called Paneth cells), Reg3γ and Cathelicidins to direct kill approaching microbes. Another group of specialized epithelial cells, called Goblet cells, secret Mucin proteins to form a viscous barrier and physically separate luminal microbes from host tissues. B cells residing in the lamina propria produce large quantity of antibodies such as IgA to protect host tissues. Phagocytes engulf and kill invading bacteria, followed by cytokine secretion. The critical role of T helper cells in coordinating the actions of these immune cells is highlighted. **(B)** Fluorescence microscopy imaging showing the localization of T_H17 cells in the small intestine. T_H17 cells were detected by co-staining with α–RORγt and α–CD4 antibodies and pseudocolored in green.

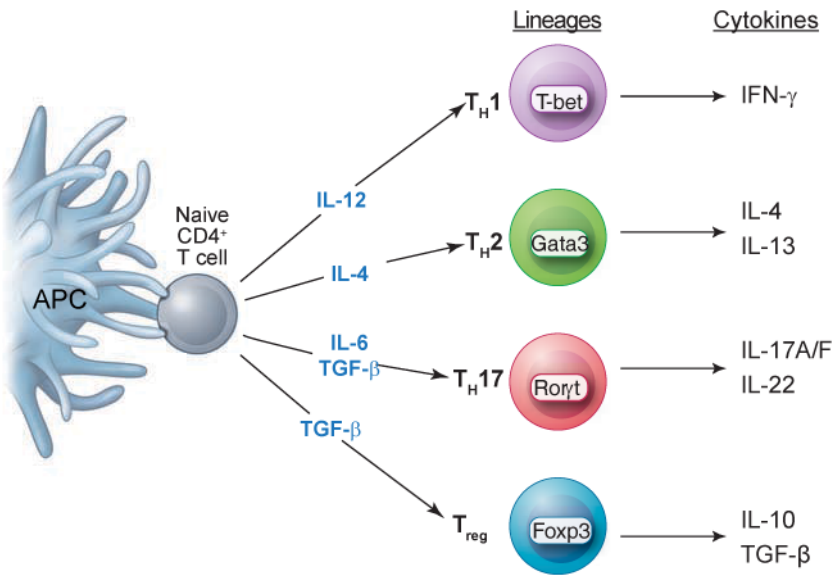

FIGURE 4: Lineage specification of T helper cells.

Schematic showing lineage specification of T_H1, T_H2, T_H17 and T_{reg} cells from naïve $CD4^+$ T helper cells (adapted from [121]). Lineage specification requires proper cytokines during initial antigen encounter, followed by expression of lineage-specifying transcription factors.

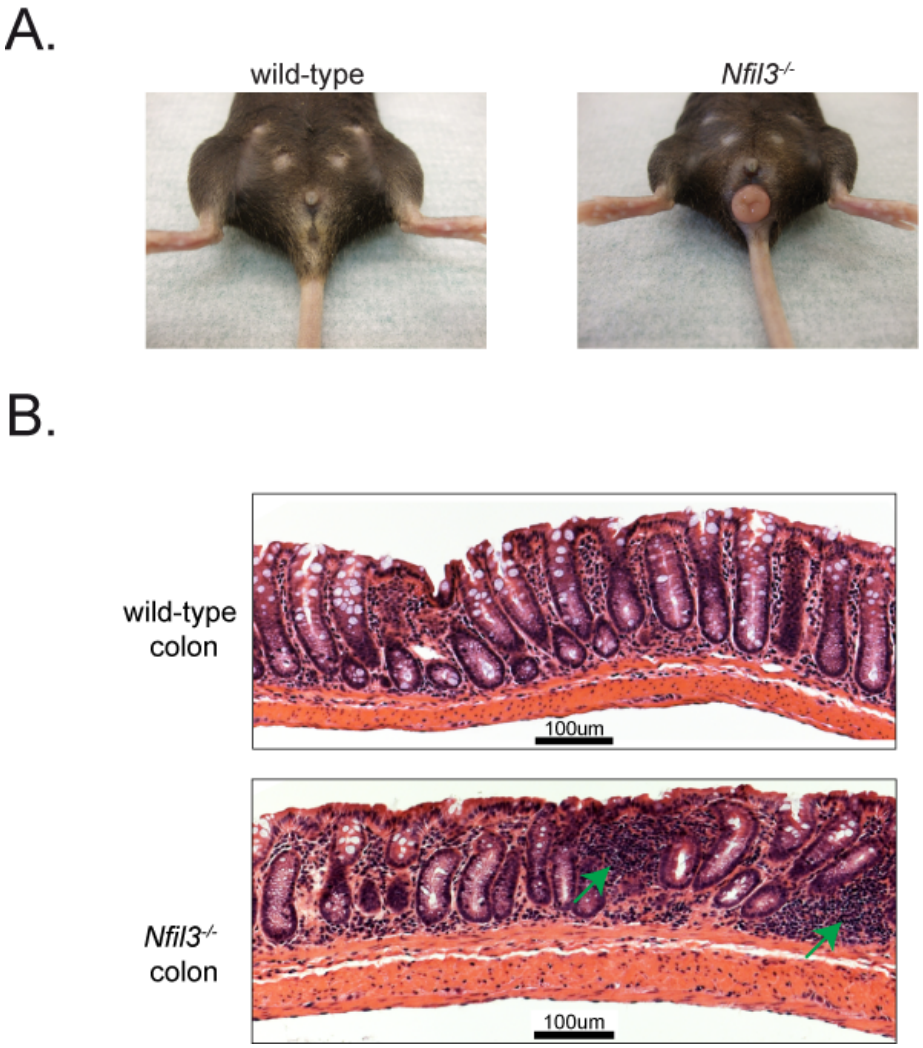

FIGURE 5: Spontaneous colitis in *Nfil3*$^{-/-}$ mice.

(A) Rectal prolapse, one hallmark of murine spontaneous colitis, was observed in 10% of *Nfil3*$^{-/-}$ mice aged 6 to 9 weeks. **(B)** Hematoxylin and eosin (H&E) staining revealed more immune cell infiltration in the colons of *Nfil3*$^{-/-}$ mice (indicated by arrows).

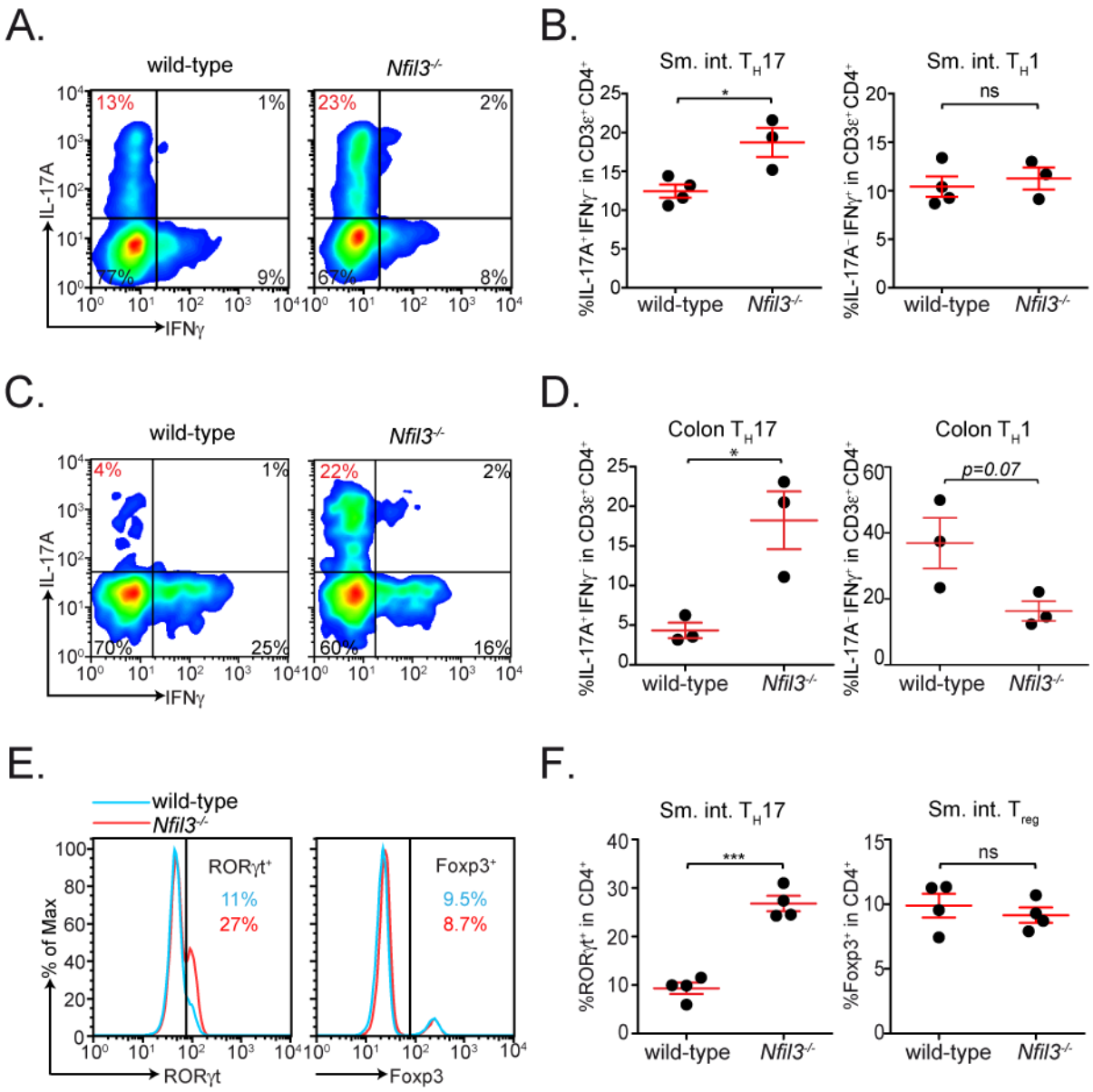

FIGURE 6: T_H17 cell frequencies are selectively elevated in *Nfil3*$^{-/-}$ mice.

(A-D) T_H17 cell frequencies were examined by intracellular cytokine staining. Lamina propria lymphocytes (LPLs) were isolated from the small intestines (A,B) and colons (C,D) of age-matched, co-housed wild-type and *Nfil3*$^{-/-}$ mice (littermates) and stimulated with PMA and ionomycin in the presence of brefeldin A. Representative flow cytometry plots of IL-17A

and IFNγ staining are shown in (A,C) and combined data are shown in (B,D). **(E,F)** T_H17 cell and T_{reg} frequencies were examined by detection of nuclear transcription factors RORγt and Foxp3. Representative histograms are shown in (E**)** and combined data are shown in (F). Groups were compared with two-tailed student's t-test. ∗, $p<0.05$; ***, $p<0.001$; ns, not significant.

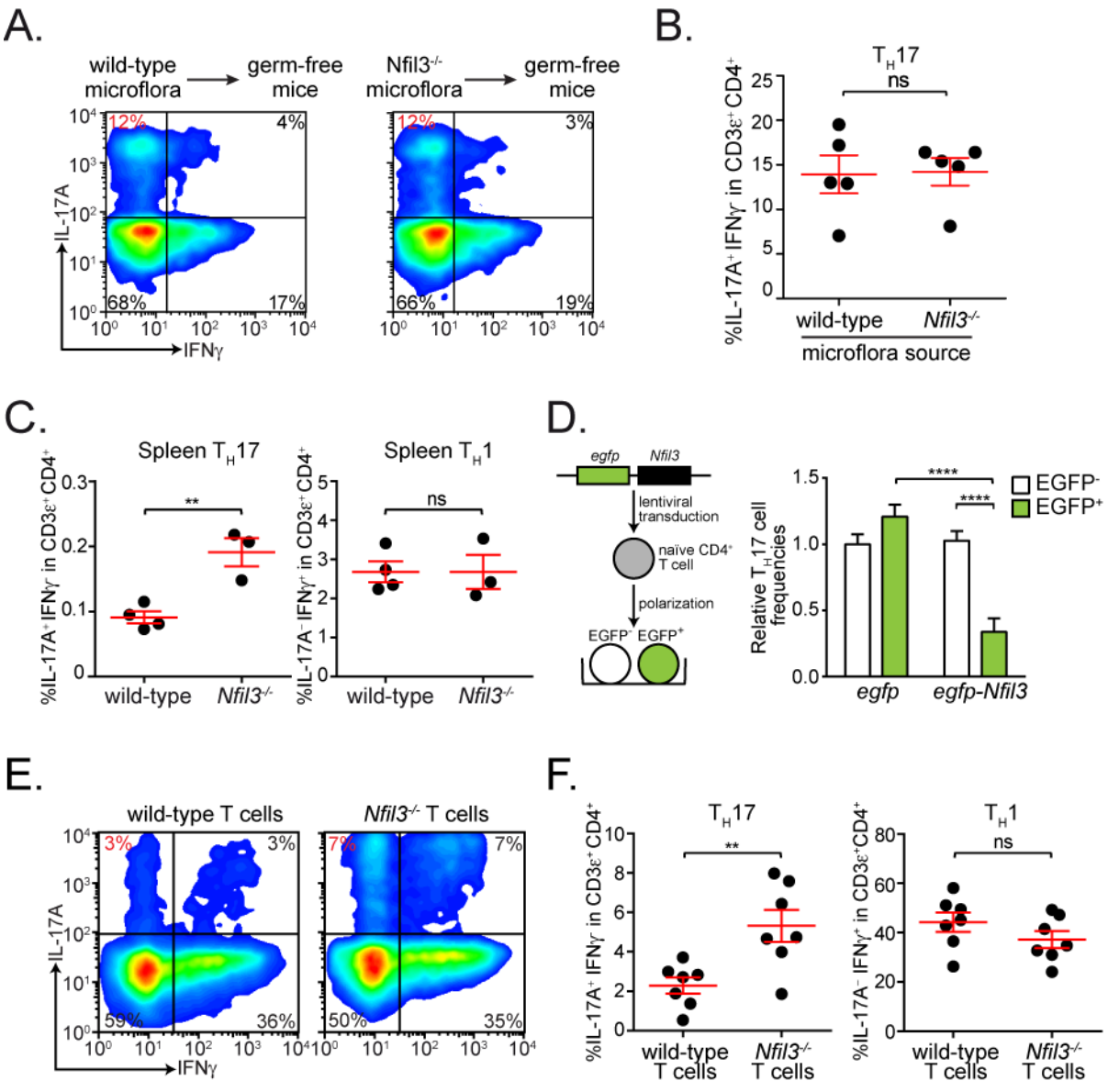

FIGURE 7: NFIL3 suppresses T_H17 development in a cell-intrinsic manner.

(A-B) Increased T_H17 cell frequencies in *Nfil3*$^{-/-}$ mice are not transferrable to wild-type germ-free mice by microflora transplantation. Microflora from wild-type or co-housed *Nfil3*$^{-/-}$ mice were transplanted into wild-type germ-free recipients and lamina propria lymphocytes were analyzed 6 weeks later. Representative FACS plots are shown in (A) and data from

multiple mice are shown in (B). **(C)** T_H17 and T_H1 cell frequencies in the spleens of wild-type and *Nfil3*$^{-/-}$ mice were examined by cytokine production. **(D-F)** NFIL3 suppresses T_H17 cell development in a T cell-intrinsic manner. (D) NFIL3 suppresses T_H17 cell development *in vitro*. Naïve wild-type $CD4^+$ T cells were transduced by lentivirus encoding EGFP only or EGFP-tagged NFIL3, followed by polarization with IL-6 and TGF-β1. T_H17 cell frequencies were compared between transduced ($EGFP^+$) and non-transduced ($EGFP^-$) $CD4^+$ T cell populations in the same sample (well). (E,F) Naïve wild-type and *Nfil3*$^{-/-}$ $CD4^+$ T cells were transferred intravenously into *Rag1*$^{-/-}$ mice and LPLs were analyzed 4 weeks later. Representative FACS plots are shown in (E) and T_H17 and T_H1 frequencies from two independent experiments are summarized in (F). Comparison between groups was done by two-tailed student's t-test (B, C, F) or one-way ANOVA (D). **, $p<0.01$; ****, $p<0.0001$; ns, not significant.

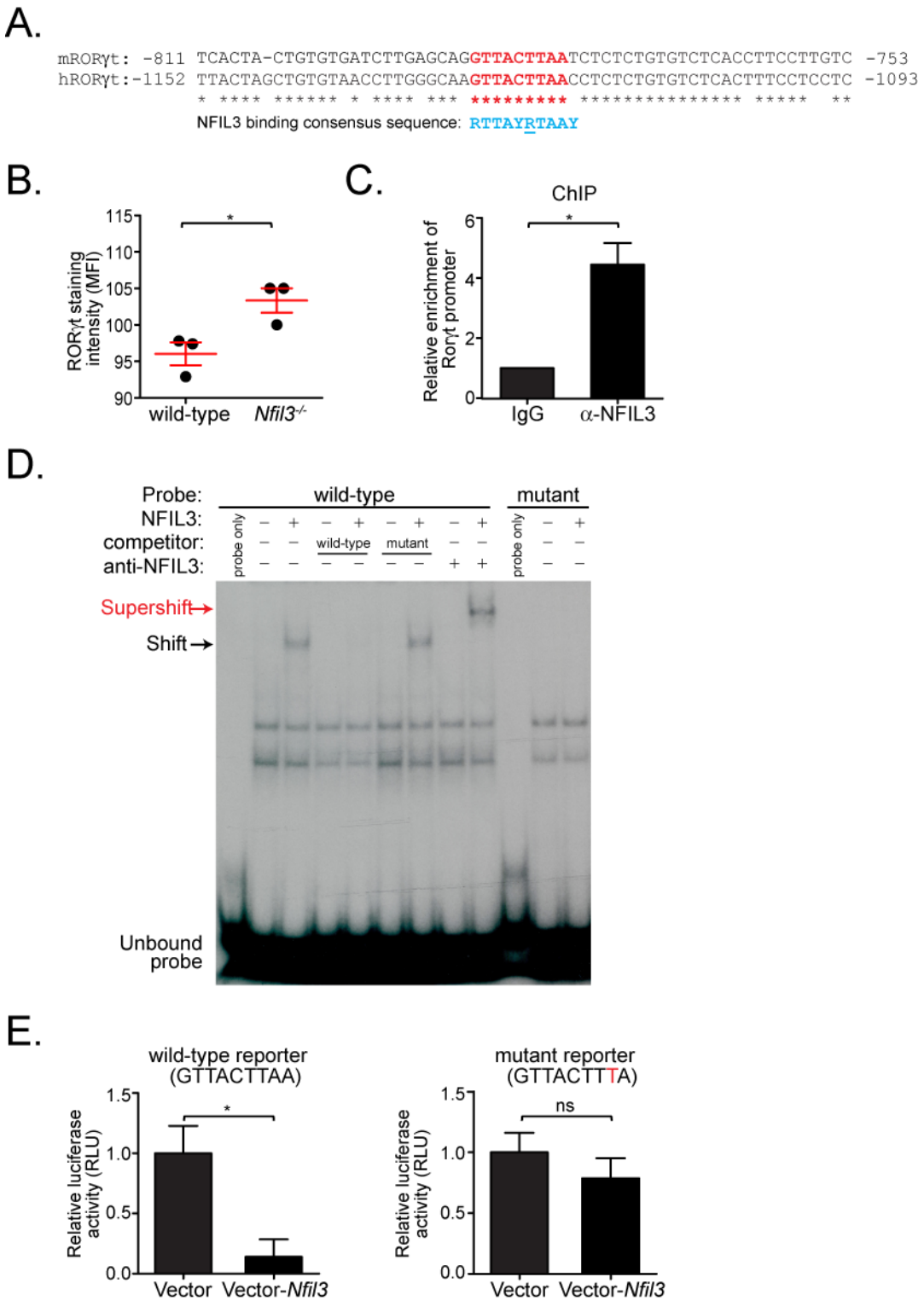

FIGURE 8: NFIL3 directly represses *Rorγt* by binding its promoter.

(A) Sequences of the mouse and human *Rorγt* promoter regions encompassing a putative NFIL3 binding site (red) were aligned. The position that does not agree with the known NFIL3 consensus sequence is underlined. **(B)** *Rorγt* expression is higher in *Nfil3*$^{-/-}$ T_H17 cells. LPLs from wild-type and *Nfil3*$^{-/-}$ mice were analyzed by nuclear staining of RORγt and mean fluorescence intensities (MFI) were plotted. **(C)** NFIL3 binds to the *Rorγt* promoter *in vivo*. Total $CD4^+$ T cells were enriched from the spleen and lymph nodes of wild-type mice by negative selection. Cells were then fixed and used for chromatin-immunoprecipitation (ChIP) with IgG or α–NFIL3 antibody. Enrichment was calculated as the ratio of DNA pulled-down by α–NFIL3 antibody to that by IgG control. **(D)** NFIL3 binds to the *Rorγt* promoter *in vitro*. HEK293T cells were transfected with an empty vector or an NFIL3-encoding vector. Nuclear extracts were then prepared for EMSA. A 30-bp DNA fragment with the same sequence as the *Rorγt* promoter encompassing the NFIL3-binding site was synthesized as probe (wild-type probe) while another fragment with the NFIL3 binding site mutated to GCGGCGGCG was used as control (mutant probe). NFIL3 binding specificity was demonstrated by using non-radioactively labeled wild-type and mutant probes as competitors and supershift with α–NFIL3 antibody (red arrow). **(E)** NFIL3 represses *Rorγt* promoter activity. A 1018 bp (from -1013 to +5) fragment of the *Rorγt* promoter was fused with firefly luciferase and a mutant was generated by incorporating an A→T mutation at position 8 of the NFIL3 binding site. Jurkat T cells were transfected with reporters and an empty vector or an NFIL3-encoding vector. Luciferase activity was normalized to cells transfected with vector-only controls. Comparison between groups was done by two-tailed student's t-test. ∗, $p<0.05$; ns, not significant.

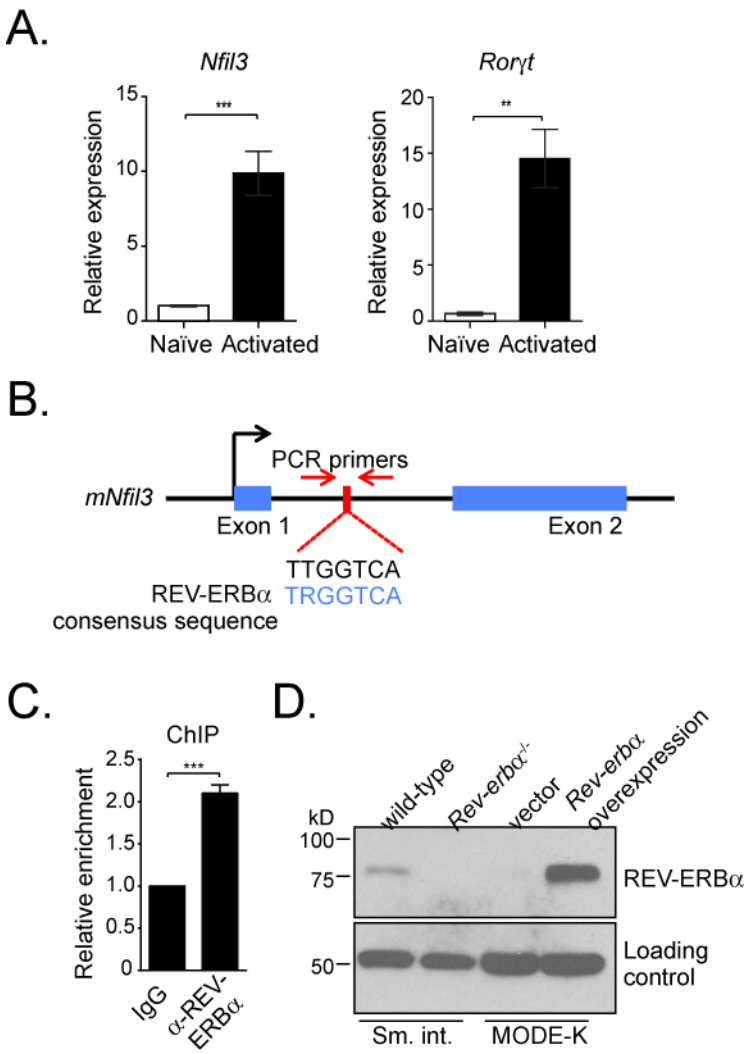

FIGURE 9: *Nfil3* is regulated by REV-ERBα in T cells.

(A) *Nfil3* and *Rorγt* expression are elevated in activated CD4$^+$ T cells. Naïve and activated T helper cells were purified by FACS sorting. Gene expression was determined by real-time PCR. Elevated *Nfil3* expression is attributable to the fact that activated T cells show a global increase in transcriptional activity relative to naïve T cells [132]. **(B-D)** REV-ERBα binds directly to the *Nfil3* gene locus in CD4$^+$ T cells. (B) Schematic presentation of the REV-ERBα binding site in the *Nfil3* gene locus and primers designed for ChIP-PCR. (C) REV-

ERBα binds to the *Nfil3* gene locus in $CD4^+$ T cells as demonstrated by ChIP with a REV-ERBα-specific antibody. Data were pooled from five independent experiments. (D) The specificity of REV-ERBα antibody was examined by Western blotting of small intestinal protein extracts from wild-type and *Rev-erbα*$^{-/-}$ mice (left) and MODE-K cells transfected with empty vector or a REV-ERBα-encoding plasmid (right). Statistical analysis was performed with two-tailed student's t-test. **, $p<0.01$; ***, $p<0.001$.

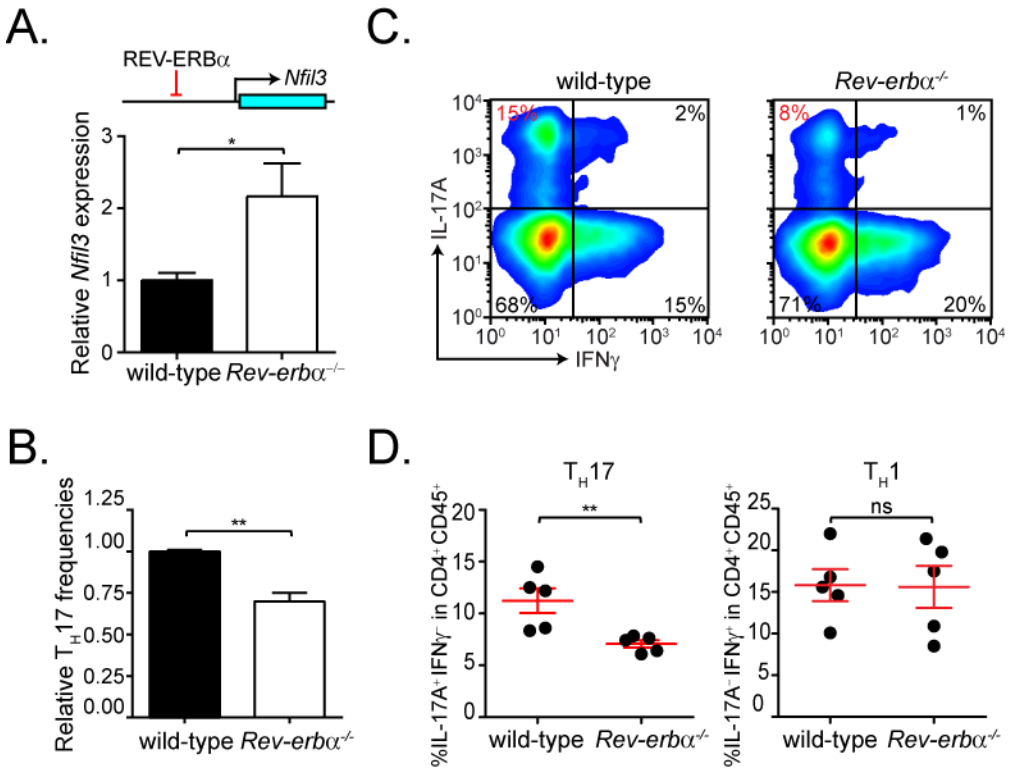

FIGURE 10: *Rev-erbα*$^{-/-}$ T cells exhibit T_H17 differentiation deficiency.

(A) *Nfil3* expression is higher in activated *Rev-erbα*$^{-/-}$ CD4$^+$ T cells than in wild-type cells, consistent with the fact that REV-ERBα is a direct repressor of *Nfil3* transcription (diagram) [36, 131]. Activated CD4$^+$ T cells were isolated by negative selection from wild-type and *Rev-erbα*$^{-/-}$ littermates. *Nfil3* expression levels were determined by real-time PCR. **(B)** Naïve *Rev-erbα*$^{-/-}$ CD4$^+$ T cells are less likely to develop into T_H17 cells after *in vitro* polarization. **(C,D)** T_H17 cell frequencies are reduced in the small intestines of *Rev-erbα*$^{-/-}$ mice. Representative flow cytometry plots are shown in (C) and combined data are shown in (D). Statistics were performed with the two-tailed student's t-test. ∗, $p<0.05$; ∗∗, $p<0.01$; ns, not significant.

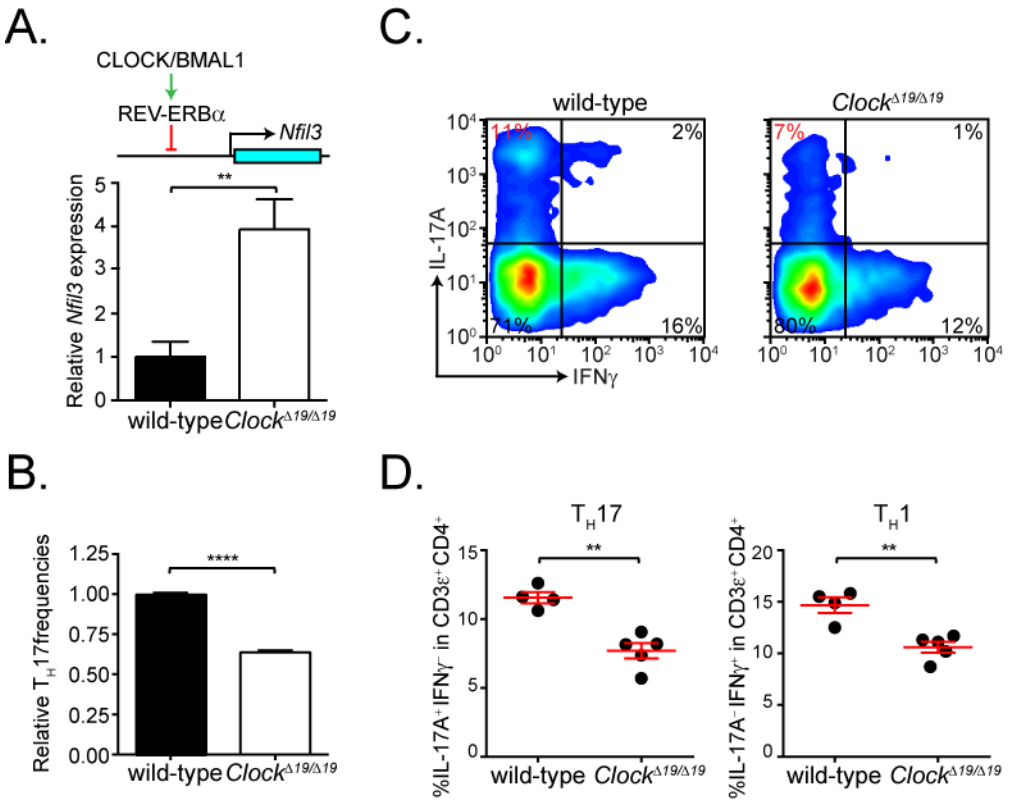

FIGURE 11: *Clock*$^{\Delta 19/\Delta 19}$ T cells show T_H17 differentiation deficiency.

(A) *Nfil3* expression is higher in activated *Clock* $^{\Delta 19/\Delta 19}$ $CD4^+$ T cells than in wild-type cells, consistent with the fact that CLOCK is a direct activator of *Rev-erbα* transcription (diagram) [22, 130]. **(B)** Naïve *Clock* $^{\Delta 19/\Delta 19}$ $CD4^+$ T cells are less likely to develop into T_H17 cells *in vitro*. **(C,D)** T_H17 cell frequencies are reduced in the small intestines of *Clock* $^{\Delta 19/\Delta 19}$ mice. Representative flow cytometry plots are shown in (C) and combined data are shown in (D). Statistics were performed with the two-tailed student's t-test. **, $p<0.01$; ***, $p<0.001$.

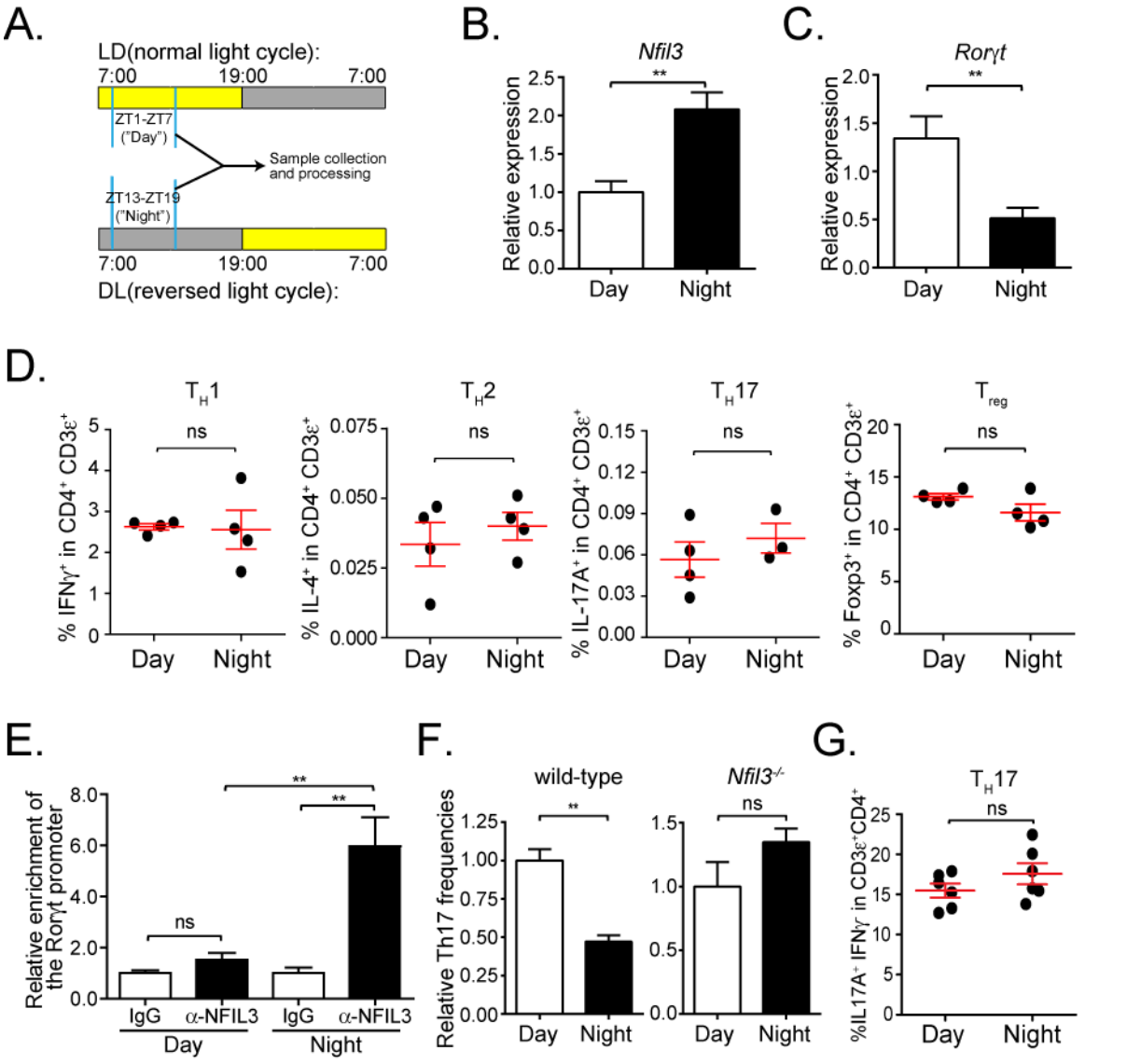

FIGURE 12: T_H17 differentiation is regulated in a circadian manner.

(A) Experimental setup for diurnal studies. Two groups of mice were maintained under opposite light cycles and analyzed at the same time. Samples processed during Zeitgeber Time (ZT)1-ZT7 are labeled as "Day" and those during ZT13-ZT19 as "Night". **(B,C)** *Nfil3* (B) and *Rorγt* (C) expression in activated $CD4^+$ T cells during the circadian cycle. **(D)** The frequencies of T_H1, T_H2, T_H17 and T_{reg} in splenic $CD4^+$ T cells were examined during the

day and at night. **(E)** Diurnal NFIL3 binding to the *Rorγt* promoter as determined by ChIP assay. **(F)** Relative percentage of IL-17A-producing T_H17 cells after *in vitro* polarization of naïve wild-type (left) or *Nfil3*$^{-/-}$ (right) CD4^{+} T cells. **(G)** The frequencies of T_H17 cells were examined in the intestine of mice during the day and at night. Statistics were performed with the two-tailed student's t-test. **, $p<0.01$; ns, not significant.

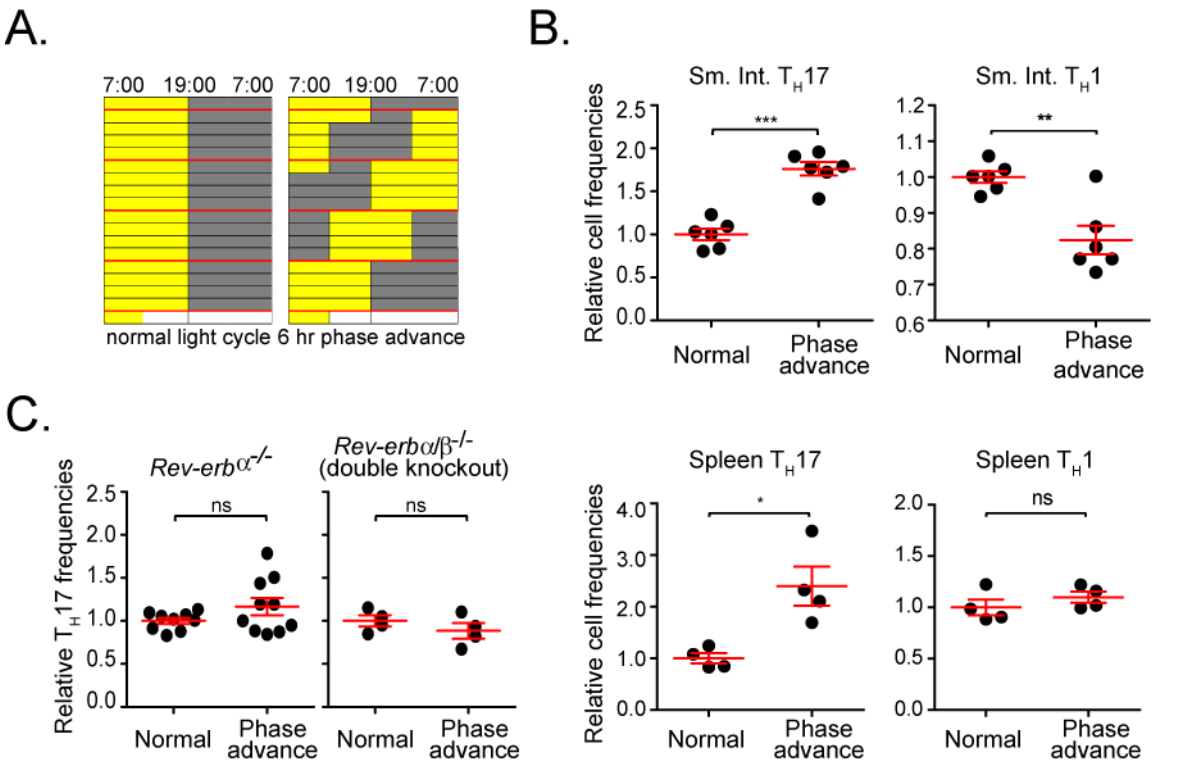

FIGURE 13: Disturbing the circadian clock disrupts T_H17 cell homeostasis in the mouse intestine.

(A) Experimental setup: wild-type, *Rev-erbα*$^{-/-}$ or *Rev-erbα/β*$^{-/-}$ double knockout mice were maintained under a normal light cycle (left) or were subjected to a 6 hour phase advance in which the light cycle changed every 4 days, followed by maintenance under a normal light cycle for 4 days (right). T_H17 and T_H1 cells were then examined and calculated relative to the age-matched, co-caged controls in each experiment. **(B)** T_H17 and T_H1 cell frequencies in the small intestine (upper) and spleen (lower) of wild-type mice under normal or disturbed light cycles were examined. **(C)** Intestinal T_H17 frequencies in *Rev-erbα*$^{-/-}$ and *Rev-erbα/β*$^{-/-}$ mice

under normal or disturbed light cycles were examined. Statistics were performed with the two-tailed student's t-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant.

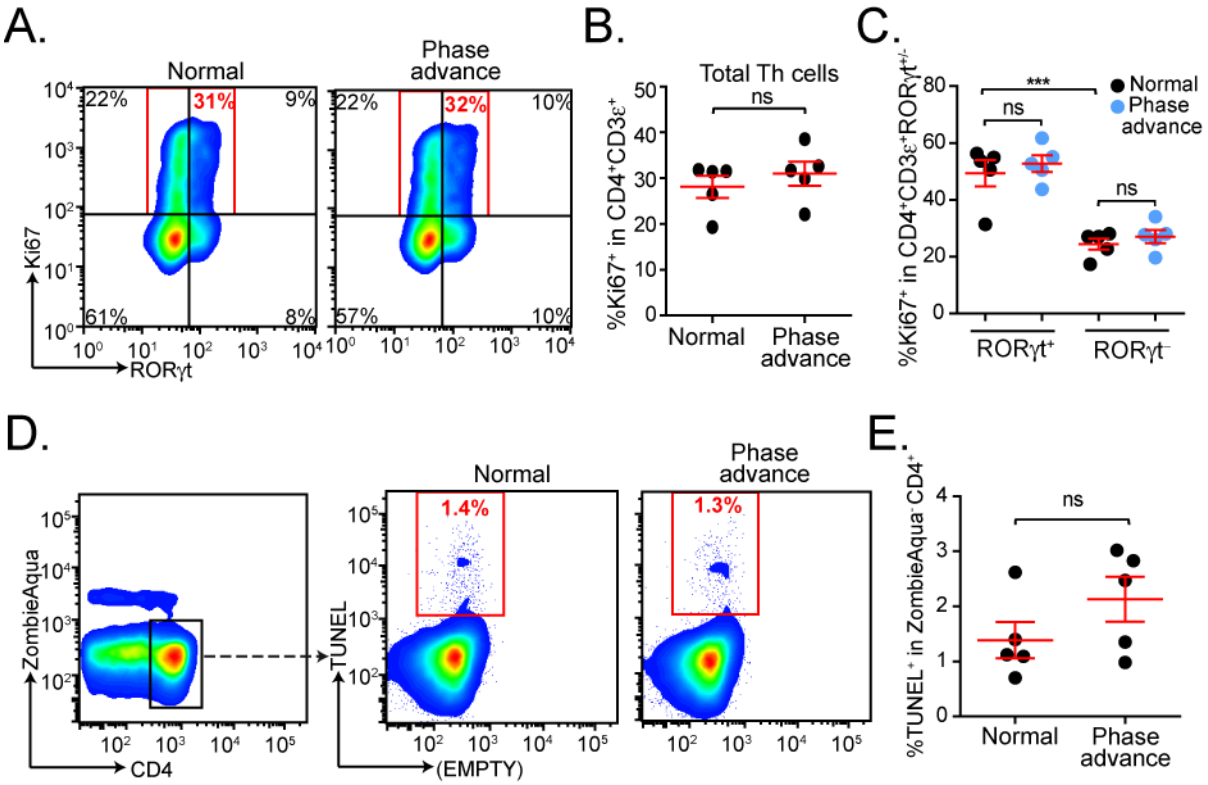

FIGURE 14: Circadian disruption does not impact proliferation or apoptosis of intestinal T helper cells.

(A-C) Proliferation of intestinal T helper cells was analyzed by nuclear staining of Ki67 and RORγt. Representative flow plots are shown in (A). Percentages of $Ki67^+$ cells in total T helper cells and in $ROR\gamma t^+$ and $ROR\gamma t^-$ fractions are shown in (B) and (C), respectively. **(D-E)** Apoptosis of intestinal T cells was analyzed by TUNEL staining. The gating strategy and representative flow plots are shown in (D). Percentages of $TUNEL^+$ cells in total T helper cells are summarized in (E). Statistical analysis was performed with the two-tailed student's t-test. ***, $p<0.001$; ns, not significant.

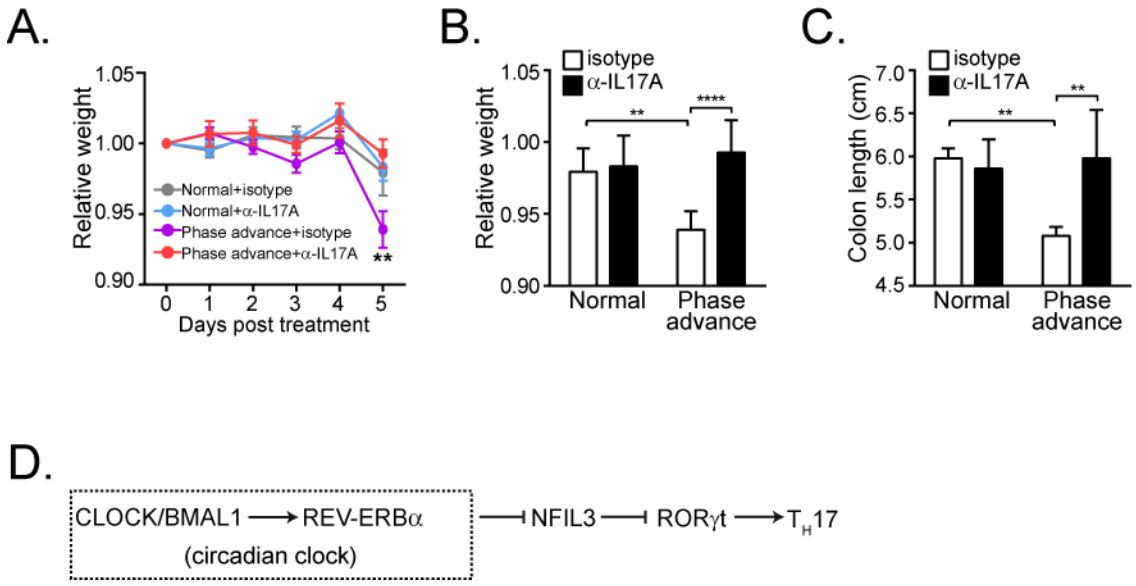

FIGURE 15: Circadian disruption increases mouse susceptibility to DSS-induced colitis, which is mediated by IL-17.

(A-C) Wild-type mice subjected to normal or perturbed light cycles were treated with DSS to induce colitis. An IL-17A-neutralizing antibody or IgG control was delivered by intraperitoneal injection on Day 0, 2 and 4. Disease severity was monitored by mouse weight loss during the treatment (A). Disease severity on Day 5 was assessed by weight loss (B) and colon shortening (C). **(D)** Schematic diagram summarizing how NFIL3 links the circadian clock circuitry to T_H17 cell development. Statistical analysis was performed with the two-way ANOVA with posttests. **, $p<0.01$; ****, $p<0.001$.

CHAPTER FOUR

NFIL3 IS REQUIRED FOR INNATE LYMPHOID CELL DEVELOPMENT

INTRODUCTION TO INNATE LYMPHOID CELLS

Discovery of innate lymphoid cells

Innate lymphoid cells (ILCs) have been recently recognized as important players in organogenesis and early immune defense against infection [138]. ILCs share morphological characteristics of lymphoid cells with commonly known lymphocytes such as T cells and B cells: they lack of granules, have a compact nucleus, and a transparent cytoplasm. In contrast to T and B cells that express rearranged antigen-specific receptors and require priming during immune responses, ILCs lack antigen-specific receptors and respond promptly to a wide-range of immune signals.

The first known ILCs were natural killer (NK) cells [139, 140]. NK cells recognize proteins expressed on virally-infected or stressed cells as well as cancer cells and exert cytotoxic (cell-killing) activities and secret the cytokine IFN-γ. Development of NK cells requires the T-box transcription factors T-bet and EOMES [141]. Another group of well-known ILCs are lymphoid tissue inducer (LTi) cells [142]. LTi cells express membrane bound lymphotoxin $LT\alpha_1\beta_2$, which interacts with LTβR on stromal cells to activate expression of adhesion molecules and chemokines necessary for recruitment of more LTi

cells, B cells, T cells and DCs. LTi cells thus are critical for prenatal formation of secondary lymphoid organs such as lymph nodes and Peyer's patches as well as postnatal development of tertiary lymphoid tissues such as isolated lymphoid follicles (ILFs) in the intestine [143]. Upon stimulation by the cytokine IL-23 or microbial signals such as zymosan, LTi cells can also produce the pro-inflammatory cytokines IL-17 and IL-22 [144]. Development of LTi cells has been shown to depend on the transcription factor RORγt [145].

Recently, multiple new types of ILCs have been discovered, which differ in tissue distribution, immune functions and developmental pathways. These cells include, but are not limited to, an $NKp46^+$ subset in the intestine that produces IL-22 [146], a $cKit^+$ $Sca1^+$ subset in the mesenteric adipose tissue that secrets T_H2 cytokines IL-5 and IL-13 [147], nuocytes that express IL-4/5/13 in the intestine [148], a lung-resident $CD127^+$ $CD25^+$ $ST2^+$ subset that produces IL-5/13 and amphiregulin [149], an intraepithelial subset that produces IFN-γ [150] and another $NK1.1^+$ $NKp46^+$ $EOMES^-$ $T\text{-}bet^+$ subset in the intestine that secrets IFN-γ and exhibits poor cytotoxic activity [151].

Functional and developmental categorization of ILCs

As discussed above, the functions of ILCs resemble those of T helper cells in term of cytokine production. These cytokines are not only critical for immune defense against bacteria [146], virus [149], Toxoplasma [151] and helminth [147, Neill, 2010 #967] infections but are also important for immune homeostasis. For example, IL-5 and IL-13 produced by ILCs control eosinophil homeostasis and diurnal oscillation in mice [151].

RORγt$^+$ IL-22-producing ILCs have been shown to contain symbiotic *Alcaligenes* species in lymphoid tissues such as Peyer's patches and lymph nodes [152]. In addition to cytokine production, RORγt$^+$ ILCs also inhibit T cell activation by antigen presentation on MHC-II in order to limit immune responses to intestinal commensal bacteria [153].

The development of ILCs is not well understood, though transcriptional programs required for some ILC subsets have been identified [154]. In particular, it is not clear whether ILCs develop from a common progenitor population. Such a common ILC progenitor (CILP) has been hypothesized based on dependence of ILC development on *Id2* and IL-7, with the notable exception of NK cells.

Nevertheless, it has been suggested that ILCs are categorized into three different types, according to their function and developmental requirement (FIGURE 16) [138]. ILC1 (including NK cells) require the transcription factors T-bet and/or EOMES and produce interferon-γ (IFNγ) [140, 141, 150], thereby resembling T_H1 cells. ILC2 require the transcription factor GATA-3 and produce IL-5/13 and amphiregulin [147-149, 155], mimicking T_H2 cells. ILC3, including lymphoid tissue inducer (LTi) cells, depend on the transcription factor RORγt and secrete IL-17/22 [144, 146, 156, 157], similar to T_H17 cells. However, it is still under debate whether NK cells should be considered as a type of ILCs distinct from other ILC1 cells, because i) NK cells possess cytotoxic activity while other ILC1 don't, which makes NK cells functionally more similar to cytotoxic T cells instead of T helper cells [150, 151]; ii) NK cell development strictly relies on EOMES, but mildly on T-

bet, while other ILC1 cells depend on T-bet; and iii) NK cell development and functional maturation requires IL-15 while other ILCs require IL-7 [158].

Dysregulation of ILCs has been implicated in human diseases such as inflammatory bowel disease and allergy [138]. Despite the essential role of ILCs in human health and disease, the pathways underlying ILC development remain unclear. Specifically, prior studies have identified precursors that express the transcription factor *Id2* or *Zbtb16* (encoding PLZF) and give rise to ILC2 and several subtypes of ILC3 and ILC1, but do not differentiate into NK cells [151, 159], suggesting that developmental pathways common to all ILCs remain to be identified.

RESULTS

NFIL3 is required for innate lymphoid cell development in mice

We previously showed that the transcription factor NFIL3 represses *Rorγt* expression in $CD4^+$ T cells and thus suppresses T_H17 cell development [160]. Because RORγt is also essential for ILC3 development [138, 154], we predicted that ILC3 frequencies would be elevated in *Nfil3*$^{-/-}$ mice, similar to T_H17 cells [160]. However, we observed lower frequencies of total ILC3 ($CD45^+$ $CD3^-$ $CD19^-$ $ROR\gamma t^+$ $CD127^+$) in the small intestinal lamina propria of *Nfil3*$^{-/-}$ mice than in wild-type mice (FIGURE 17A,B). Among ILC3, the $NKp46^+$ subtype (also known as NK22) [146, 161], was markedly decreased in *Nfil3*$^{-/-}$ mice (FIGURE 17A,B), consistent with previous findings [128, 151]. ILC3 also include lymphoid tissue inducer cells (LTi), which are critical for prenatal formation of secondary lymphoid

tissues such as the Peyer's patches in the small intestine [156]. *Nfil3*$^{-/-}$ mice had fewer and smaller Peyer's patches in the small intestine and remaining Peyer's patches contained fewer LTi cells (RORγt^{+} LTβ^{+}) than wild-type mice (FIGURE 17C,D). Thus, *Nfil3*$^{-/-}$ mice have a general deficiency in ILC3 development.

Similar to ILC3, *Nfil3*$^{-/-}$ mice had markedly lower frequencies of ILC2 (CD45^{+} CD3^{-} CD19^{-} GATA3^{+} Sca1^{+}; FIGURE 18A,B) in the small intestine, which was further confirmed by gating on another ILC2 marker KLRG1 [155] (FIGURE 18A,B). Reduction of GATA3^{+} ILC2 in *Nfil3*$^{-/-}$ mice coincided with a decrease in the frequency of ILCs that produced the ILC2 signature cytokine, IL-5 (FIGURE 18C,D). Thus, both ILC2 and ILC3 are deficient in *Nfil3*$^{-/-}$ mice. Given that *Nfil3*$^{-/-}$ mice are known to be deficient in ILC1 [79, 80, 151], including NK cells, our data indicate that NFIL3 is required for the development of all three types of innate lymphoid cells.

NFIL3 is required for ILC development in a cell-intrinsic manner.

ILCs develop from common lymphoid progenitors (CLPs) [155, 162], which are present in normal numbers in *Nfil3*$^{-/-}$ mice [85, 86]. To test whether the requirement for NFIL3 is CLP-intrinsic, we co-transferred wild-type and *Nfil3*$^{-/-}$ CLPs (Lin^{-} cKit^{low} CD127^{+} Sca1^{low} Flt3^{+}) into sublethally irradiated alymphoid *Rag2*$^{-/-}$;*Il2rg*$^{-/-}$ mice and examined ILC subsets in the small intestine 5 weeks later (FIGURE 19A). *Nfil3*$^{-/-}$ CLPs generated fewer small intestinal ILCs in recipient mice than wild-type CLPs (FIGURE 19B), suggesting a CLP-intrinsic requirement for NFIL3 in ILC development.

Nfil3$^{-/-}$ mice are deficient in bone marrow ILC precursors downstream of CLP.

CLPs give rise to all lymphoid cells, including ILCs, T cells, and B cells. In contrast to ILCs, overall T and B cell numbers are not altered in *Nfil3*$^{-/-}$ mice [69]. The general requirement for NFIL3 in ILC development therefore suggested that NFIL3 might be essential for the development of ILC-committed precursors downstream of CLPs. To further investigate the cellular origin of the ILC developmental deficiency in *Nfil3*$^{-/-}$ mice, we analyzed various bone marrow precursor populations in wild-type and *Nfil3*$^{-/-}$ mice. Due to low frequencies of these precursors in adult mouse bone marrow, we enriched undifferentiated cells (lineage marker-negative) by negative selection prior to analysis (FIGURE 20A,B). In agreement with previous findings [85, 86], wild-type and *Nfil3*$^{-/-}$ littermates harbored similar frequencies of LSK cells (Lin^{-} Sca1^{+} cKit^{+}) (FIGURE 21A,B), which include hematopoietic stem cells (HSC) that give rise to all hematopoietic cells, and CLPs that produce all lymphoid lineages (FIGURE 22A,C). This accords with the fact that *Nfil3*$^{-/-}$ mice do not exhibit marked deficiencies in myeloid cells, B cells or T cells [69]. However, *Nfil3*$^{-/-}$ mice had markedly fewer Flt3^{-} $\alpha4\beta7^{+}$ CLPs (αLPs) (FIGURE 22A,D), which have been shown to differentiate into ILC3 and NK cells [162]. *Nfil3*$^{-/-}$ mice also had fewer previously identified ILC2 progenitor cells (ILC2P, Lin^{-} cKit^{low} CD127^{+} Sca1^{+} CD25^{+}) [155] (FIGURE 22A,E). Consistent with a recent report that *Nfil3*$^{-/-}$ mice show impaired development of non-NK ILC1 [151], the frequencies of the precursors (CHILP) that can differentiate into this ILC1 subtype were lower in *Nfil3*$^{-/-}$ mice than in wild-type mice (FIGURE 22B,F). Similarly, NFIL3 has been found to be critical for generation of the earliest NK-committed precursors (PreNKP) [85, 86]. Thus, *Nfil3*$^{-/-}$ mice have reduced numbers of precursors that give rise to

ILC1 (including NK), ILC2 and ILC3. These data suggest that NFIL3 is required for generation of ILC precursors in the bone marrow.

αLP can differentiate to ILC2, in addition to previously-reported NK and ILC3.

It has been hypothesized that ILCs differentiate from a common ILC progenitor population [154, 163]. Fate-mapping studies with *Id2* and *Zbtb16* (encoding PLZF) reporter mice led to the discovery of two progenitor populations that develop into most, but not all, subtypes of known ILC lineages [151, 155, 159]. These findings accorded with the partial ILC deficiencies seen in mice lacking *Id2* and *Zbtb16* [164, 165]. In particular, NK cell development is not impaired in *Zbtb16*$^{-/-}$ mice while *Id2*$^{-/-}$ mice only show NK developmental defects during NK maturation. The broad ILC (including NK) deficiency and impaired ILC precursor development in *Nfil3*$^{-/-}$ mice thus suggested that the common ILC progenitor might be NFIL3-dependent. We therefore sought to identify NFIL3-dependent precursor populations that differentiate into all three ILC lineages. ILC2Ps are lineage-specified progenitors of ILC2s with no appreciable potential to differentiate into NK cells or ILC3 [155], indicating that ILC2Ps lie developmentally downstream of the common ILC progenitor. The common "helper-like" innate lymphoid progenitor (CHILP) can differentiate into non-NK ILC1, ILC2 and NK1.1^{+} NKp46^{+} ILC3 but not NK cells [151]. In contrast, αLP cells are considered to be α4β7^{+} integrin -expressing CLPs, and likely represent an early stage of ILC development as they can differentiate into both NK cells and ILC3 [162, 166].

To determine whether αLP can also give rise to ILC2, we co-cultured purified αLP with bone marrow stromal OP9 cells (OP9-GFP) or OP9 cells expressing the Notch ligand Delta-like 1 (OP9-DL1), which support ILC differentiation *in vitro* [155, 162, 167]. When co-cultured with OP9-DL1 cells in the presence of ILC2-inducing cytokines, αLPs readily developed into ILC2 as the majority of progeny cells expressed ILC2 markers ($GATA3^+$ $Sca1^+$) (FIGURE 23A,B). When OP9-GFP cells (not expressing Notch ligand) were used in this assay, only a small fraction of progeny cells became ILC2 (FIGURE 23A,B), confirming that Notch signaling is also important for ILC2 differentiation *in vitro* [168, 169]. In agreement with a prior study [162], αLP differentiated into ILC3 and $ROR\gamma t^-$ $NK1.1^+$ cells under ILC3-inducing conditions (FIGURE 23C,D).

To assess the potential of αLPs to differentiate into ILC2 *in vivo*, we transferred ~1000 purified αLPs ($CD45.1^+$) into sublethally irradiated *Rag2*$^{-/-}$*;Il2rg*$^{-/-}$ mice ($CD45.2^+$). After 5 weeks, ILC2 differentiated from engrafted αLPs were detected in small intestine and colon of the recipient mice (FIGURE 24A,B). Interestingly, $GATA3^+$ ILC2 comprised a small fraction of $CD127^+$ ILCs in the small intestine but were the majority in the colon while $ROR\gamma t^+$ ILC3 showed an opposite tissue distribution pattern, suggesting that tissue-specific microenvironments influences ILC development and/or recruitment (FIGURE 24A,B). Consistent with the previously reported NK cell differentiation potential of αLPs [162], donor cells gave rise to a large population of $NK1.1^+$ cells in the spleen (FIGURE 24C). Differentiation of ILC2 from αLPs was not caused by contamination of αLPs with CLPs, as no donor-derived B cells were detected in the spleen and small intestine of recipient mice

(FIGURE 24C), consistent with the loss of B cell differentiation potential in αLPs [162, 166]. Thus, αLPs can give rise to all three ILC lineages *in vitro* and *in vivo*. Given the more restricted differentiation potential of ILC2P and CHILP, αLPs are likely to be developmentally upstream of ILC2P and CHILP, and defective αLP development in *Nfil3*$^{-/-}$ mice thus explains the general ILC deficiency in these mice.

NFIL3 drives ILC development by regulating *Tox* expression in CLPs.

To identify potential mechanisms underlying NFIL3-dependent ILC development, we isolated CLPs from wild-type and *Nfil3*$^{-/-}$ mice and surveyed their transcriptomes by Illumina BeadArrays. *Nfil3* expression could be readily detected in CLPs (FIGURE 25A), which accords with a previous report [86] and is consistent with our finding that NFIL3 regulates ILC development in a CLP-intrinsic manner. However, there was no detectable expression of other transcription factors that are known to govern ILC development, such as *Id2* [85, 86, 155], *Zbtb16* [159], *Eomes* [85, 86], *Tcf7* (encoding TCF-1) [168], *Rora* [169], *Rorc* [156, 170], *Gata3* [155] and *Tbx21* [141, 171] in CLPs. In contrast, the high mobility group (HMG) transcriptional regulator *Tox*, which is known to regulate NK and ILC3 development, was expressed at a detectable level in wild-type CLPs and was down-regulated in *Nfil3*$^{-/-}$ CLPs (FIGURE 25A,B). This suggested that NFIL3 may regulate *Tox* expression in CLPs.

CLPs are present in small numbers in adult mice [151], making it challenging to perform biochemical studies of *Tox* regulation by NFIL3 using these cells. As an alternative,

we found that NFIL3 regulates *Tox* expression in EL4 cells, a mouse lymphoma cell line (FIGURE 25C,D). Knockdown of NFIL3 in EL4 cells with two independent shRNA constructs led to dose-dependent down-regulation of *Tox* expression (FIGURE 25C). Conversely, overexpression of NFIL3 in EL4 cells increased *Tox* expression (FIGURE 25D), indicating that *Tox* expression is sensitive to NFIL3 levels in EL4 cells in a manner similar to CLPs. A chromatin immunoprecipitation (ChIP) assay with an NFIL3-specific antibody [160] demonstrated that NFIL3 directly bound to the *Tox* promoter and that overexpression of NFIL3 enhanced this binding (FIGURE 25E). Finally, NFIL3 activated *Tox* promoter activity as assessed by a luciferase reporter assay (FIGURE 25F). Thus, NFIL3 activates *Tox* expression by directly binding to its promoter.

Because *Tox* is known to be essential for NK and ILC3 development [172], we postulated that lowered *Tox* expression leads to the broad ILC deficiency in *Nfil3*$^{-/-}$ mice and that restoring *Tox* expression would rescue ILC development. To test this idea, we cloned *Tox* coding sequences into a bicistronic vector (MSCV-ires-hCD2), which allowed expression of the native form of TOX and also marked cells with the cell surface marker hCD2. We then delivered the TOX-encoding plasmid or the empty vector into highly purified *Nfil3*$^{-/-}$ LSK cells (CD45.2^{+}) by retroviral transduction [173], followed by transfer of these cells into lethally irradiated wild-type mice (CD45.1^{+}) (FIGURE 26A,B). Compared to the empty vector control, transduction of the TOX-encoding plasmid led to increased numbers of ILCs in the small intestines of recipient mice (FIGURE 26C). Thus, ILC development is rescued by restoring *Tox* expression in *Nfil3*$^{-/-}$ progenitors, indicating that NFIL3 drives ILC development in part by regulating *Tox* expression. Recent studies have shown that forced

expression of *Eomes* can rescue NK cell development from *Nfil3*$^{-/-}$ hematopoietic progenitors [85, 86]. However, because *Eomes* is not expressed in CLPs (FIGURE 25A) and *Eomes* deficiency only impacts NK cells but no other ILCs [151], *Eomes* is unlikely to mediate the NFIL3-dependent development of non-NK ILCs.

To assess the physiological role of NFIL3-dependent ILC development in host immunity, we challenged *Nfil3*-deficient mice with *Citrobacter rodentium,* a mouse enteric pathogen. IL-22-mediated immune defense is essential for protection against *C. rodentium* infection [146, 174]. To rule out confounding effects of T_H17 cells, which also produce IL-22 and are elevated in *Nfil3*$^{-/-}$ mice [160], we crossed *Nfil3*$^{-/-}$ mice with *Rag1*$^{-/-}$ mice to create *Nfil3*$^{-/-}$;*Rag1*$^{-/-}$ mice, which lack all T and B cells. *Nfil3*$^{-/-}$;*Rag1*$^{-/-}$ mice were more susceptible to oral *C. rodentium* infection than *Rag1*$^{-/-}$ mice as measured by weight loss (FIGURE 27). These data thus establish that NFIL3-dependent ILC development is essential for host immune defense against a mucosal pathogen.

DISCUSSION

Our findings reveal an essential role for NFIL3 in the development of all known innate lymphoid cell subsets, including NK cells, and identify a key transcriptional cascade (*Nfil3*-*Tox*) that regulates ILC development. The requirement for NFIL3 is intrinsic to the CLP and impacts the generation of downstream ILC precursors in the bone marrow. Because *Id2* is not expressed in CLPs [155], NFIL3 represents the earliest-acting known transcription factor that selectively regulates ILC development and may thus serve as a marker for the long sought-

after common ILC progenitor. We have identified αLP as a NFIL3-dependent bone marrow precursor population that can differentiate into all known types of ILCs *in vitro* and *in vivo*. Because this population may be heterogeneous, additional studies will be required to determine whether αLP represents a true common ILC progenitor population or whether it includes multiple lineage-specified precursor subpopulations. Because of the general importance of ILCs in mucosal defense, NFIL3-dependent pathways may provide new targets for inflammatory and infectious diseases of mucosal tissues.

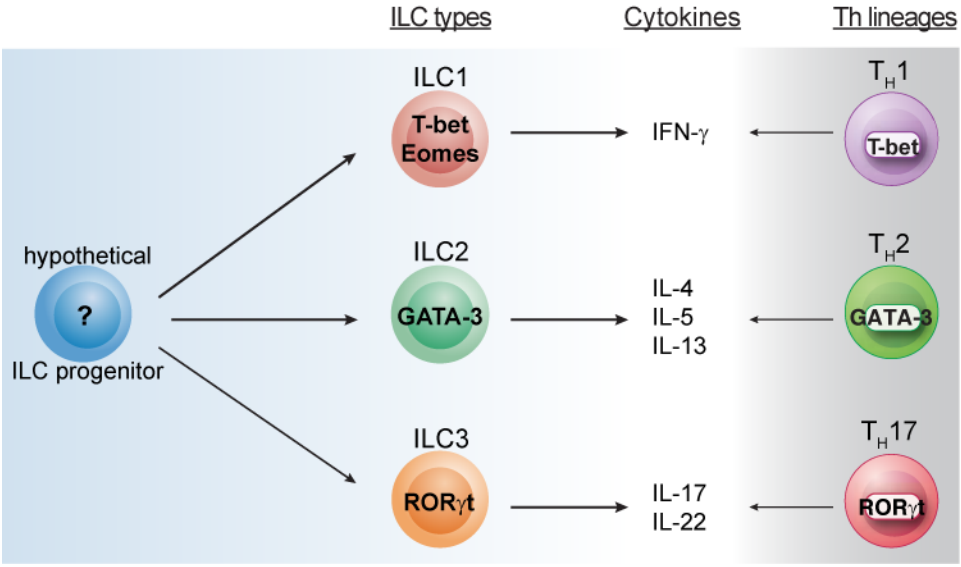

FIGURE 16: Functional and developmental classification of ILCs.

ILCs are categorized into three distinct groups based on their function and developmental requirement (left), which mirror those of T helper cell lineages (right). The schematic is adapted from [163] and [121].

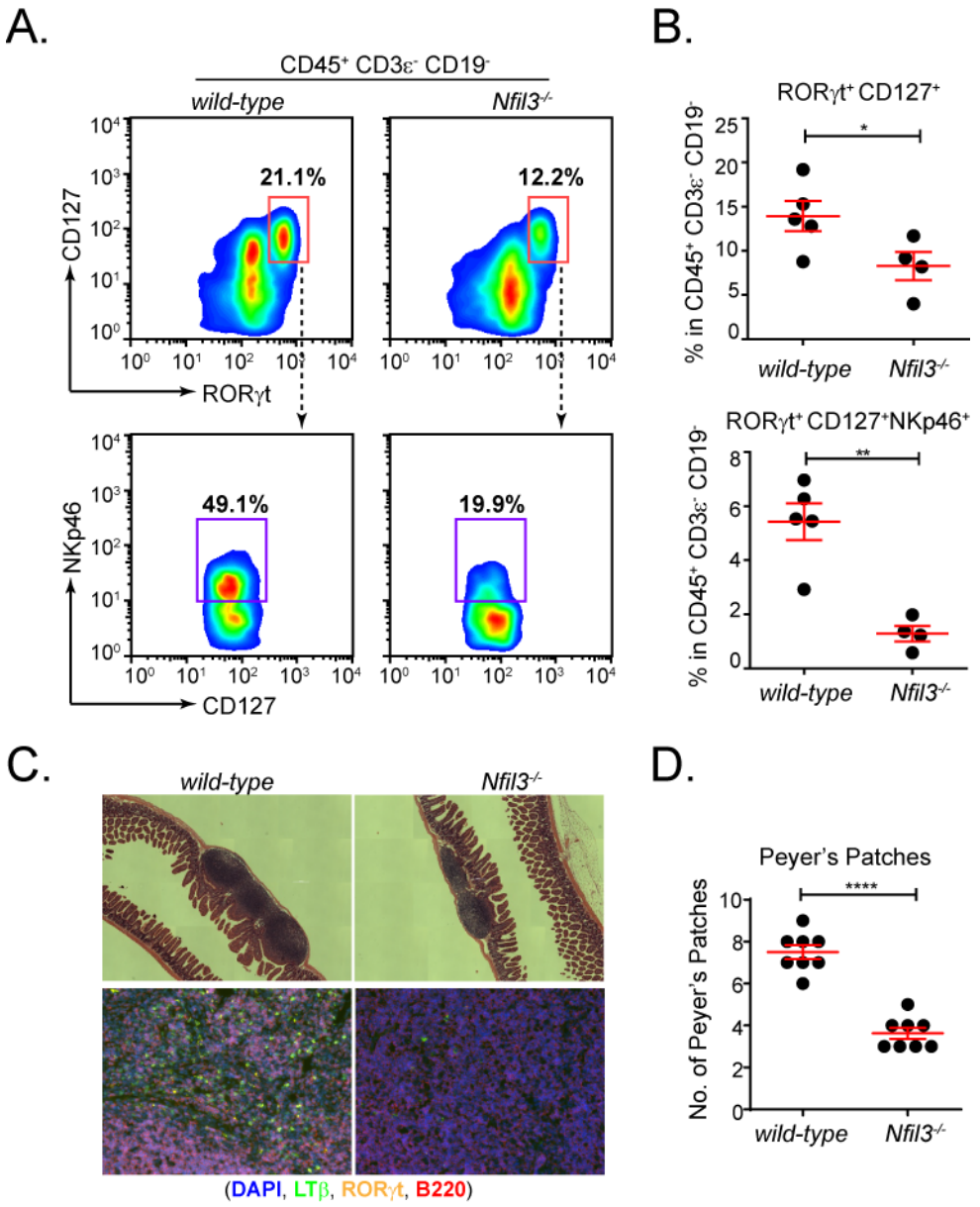

FIGURE 17: *Nfil3*$^{-/-}$ mice are deficient in ILC3.

(A-B) *Nfil3*$^{-/-}$ mice show reduced ILC3 frequencies in the small intestinal lamina propria. Small intestinal lamina propria lymphocytes (LPL) were isolated and stained as described before. Cells were electronically gated on CD45^{+} to remove non-hematopoietic cells and then on CD3ε^{-} and CD19^{-} to exclude T cells and B cells, respectively. Total ILC3 cells were

detected as CD127^{+} RORγt^{+}. The NK receptor-expressing subtype of ILC3 (also known as NK22 cells) was identified by additional staining for NKp46. Representative flow plots are shown in (A) and data from multiple mice are summarized in (B). **(C-D)** *Nfil3*$^{-/-}$ mice show smaller and fewer Peyer's patches in the small intestine. (C) Peyer's patches from wild-type and *Nfil3*$^{-/-}$ mice were cut into 0.7mm sections and stained with either hematoxylin and eosin (H&E, upper) or fluorophore-conjugated antibodies against B cells (B220) and LTi cells (RORγt and LTβ) (lower). (D) The number of Peyer's patches in the small intestines of wild-type and *Nfil3*$^{-/-}$ mice were enumerated and plotted. Groups were compared by two-tailed student's t-test. *, $p<0.05$, **, $p<0.01$, ****, $p<0.0001$.

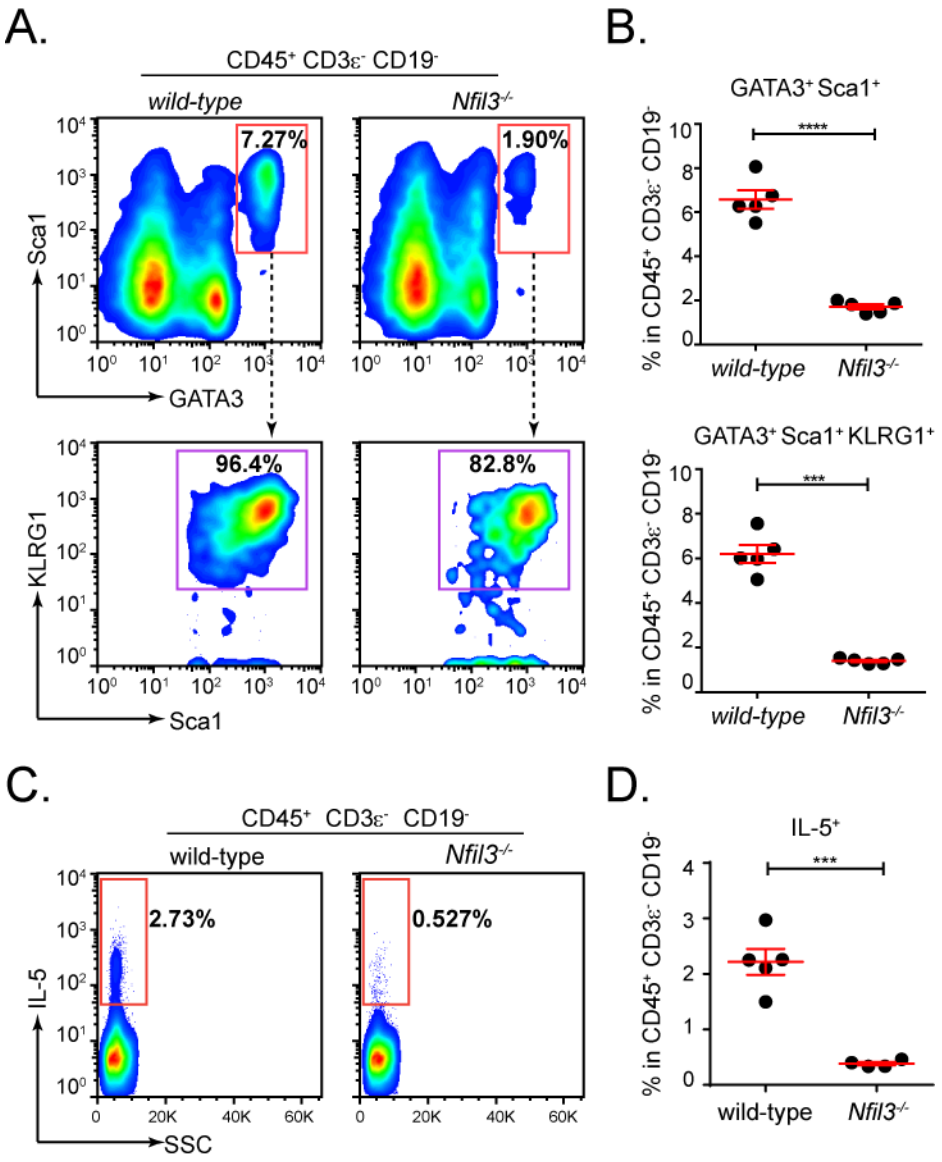

FIGURE 18: *Nfil3*$^{-/-}$ mice are deficient in ILC2.

(A,B) ILC2 were examined by nuclear staining of GATA3. ILC2 cells were identified among LPLs as GATA3^{+} Sca1^{+} or, more stringently, GATA3^{+} Sca1^{+} KLRG1^{+}. Representative flow plots are shown in (A) and data from multiple mice are summarized in (B). **(C,D)** IL-5-producing ILCs were analyzed after *in vitro* stimulation. Representative flow plots are shown

in (C) and data from multiple mice are summarized in (D). Groups were compared by two-tailed student's t-test. ***:$p<0.001$, ****:$p<0.0001$.

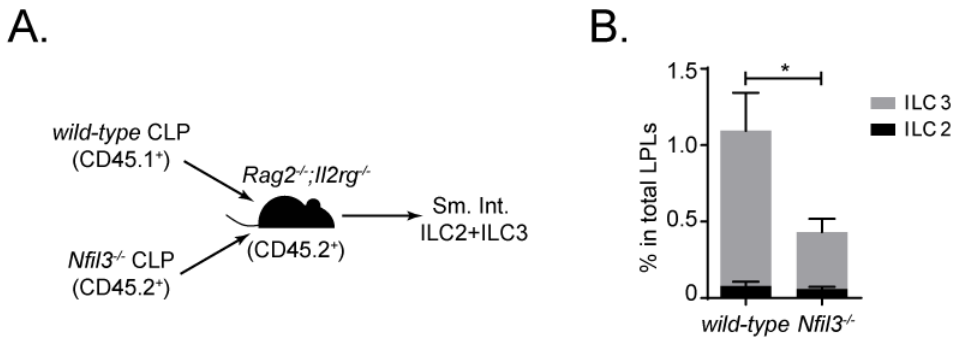

FIGURE 19: NFIL3 regulates ILC development in a cell-intrinsic manner.

(A) Experimental setting. Equal numbers of wild-type (CD45.1$^+$) and *Nfil3*$^{-/-}$ (CD45.2$^+$) CLPs were co-transplanted into sublethally irradiated alymphoid *Rag2*$^{-/-}$*;Il2rg*$^{-/-}$ mice and intestinal ILCs were analyzed 5 weeks later. **(B)** The frequencies of ILCs (ILC2+ILC3) derived from wild-type (CD45.1$^+$) and *Nfil3*$^{-/-}$ (CD45.2$^+$) donor cells among total LPLs were calculated and plotted. Groups were compared by two-tailed student's t-test. *, $p<0.05$.

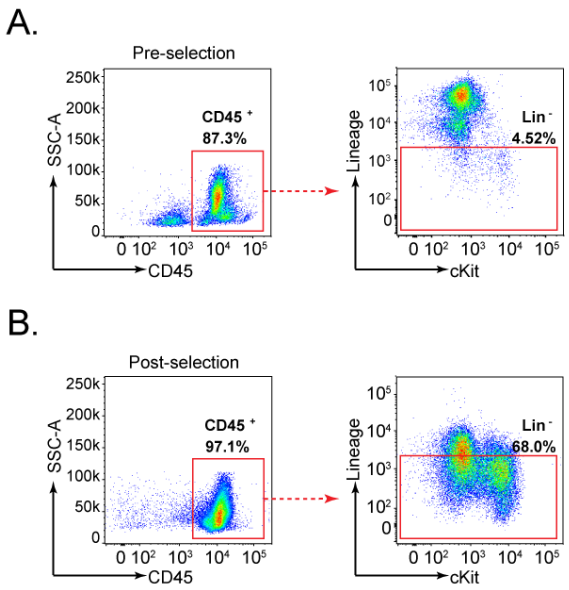

FIGURE 20: Enrichment of undifferentiated bone marrow cells by negative selection. Due to low frequencies of hematopoietic progenitor cells in the bone marrow of adult mice, undifferentiated (lineage marker-negative) cells were enriched by MACS-mediated negative selection prior to analysis or purification by FACS. To examine the efficiency of negative selection, samples before selection **(A)** and after selection **(B)** were subjected to CD45 and Lineage marker staining. More than 10-fold enrichment of Lineage-negative cells was routinely obtained during this process. Lineage markers used here include CD3ε, B220, CD11b, Gr-1, Ter119, CD5, TCRγδ, and NK1.1.

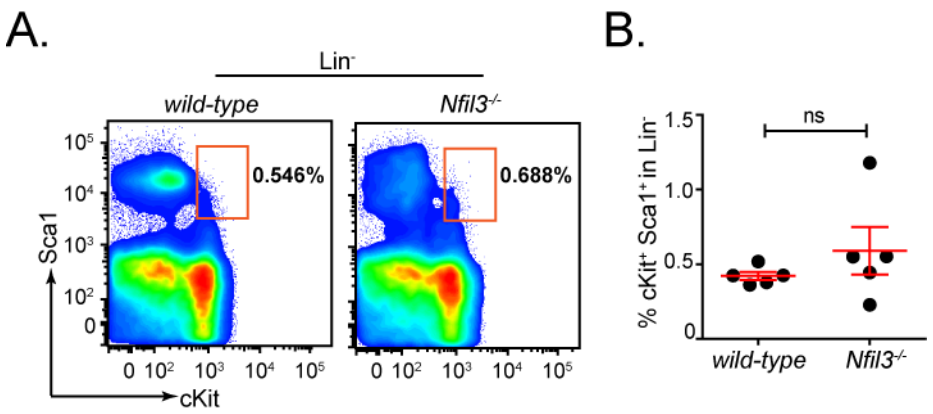

FIGURE 21: *Nfil3*$^{-/-}$ mice are not deficient in LSK cells.

Lin$^-$ cKit$^+$ Sca1$^+$ (LSK) cells roughly represent the hematopoietic stem cells (HSC), multipotent progenitors (MPPs) and lymphoid-primed multipotent progenitors (LMPPs). Bone marrow cells were isolated from femur and tibia from wild-type and *Nfil3*$^{-/-}$ mice. Lineage marker (Lin)-negative cells were first enriched by negative selection and then stained with antibodies against Sca1 and cKit. Typical flow plots are shown in **(A)** and data from multiple mice are pooled in **(B)**. Comparison between the genotypes was done with the two-tailed student's t-test. ns, not significant.

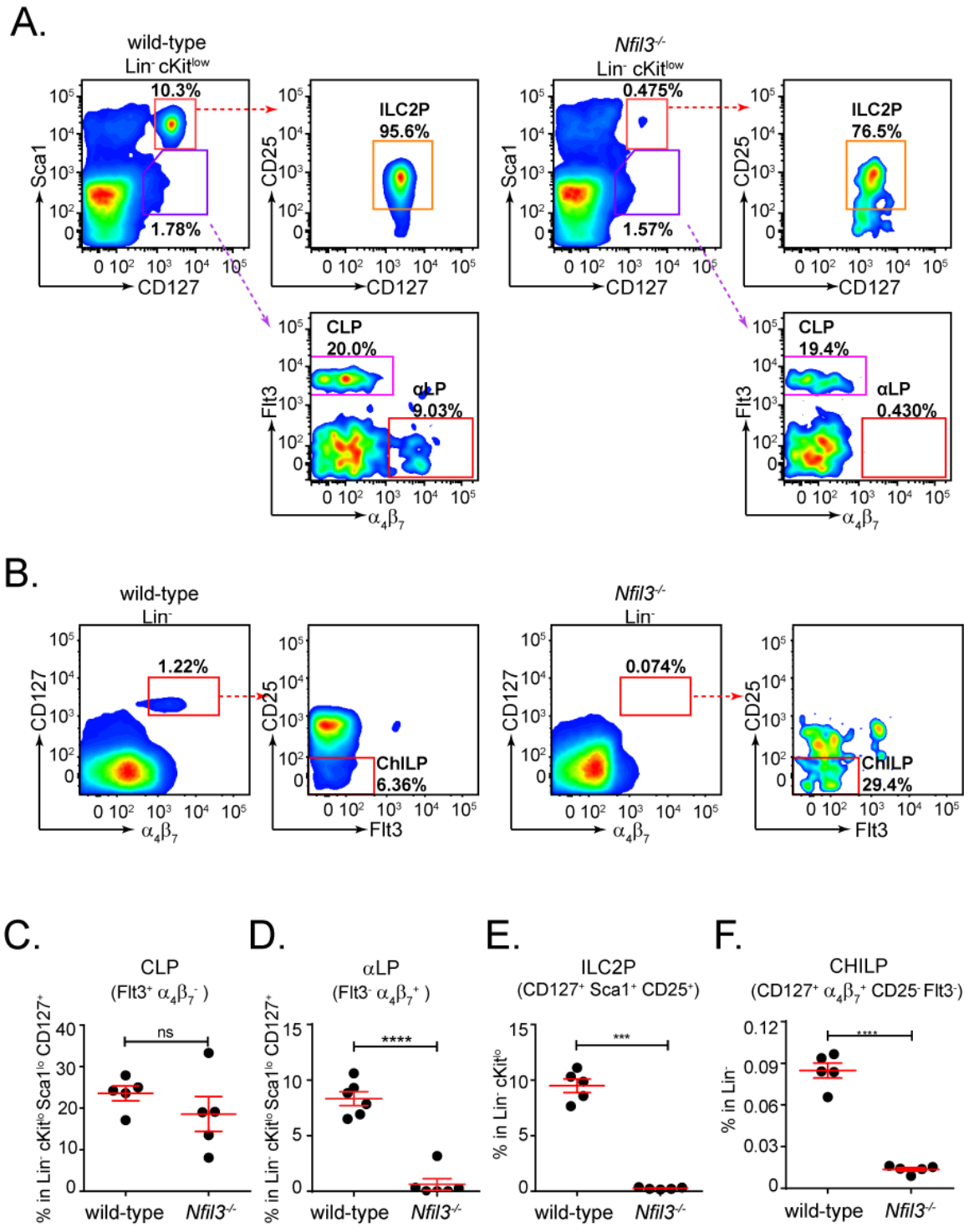

FIGURE 22: *Nfil3*$^{-/-}$ mice are deficient in bone marrow ILC precursors.

(A, C, D, E) *Nfil3*$^{-/-}$ mice have comparable CLP frequencies but show deficiencies in αLP and ILC2P. Bone marrow cells were isolated from femur and tibia from wild-type and *Nfil3*$^{-/-}$ mice. Lineage marker (Lin)-negative cells were first enriched by negative selection and then stained with antibodies to identify CLP (Lin^{-} cKit^{low} CD127^{+} Sca1^{low} Flt3^{+} α4β7^{-}), αLP (Lin^{-} cKit^{low} CD127^{+} Sca1^{low} Flt3^{-} α4β7^{+}) [162] and ILC2P (Lin^{-} cKit^{low} CD127^{+} Sca1^{+} CD25^{+}) [155]. Gating strategy and representative flow plots are shown in (A) and combined data for CLP, αLP and ILC2P are shown in (C), (D) and (E), respectively. **(B, F)** *Nfil3*$^{-/-}$ mice are deficient in common "helper-like" innate lymphoid progenitor (CHILP) cells [151]. Bone marrow cells were processed as above and CHILPs were identified as Lin^{-} CD127^{+} α4β7^{+} CD25^{-} Flt3^{-}. Typical flow plots are shown in (B) and data from multiple mice are pooled in (F). Statistical analysis was performed with two-tailed student's t-test. ns, not significant; ***, $p<0.001$; ****, $p<0.0001$.

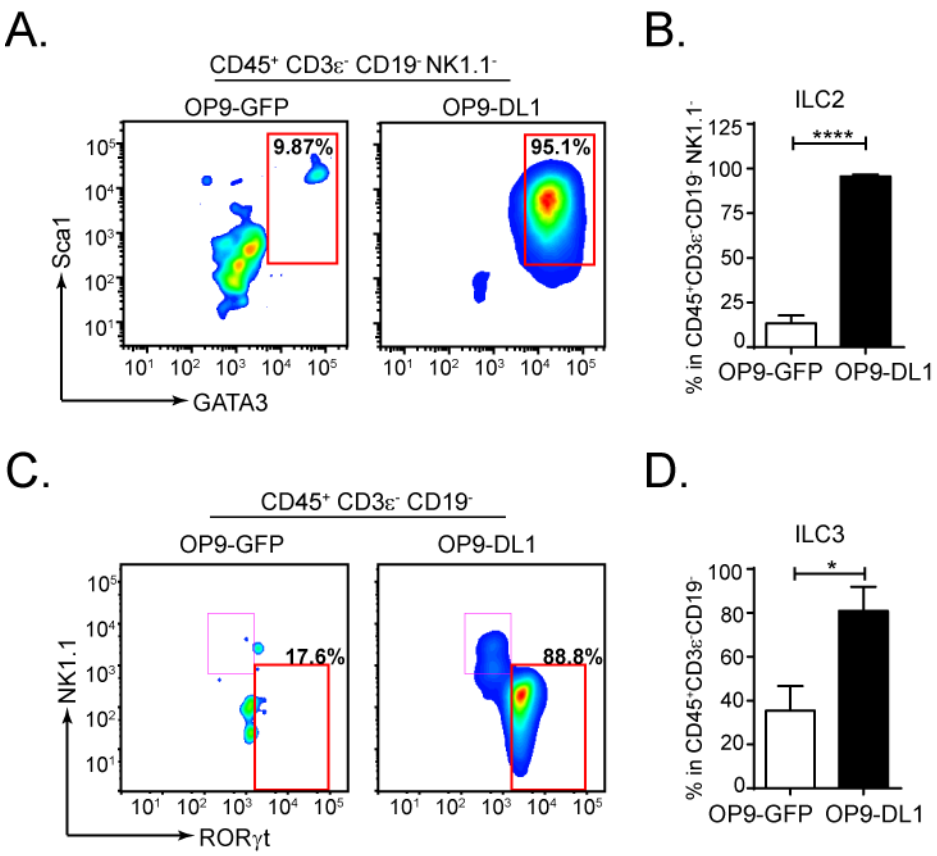

FIGURE 23: αLPs differentiate into ILC2 and ILC3 *in vitro*.

(A,B) αLPs can differentiate into ILC2 cells *in vitro*. αLPs were purified by FACS and ~25 cells were co-cultured with a bone marrow stromal cell line OP9 (OP9-GFP) or OP9 cells stably expressing the Notch ligand Delta-like 1 (OP9-DL1) for 14 days in the presence of ILC2-inducing (IL-7 and IL-2) cytokines. ILC2 cells were identified as GATA3^{+} Sca1^{+}. Typical flow plots are shown in (A) and combined data are shown in (B). **(C,D)** αLPs can differentiate into ILC3 cells *in vitro*. αLPs were co-cultured with OP9-GFP or OP9-DL1 cells in the presence of ILC3-inducing (IL-7 and IL-23). ILC3 were detected as RORγt^{+} NK1.1^{-}. Representative flow plots are shown in (C) and combined data are shown in (D). Statistical analysis was performed with two-tailed student's t-test. *, $p<0.05$; ***, $p<0.001$.

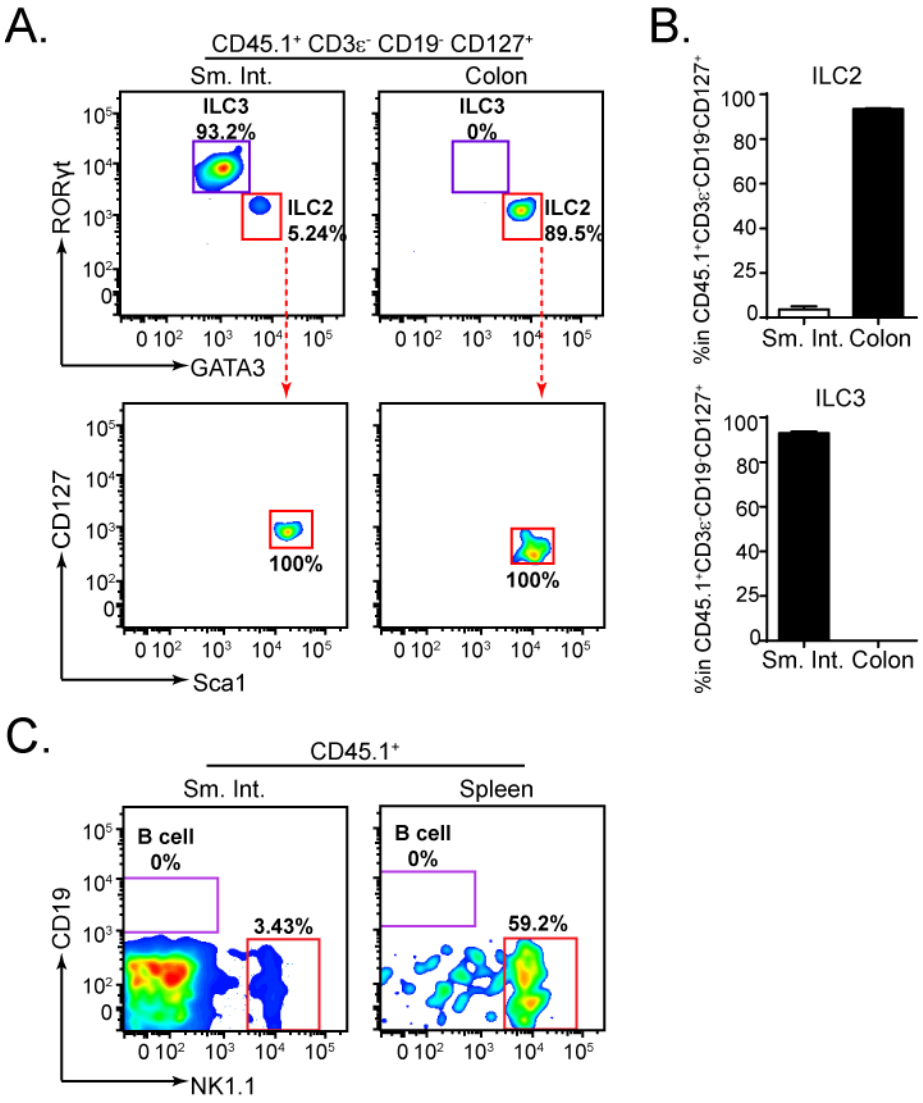

FIGURE 24: αLPs differentiate into ILC2 *in vivo*.

(A,B) ILC2 and ILC3 were detected in the small intestines and colons of recipient mice. αLP cells were purified from wild-type ($CD45.1^+$) mice and ~1000 αLP cells were transplanted into sublethally irradiated *Rag2*$^{-/-}$*;Il2rg*$^{-/-}$ ($CD45.2^+$) mice. ILCs in the small intestine and colon were examined 5-6 weeks later. ILC2 were detected as $CD127^+$ $GATA3^+$, of which all were also $Sca1^+$. Representative flow plots are shown in (A) and differential ILC distribution in the small intestine and colon is summarized in (B). **(C)** Purified αLPs were

not contaminated with CLPs as no donor-derived B cells ($CD45.1^+$) were detected in the spleen and intestine of recipient mice. αLPs were purified and transplanted as above. In agreement with a previous report [162], αLPs failed to differentiate into B cells both in the small intestine and spleen. In contrast, a large population of $NK1.1^+$ cells was observed in the spleen.

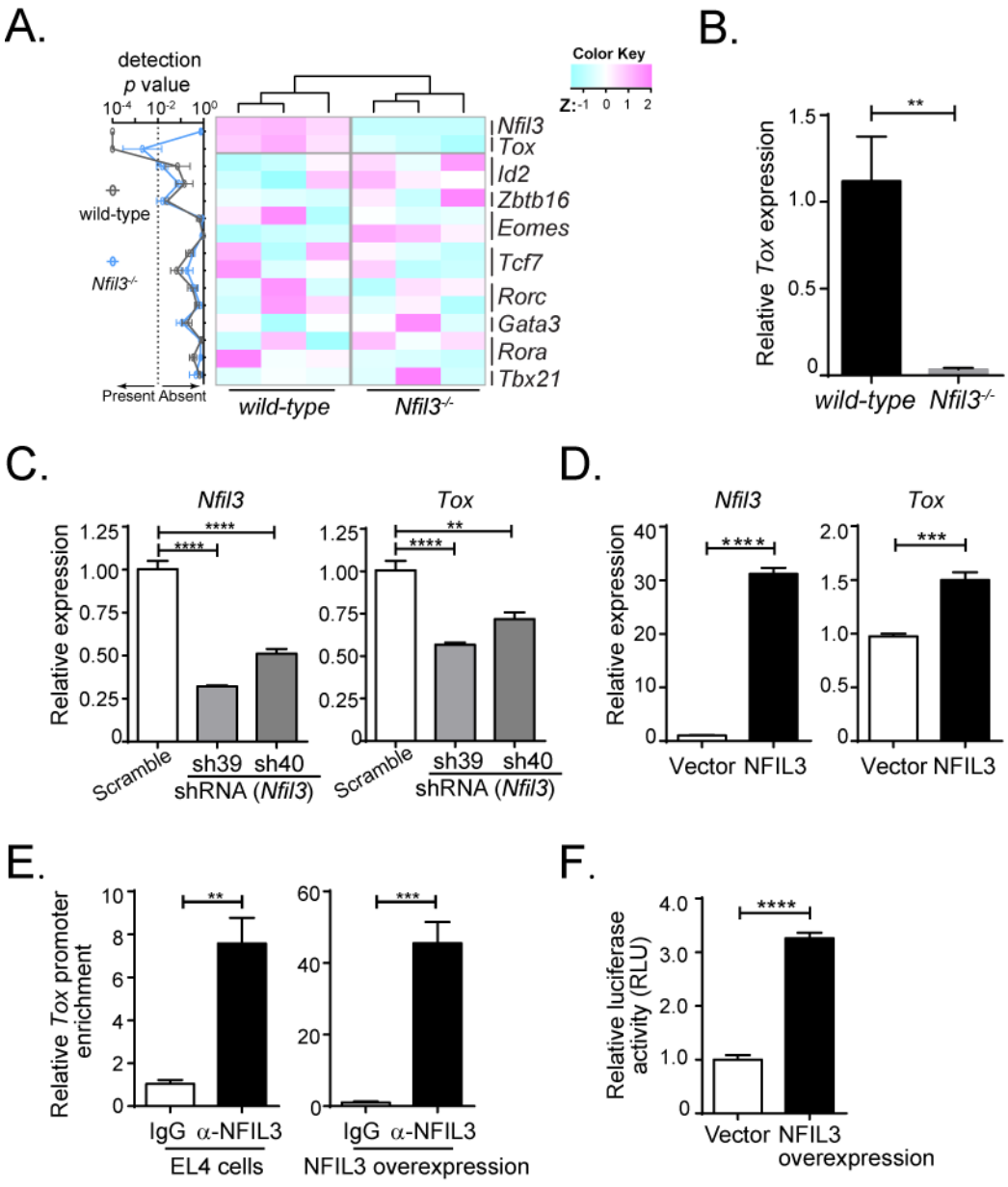

FIGURE 25: NFIL3 activates *Tox* expression by binding directly to the *Tox* promoter.

(A-B) *Tox* expression is lower in *Nfil3*$^{-/-}$ CLPs than in wild-type cells. (A) Heatmap comparing expression levels of transcription factors in wild-type and *Nfil3*$^{-/-}$ CLPs. Each column represents a biological sample and each row represent one probe set of the given gene. Factors included *Nfil3*, *Tox* and other transcription factors that are known to be

involved in ILC development. The detection *p* values for each transcription factor in Illumina BeadArrays are also shown (left). (B) Q-PCR analysis of *Tox* expression in wild-type and *Nfil3*$^{-/-}$ CLPs. **(C-F)** NFIL3 activates *Tox* expression by binding directly to the *Tox* promoter. *Tox* expression was determined by Q-PCR following shRNA knockdown of NFIL3 (C), and NFIL3 overexpression (D) in EL4 cells. (E) ChIP analysis of EL4 cells using an NFIL3-specific antibody or IgG control. Enrichment of the *Tox* promoter was calculated as the ratio of the NFIL3-specific antibody pull-down to the IgG control pull-down. The left panel shows results with endogenous NFIL3 levels and the right panel shows results with NFIL3 overexpression. (F) Luciferase reporter assay. A 2.3kb fragment of the *Tox* promoter was cloned and fused with the firefly *luciferase* gene to generate a *Tox-luciferase* reporter. HEK293T cells were co-transfected with the reporter and an empty vector or an NFIL3-encoding vector. Luciferase activity was normalized to cells transfected with vector-only controls. Statistical analysis was performed with two-tailed student's t-test. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

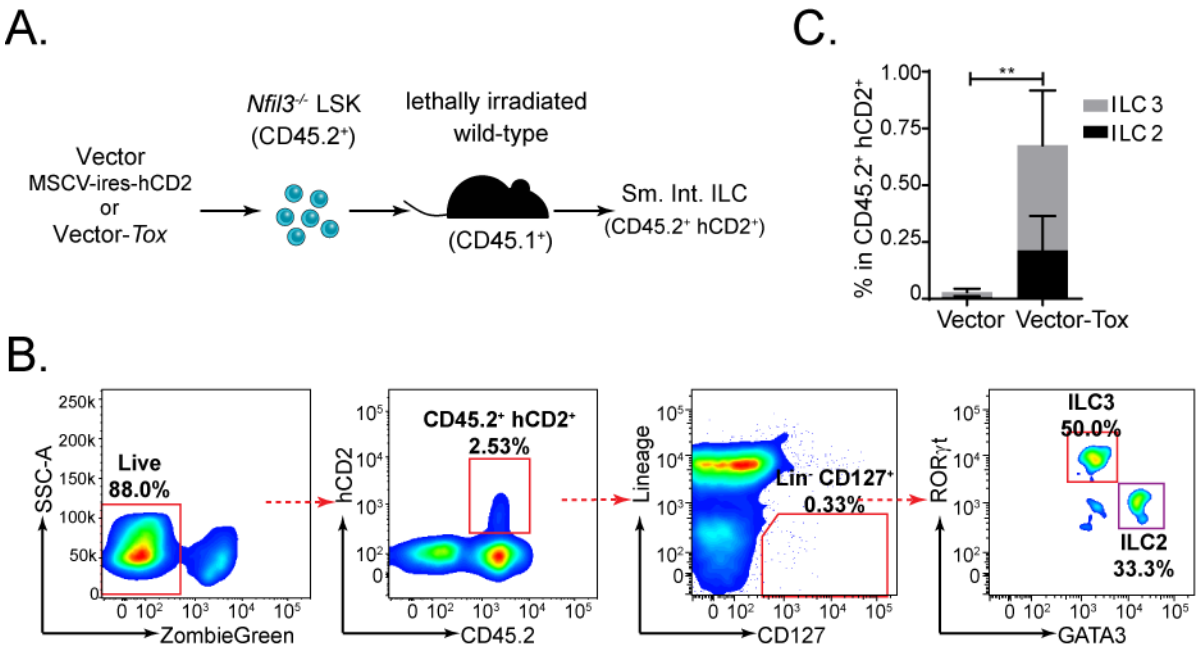

FIGURE 26: Restoring *Tox* expression in *Nfil3*$^{-/-}$ LSK cells rescues ILC development.

(A) Diagram illustrating the experimental design. *Nfil3*$^{-/-}$ LSK (CD45.2^{+}) cells were retrovirally transduced with either TOX-encoding (Vector-*Tox*) or empty (MSCV-ires-hCD2) vectors. Cells were then transplanted into lethally irradiated wild-type mice (CD45.1^{+}) and ILCs were examined 5-6 weeks later. **(B)** Gating strategy for examining ILCs in the recipient mice. Live cells were first electronically gated as ZombieGreen-negative and cells transduced by retrovirus were identified as CD45.2^{+} hCD2^{+}. ILC2 and ILC3 were gated as Lineage (CD3, CD19, CD5, TCRβ, TCRγδ)$^{-}$ CD127^{+} GATA3^{+} and Lin^{-} CD127^{+} RORγt^{+}. **(C)** The sum of ILC2 and ILC3 frequencies were plotted. Statistical comparisons between groups were performed with two-tailed Mann-Whitney test. **, $p<0.01$.

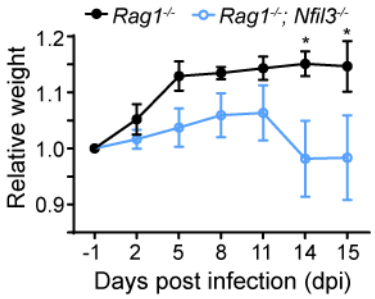

FIGURE 27: *Nfil3* deficiency results in increased susceptibility to *Citrobacter rodentium* infection in mice.

Nfil3$^{-/-}$ mice were crossed with *Rag1*$^{-/-}$ mice to generate *Nfil3*$^{-/-}$;*Rag1*$^{-/-}$ mice to eliminate the effects of adaptive immune cells, especially T_H17 cells. *Rag1*$^{-/-}$ and *Nfil3*$^{-/-}$;*Rag1*$^{-/-}$ mice were orally challenged with 5 x 10^9 CFU of *C. rodentium* and mouse weight loss was monitored. Statistical comparisons between groups were performed with two-way ANOVA with posttests. *, $p<0.05$.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

It has been recently recognized that the immune system is not only important for limiting and resolving infections, but is also critical for maintaining homeostasis in the body. Dysregulation of the immune function has been implicated in diseases that were thought unrelated to immunity, such as metabolic syndromes [175]. For example, inflammatory cytokines secreted by macrophages have been shown to play an important role in development of insulin resistance. Due to the wide influence of the immune system in the body, it has been of great interest to study the development and regulation of the immune system in response to environmental cues.

The day-night transition is a fundamental feature of life on Earth. To adapt to rhythmic environmental changes caused by the day-night cycle, living organisms have developed internal circadian clocks to coordinate behaviors and physiology accordingly. Meanwhile, a functional immune system helps maintain the integrity of life forms for long enough to allow evolution to occur. Therefore, it is not unexpected that the circadian clock and immunity are interconnected. In fact, disturbing the circadian clock by shift work has been implicated in immune disorders in humans [136]. A recent GWAS study also discovered that a circadian clock gene *Period 3* is associated with a human immune disorder, IBD [176].

In spite of mounting evidence that immunity is influenced by the circadian clock, the underlying mechanisms are largely unknown. My work reveals that the transcription

factor NFIL3 is at the interface between the circadian clock and the immune system and therefore provides novel insights into the mechanism by which the circadian clock regulates immunity (FIGURE 28).

REGULATION OF T_H17 CELL DEVELOPMENT BY NFIL3

T_H17 cells are an important player in host heath and disease. Cytokines produced by T_H17 cells regulate the functions of other immune cells to mount coordinated immune responses against extracellular pathogens such as bacteria and fungi [107]. In the meantime, T_H17 cells are also implicated in immune disorders such as IBD, multiple sclerosis, psoriasis, and rheumatoid arthritis [118, 119, 122]. Interestingly, NFIL3 has recently been identified as an IBD-risk gene [127], though the mechanism is not understood. Thus, my work potentially provides mechanistic insights into the genetic association of T_H17 and NFIL3 with IBD.

NFIL3 suppresses T_H17 cell development by regulating *Rorγt* expression.

RORγt is the master regulator of T_H17 lineage specification from naïve $CD4^+$ T cells, together with a transcriptional regulatory network consisting of IRF4, BATF, STAT3 and c-Maf [92, 122]. However, this transcriptional network was discovered by a candidate approach, i.e. choosing a set of transcription factors to start with, which is a powerful way to study the regulation between and downstream of the pre-chosen factors, but is limited in its ability to uncover factors functioning upstream. Indeed, my results demonstrated that NFIL3 is an upstream repressor of RORγt by binding directly to the GTTACTTAA motif in the *Rorγt* promoter, which has a profound impact on T_H17 development. Loss of NFIL3 led to

increased expression of *Rorγt* and, accordingly, elevated frequencies of T_H17 cells in mice. Conversely, overexpression of NFIL3 resulted in reduced T_H17 differentiation.

The circadian clock regulates T_H17 cell development through NFIL3.

The circadian clock has been shown to influence various immune functions such as cytokine production by macrophages, proliferation of T helper cells and migration of monocytes [40, 43, 50, 177]. But little was previously known about the role of the circadian clock in lineage specification of T cells. Regulation of RORγt by NFIL3 thus provides an opportunity to examine the role of the circadian clock in T helper cell differentiation. My study revealed two lines of evidence supporting this hypothesis. First, mice genetically deficient in circadian clock components *Rev-erbα* and *Clock* showed impaired T_H17 differentiation *in vitro* and *in vivo*, in line with altered *Nfil3* expression in these cells. Second, consistent with oscillation in *Nfil3* expression, naïve $CD4^+$ T cells from wild-type mice showed circadian variation in their potential for T_H17 differentiation, which was abolished in *Nfil3*$^{-/-}$ cells. Thus, my work demonstrated that T_H17 cell differentiation is regulated by the circadian clock and timing of antigenic stimulation could influence the lineage specification choice of naïve T helper cells. To highlight the physiological role of circadian-regulated T_H17 differentiation, I also provided evidence that disrupting the circadian clock by light cycle disturbance, a procedure mimicking shift work or jet-lag, elevated T_H17 cell frequencies in the intestine and increased mouse susceptibility to DSS-induced colitis. These data correlate well with human studies [178, 179]. Note that the impacts of circadian disruption accumulate over time as both human and mice require prolonged circadian disruption to show pathological effects.

REGULATION OF INNATE LYMPHOID CELL DEVELOPMENT BY NFIL3

Our understanding of innate lymphoid cells can be traced back to about 40 years ago when the first group of innate lymphoid cells, NK cells, was discovered [140]. In the past few years, several new groups of ILCs have been discovered, which motivated researchers to attempt to create a unified view of ILC development and function. This idea is based on the observation that ILCs are all of lymphoid origin, but do not bear rearranged specific antigen receptors as T and B cells do [138, 163]. This suggests that there may be a common ILC progenitor (CILP), distinct from B and T cell progenitors, that differentiates from the common lymphoid progenitor (CLP). Studies searching for the CILP have proven fruitful, as several progenitor populations have been identified with varied ILC differentiation potential [151, 155, 159, 162]. However, none of these progenitor populations has been shown to differentiate into all known ILCs. For example, the CHILP marked by expression of *Id2*, can differentiate into a non-NK ILC1 subtype, ILC2 and an NK1.1^{+} NKp46^{+} RORγt^{+} ILC3 subtype, but not NK cells. This agrees with the observation that *Id2* deficiency doesn't impact NK cell lineage specification but rather maturation [164]. Therefore, finding a factor that is essential for all ILC development at early differentiation stages will be critical for identifying the hypothesized CILP.

NFIL3 is required for generation of bone marrow progenitors of all known ILCs.

My study revealed that NFIL3 is required for development of mature ILC2 and ILC3 in peripheral tissues, such as the intestine. Together with published studies showing that NFIL3

is required for NK and non-NK ILC1 development [79, 80, 150, 151], NFIL3 is essential for the development of all known ILCs in mice. My study has also shown that deficiency of all known ILCs in *Nfil3*$^{-/-}$ mice coincides with impaired development of bone marrow precursors, such as ILC2P, αLP and CHILP, which can collectively give rise to ILC1 (including NK cells), ILC2 and ILC3. In addition, two recent studies have also shown that the requirement for NFIL3 occurs very early during NK cell development and is involved in generation of the earliest known NK precursor (PreNKP) [85, 86]. Thus, NFIL3 is the first known transcription factor that is essential for generation of bone marrow precursors of all known ILCs.

Interestingly, CLPs, which differentiate into all lymphoid lineages including ILC, T and B cells, are not impacted in *Nfil3*$^{-/-}$ mice, consistent with a previous report that development of T and B cells in *Nfil3*$^{-/-}$ mice is largely intact [69]. This is not due to lack of *Nfil3* expression in CLPs. Instead, both my work and other studies demonstrate that *Nfil3* is expressed at considerable levels in CLPs [85, 86]. In contrast, many transcription factors that are known to regulate ILC development including *Id2*, *Zbtb16*, *Tcf7*, *Rorγt*, *Gata3*, *Tbx21* and *Rorα*, are not expressed in CLPs. Therefore, NFIL3 is the earliest acting transcription factor that we ever known to selectively regulate ILC differentiation.

Taken together, NFIL3 is the first ever known transcription factor that is required for all ILC development and is probably the earliest-acting transcription factor that specifically regulates generation of ILC precursors in the bone marrow. Thus, NFIL3 may serve as a molecular marker when searching for the hypothesized common ILC progenitor.

αLPs differentiate into all known ILC types.

It has been hypothesized that there is a common ILC progenitor that give rise to all ILCs and expresses *Id2* [163]. However, the fact that *Id2* doesn't impact early NK precursor development undermines this hypothesis [164]. Indeed, the ILC precursor populations identified using *Id2* as a molecular marker, including CHILP, fail to develop into NK cells [151]. Similarly, another study used *Zbtb16* (encoding PLZF) as a marker and identified a $PLZF^{high}$ precursor population, which does not give rise to NK and LTi cells [159], consistent with normal development of NK and $ROR\gamma t^{+}$ ILC3 from *Zbtb16*$^{-/-}$ hematopoietic progenitors. Indeed, about half of CHILP are $PLZF^{+}$, indicating the $PLZF^{high}$ precursor may be a subpopulation of CHILP [151]. On the contrary, *Nfil3* deficiency impacts the development of all known ILCs, indicating the common ILC progenitor might be dependent on NFIL3.

Given the relatively limited differentiation potential of CHILP, $PLZF^{high}$, and ILC2P precursor populations, I tested whether αLPs can differentiate into all ILCs. αLP are considered to be $\alpha_4\beta_7$ integrin-expressing CLPs, which depend on NFIL3 for development and have been shown to differentiate into NK and ILC3. Indeed, αLPs can differentiate into ILC2 both *in vitro* and *in vivo*, making αLPs the first known precursor population that can develop into all known ILC types. However, it is not clear whether αLPs can differentiate into these ILC types at the clonal level.

NFIL3 regulates *Tox* expression.

TOX is a High Mobility Group (HMG) transcription factor and has been shown to be required for NK and LTi cell development [172]. However, it is not clear how *Tox* fits into

the molecular pathway regulating ILC development. My data demonstrated that *Tox* is expressed in CLPs and is regulated by NFIL3. *Nfil3* deficiency resulted in lower *Tox* expression. Using EL4 cells as a model, in which NFIL3 is also required for *Tox* expression as it is in CLPs, I provided evidence that NFIL3 binds directly to the *Tox* promoter and activates *Tox* expression. Furthermore, restoring Tox expression in *NFIL3*$^{-/-}$ LSK cells rescued ILC development *in vivo*, suggesting TOX functions downstream of NFIL3.

FUTURE DIRECTIONS

My work has demonstrated that NFIL3 regulates T_H17 and ILC development by controlling expression of the downstream transcription factors RORγt and TOX, respectively. However, future studies will be required to address the following questions.

Is αLP the common ILC progenitor?

Though my data showed that αLP can differentiate into ILC2, in addition to previously reported NK and ILC3, it is still not known whether αLP can develop into these ILC types at the clonal level. It is possible that αLP is a collection of early lineage-committed precursors for different types of ILCs. To address this question, individual αLP cells need to be co-cultured with OP9-DL1 cells in the presence of neutral, not lineage inducing, cytokines such as IL-7 and SCF [151], followed by lineage identification of ILC1 (including NK), ILC2 and ILC3.

If αLP does not represent the common ILC progenitor (CILP), an alternative approach will be necessary to identify CILP. A reporter mouse strain can be generated by

knocking in an *ires-egfp*-containing DNA fragment into the *Nfil3* locus, which allows identifying *Nfil3*-expressing cells in the bone marrow. An undifferentiated (Lin^-) $NFIL3^+$ population that depends on NFIL3 may include the CILP and further fractionation using other cell markers may be required to isolate the CILP.

Is ILC differentiation regulated by the circadian clock?

Because ILC development requires NFIL3 and *Nfil3* is regulated by the circadian clock, it is reasonable to speculate that ILC development is regulated by the circadian clock and the day-night cycle. To test this hypothesis, CLPs can be isolated at different times of the circadian cycle and cultured *in vitro* together with OP9 cells. Production of the common ILC progenitor (CILP) can be examined while early pro-B cells, the earliest B-lineage cells, can be used as an internal control. It may be also possible to monitor the size of the CILP population in the bone marrow around the circadian cycle. However, there are several caveats associated with this approach. First, the CILP may be long-living and therefore newly-generated CILP cells during a single circadian cycle may only account for a very small fraction of the total CILP pool. In that case, daily variation of the CILP pool may be too small to be detected. I have had a similar experience when studying circadian regulation of T_H17 cells in mice. Second, if circadian variation in the size of the CILP pool in the bone marrow is observed, it will be necessary to examine whether that is due to cell migration. For example, the hematopoietic stem cells (HSCs) pool in bone marrow shows daily oscillation as a result of oscillation of HSC-retraining signal chemokine CXCL12 in the bone marrow.

When the CXCL12 level decreases from midnight to noon, HSCs are released into blood, resulting in smaller HSC pool in the bone marrow [44, 180].

Differential regulatory mechanisms of *Rorγt* and *Tox* by NFIL3

My study revealed that NFIL3 acts as an activator on the *Rorγt* promoter, but as a repressor on the *Tox* promoter. An interesting question remains to be answered: how does NFIL3 function differently on the two promoters?

NFIL3 has been predominantly considered as a repressor as it is on the *Rorγt* promoter. For example, in the earliest studies that discovered NFIL3, NFIL3 was found to repress the E4 promoter of adenovirus by binding to a TGACGTAA motif [52]. A follow-up study identified a minimum repressive domain (aa299-aa363 of the human NFIL3 protein) [56]. Similarly, NFIL3 represses *Il12b*, *Il13*, *Fgf21 and cPer2* [58, 67, 73, 78, 181]. Meanwhile, many other studies revealed NFIL3 as an activator. For example, the other early study that independently discovered NFIL3 showed that NFIL3 activates *Il3* by binding to a TAATTACGTCTG motif [53]. Recently, NFIL3 has been shown to activate the Ig GLε transcription in B cells [69] and activates *Eomes* in NK cells [85] by binding to their promoters. Therefore, both activating and repressing activities of NFIL3 have been well documented.

It will require intensive investigation to address the mechanism underlying the differential regulatory functions of NFIL3 on these two sets of promoters, such as identifying cofactors that NFIL3 interacts with and epigenetic modification of chromatin under these two settings. However, one simple hypothesis that may be worth testing involves the binding

motifs in these promoters. The current known binding consensus for NFIL3, RTTAYRTAA, was discovered by studying the repressive effects of NFIL3. Interestingly, NFIL3 appears to bind other motifs when functioning as an activator. As mentioned before, NFIL3 binds to the TAATTACGTCTG motif to activate *Il3*, which was discovered by a DNase footprinting assay, a relatively unbiased approach to pinpoint transcription factor binding site [53]. In addition, no motif similar to the RTTAYRTAA consensus has been found in the NFIL3-responsive, minimum *Tox* promoter in my preliminary studies, suggesting a different binding motif exists in this promoter region. Further studies in this direction may yield important clues about the mechanism of NFIL3 in transcriptional regulation.

CONCLUSIONS

The studies presented in this thesis have uncovered new roles for NFIL3 in regulating innate and adaptive immunity and have highlighted NFIL3 as a critical molecule linking host immunity to the external circadian cycle. NFIL3 suppresses T_H17 cell development from naïve $CD4^+$ T cells and mediates circadian variation in T_H17 lineage specification. This provides one way to maintain T_H17 cell homeostasis and limit excess inflammation at mucosal sites where dense microflora is present. NFIL3 also drives development of ILCs by regulating generation of ILC precursors in the bone marrow, making NFIL3 the first known transcription factor that is required for all ILC development at bone marrow precursor stages. In addition, my studies also revealed two distinct transcriptional regulatory cascades employed by NFIL3 in different immune cells, suggesting that NFIL3 plays different roles in

different immune compartments. Finally, this work not only provides novel insights into circadian regulation of immunity, but also opens up new areas for future investigation.

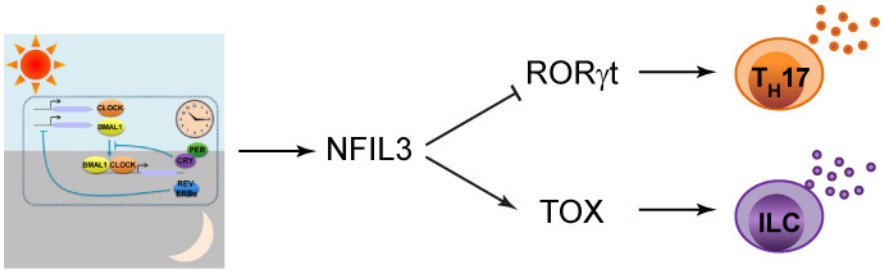

FIGURE 28: Working model.

NFIL3 regulates the development of T_H17 and ILC by controlling transcription factors RORγt and TOX, respectively. Because NFIL3 is regulated by the circadian clock, it provides a mechanistic link between external circadian cycle and internal immune functions.

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