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RUNX3 prevents spontaneous colitis by directing the differentiation of anti-inflammatory mononuclear phagocytes

**Shay Hantisteanu1, Joseph Dicken1, Varda Negreanu1, Dalia Goldenberg1, Ori Brenner2, Dena Leshkowitz3, Joseph Lotem1, Ditsa Levanon1 and Yoram Groner1\***

**Departments of Molecular Genetics1, Veterinary Resources2 and Bioinformatics Unit3, The Weizmann Institute of Science, Rehovot, Israel**

**\*Corresponding author: yoram.groner@weizmann.ac.il**

**ABSTRACT**

RUNX3 is one of three mammalian Runt-domain transcription factors (TFs) that regulate gene expression in several types of immune cells. Runx3-deficiency in mice is associated with several defects in the adaptive and innate immunity systems, including the development of early onset colitis. The present study revealed that the conditional deletion of Runx3 specifically in mononuclear phagocytes (MNPs) (MNPRunx3-/-), but not in T cells, recapitulates the early onset of spontaneous colitis in Runx3-/- mice.

We showed that Runx3 is expressed in colonic MNPs, including resident macrophages (RM) and the dendritic cell cDC2 subsets, and its loss results in impaired differentiation/maturation of both cell types. At the transcriptome level, the loss of Runx3 in RM and cDC2 was associated with the up-regulation of pro-inflammatory genes, similar to those in the early onset inflammatory bowel disease (IBD) murine model of RMIL10r-/-. The impaired maturation of RM in the absence of Runx3 was associated with a marked reduction in the expression of anti-inflammatory and transforming growth factor beta (TGF--regulated genes. Similarly, reduced expression of -catenin signaling associated genes in Runx3-deficient cDC2 indicated their impaired differentiation/maturation.

Analysis of chromatin immunoprecipitation-sequencing (ChIP-seq) data suggested that in both MNP cell types, a significant fraction of these differentially expressed genes are high-confidence Runx3 directly regulated genes. Interestingly, several of these putative Runx3 target genes harbor single nucleotide polymorphisms (SNPs) associated with IBD susceptibility in humans. Remarkably, the impaired maturation and pro-inflammatory phenotype of MNPs lacking Runx3 was associated with a substantial reduction in the prevalence of colonic lamina propria Foxp3+ regulatory Tcells and an increase in interferon (IFN)-producing CD4+ T cells, underscoring the critical role of Runx3 in establishing tolerogenic MNPs.

Together, these data emphasize the dual role of Runx3 in colonic MNPs, as a transcriptional repressor of pro-inflammatory genes and an activator of maturation-associated genes, including anti-inflammatory genes. The present study highlights the significance of the current MNPRunx3-/- model as it relates to human MNP-associated colitis. It provides new insights into the crucial involvement of Runx3 in intestinal immune tolerance and its regulation of colonic MNP maturation through TGF-R signaling, and anti-inflammatory functions through IL10R signaling. Thus, *RUNX3* has been appropriately identified as a genome-wide associated risk gene for various immune-related diseases in humans, including gastrointestinal tract diseases such as celiac and Crohn’s disease.

**INTRODUCTION**

RUNX3 is one of the three mammalian Runt-domain transcription factors (TFs) that are key gene expression regulators during development (Levanon and Groner, 2004; Lotem et al., 2017). *Runx3* was originally cloned, based on its similarity to *Runx1* (Levanon et al., 1994) and subsequently localized on human and mouse chromosomes 1 and 4, respectively (Avraham et al., 1995; Levanon et al., 1994). *Runx3*-/- mice phenotypes reflect its expression pattern and necessity for the proper functioning of important organs (Ebihara et al., 2017; Lotem et al., 2017).

The absence of Runx3 is associated with a multitude of defects in both adaptive and innate immunity including: defective proliferation and differentiation of activated cytotoxic CD8+ T cells (Cruz-Guilloty et al., 2009; Lotem et al., 2013; Taniuchi et al., 2002; Woolf et al., 2003); impaired induction of type 1 T-helper (Th1) cells (Djuretic et al., 2007) and activation of natural killer (NK) cells (Levanon et al., 2014); impaired development of intestinal innate lymphoid cells type 3 (ILC3) (Ebihara et al., 2015); and the lack of dendritic epithelial T cells (Woolf et al., 2007).

We have also reported that Runx3 plays a pivotal role in mononuclear phagocyte (MNP) homeostasis. For example, Runx3 facilitates specification of splenic CD11b+ dendritic cells (DCs) (Dicken et al., 2013) and is required for the development of TGF--dependent Langerhans cells in the skin (Fainaru et al., 2004). The Runx3-/- bone marrow derived DCs (BMDCs) are hyper-activated owing to deregulation of TGF-β-mediated maturation. Immune deficiencies in Runx3-/- mice also reportedly cause lung inflammation associated with the accumulation of hyper-activated DCs, leading to the development of major hallmarks of asthma (Fainaru et al., 2005; Fainaru et al., 2004).

Furthermore, Runx3-/- mice spontaneously develop colitis at 4–6 weeks of age (Brenner et al., 2004). This disease is known to be closely associated with an impaired immune system. Thus, it is interesting that human genome-wide association studies have linked RUNX3 to various immune-related diseases, including asthma, ankylosing spondylitis, psoriasis, psoriatic arthritis, atopic dermatitis, and gastrointestinal tract (GIT) diseases such as celiac and Crohn’s disease (Lotem et al., 2017).

Expression of Runx3 in the GIT of wild type (WT) mice is confined to leukocytes and is not detected in the epithelium of the GIT, indicating that cell-autonomous expression of Runx3 in leukocytes is involved in GIT homeostasis (Levanon et al., 2011). In view of the defects in different cell types of the innate and adaptive immune systems in Runx3-/- mice, and the association of RUNX3 with immune-related human diseases (Lotem et al., 2017), we sought to determine which of the Runx3-/- immune cell types is directly involved in the development of colitis.

Previous research has shown that conditional deletion of Runx3 in NK cells and ILCs by crossing *Runx3fl/fl*mice with *Nkp46-Cre* mice does not induce spontaneous colitis, although those mice did show more severe intestinal damage following infection with *Citrobacter rodentium* (Ebihara et al., 2015). Additionally, *Runx3-/-* mice are highly resistant to inflammation-dependent skin chemical carcinogenesis and this resistance is fully recapitulated in Runx3 conditional knockout mice, in which Runx3 has been deleted in both DCs and T cells, but not in epithelial cells (Bauer et al., 2014). Here, we show that conditional deletion of Runx3 specifically in MNPs, but not in T cells, recapitulates the spontaneous colitis seen in *Runx3-/-* mice. Specifically, Runx3 function in MNPs is crucial for intestinal immune tolerance, as it regulates the proper maturation and anti-inflammatory functions of MNPs.

**MATERIALS AND METHODS**

### Mice

Mice lacking Runx3, specifically in MNPs, were generated by crossing *Runx3fl/fl* mice (Levanon et al., 2011) to *CD11c-Cre* (Caton et al., 2007) or *Cx3cr1c-Cre* mice (Yona et al., 2013), giving rise to *Runx3fl/fl/CD11c:Cre* (hereafter called *Runx3***Δ**) and *Runx3fl/fl/Cx3cr1:Cre* (*Cx3cr1-Runx3Δ*) mice, respectively. The *Runx3***Δ** mice were crossed to *Cx3cr1-GFP* mice (Jung et al., 2000) to obtain *Runx3***Δ** Cx3cr1-GFP mice. All animals were on the C57BL/6 background.

Mice lacking Runx3, specifically in T cells, were generated by crossing *Runx3fl/fl* mice to *Lck-Cre* mice (Takahama et al., 1998) (*Runx3fl/fl/Lck:Cre)*. The *Runx3P1-AFP/P2-GFP* knock-in mice referred to as Runx3-GFP and *Runx3-/-* mice in the present study have been previously described (Levanon et al., 2011). The C57BL/6 Ly5.2 mice were purchased from Harlan Laboratories (Rehovot). In addition, C57BL/6 Ly5.1 mice were bred in the Weizmann Animal Facility. To determine the ability of Runx3-/- leukocytes to transfer colitis, we adoptively transferred intravenously 3 × 106 E13.5 fetal liver (FL) cells from WT or *Runx3-/-* C57BL/6 mice into lethally irradiated (800R and 400R, 4 h apart) C57BL/6 mice, and pathology was determined 2 months after transfer. For the generation of bone marrow (BM) chimeras, C57BL/6 CD45.1 mice were lethally irradiated (1050R) and reconstituted by intravenous injection of a 1:1 mixture of WT C57BL/6 CD45.1 and CD11c-Runx3Δ CD45.2 BM cells. Mice were analyzed 11–15 weeks after BM transfer. Animals were maintained under specific pathogen free (SPF) conditions and handled in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science (Permit #: 09750119-4).

Genotyping primers were as follows - floxed: F5′-CCCACCCATCCAACAGTTCC, R5′-GAGACCACAGAGGACTTGTA; CRE: F5′-AACATGCTTCATCGTCGG, R5′-TTCGGATCATCAGCTACACC; Cx3cr1-GFP mice WT or GFP allele: F5′-TTCACGTTCGGTCTGGTGGG, R5′- GGTTCCTAGTGGAGCTAGGG and F5′-GATCACTCTCGGCATGGACG, R5′-GGTTCCTAGTGGAGCTAGGG, respectively.

**Isolation and analysis of colonic lamina propria (LP) cells**

Isolation of colonic LP cells was performed as previously described with some modifications (Varol et al., 2009). Briefly, extra-intestinal fat tissue and blood vessels were carefully removed, and colons were then flushed of their luminal contents with cold PBS. The cecum was opened longitudinally and cut into 0.5-cm pieces. Epithelial cells and mucus were removed via 40 min of incubation at 37 °C with shaking at 250 rpm in Hank’s balanced salt solution, containing 5% fetal bovine serum (FBS) and 2 mM EDTA. Colon pieces were then digested through 40 min of incubation at 37 °C with shaking in RPMI-1640 containing 5% FBS, 1 mg/mL Collagenase II (Worthington, USA) and 0.1 mg/mL DNase I (Roche, USA).

The digested cell suspension was then washed with PBS and passed through a 100-µm cell strainer. For analysis of blood monocytes, samples were resuspended in ACK erythrocyte-lysis buffer (0.15 M NH4Cl, 0.1 M KHCO3, and 1 mM EDTA in PBS). For intracellular staining, a Foxp3 buffer set (Invitrogen, USA) was used, according to the manufacturer’s instructions. To induce T cell activation for the determination of intracellular IFN-, LP cells were incubated for 5 h with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 µg/mL ionomycin in the presence of 10 µg/mL Brefeldin A (Sigma, IL, USA).

For fluorescence-activated cell sorting (FACS) analysis, single cell suspensions were stained with the following antibodies (Abs): EpCAM G8.8, CD16/32 clone 93, CD45 30-F11, MHCII M5/114.15.2, CD11c N418, Ly6c HK1.4, CD11b M1/70, CD103 2E7, F4/80 BM8, CD64 X54-5/7.1, CD115 AFS98, CD43 eBioR2/60, Foxp3 MF-14, CD25 PC61, CD45RB C363-16A, CD4 RM4-5, Clec12a 5D3, PD-L2 TY25, CD24a M1/69, T-bet 4B10, IFN- XMG1.2, CD45.1 A20, and CD45.2 104. All Abs were purchased from BioLegend, USA or eBiosciences, USA, unless otherwise indicated.

An LSRII flow cytometer (BD Biosciences, USA) equipped with the FACSDiva Version 6.2 software (BD Biosciences) was used to examine cells, and further data analysis was conducted using the FlowJo Version 9.7.6 software (TreeStar, BD Biosciences). A FACSAria flow cytometer (BD Biosciences) was used for cell sorting and the forward scatter height versus forward scatter width appearance was used to exclude doublets.

**Immunofluorescence (IF) and Immunohistochemistry (IHC)**

After the colon was removed, it was frozen in liquid nitrogen. Cryosections of 12–14 µm were prepared on glass slides, fixed for 3 min in acetone at -20 °C, and air dried for 20 min. Slides were blocked with PBS containing 20% horse serum and stained overnight at room temperature with rabbit anti-MHCII-biotin Ab, followed by incubation with SA-cy3 Ab. The major histocompatibility complex II (MHCII) staining and endogenous Cx3cr1-GFP signal were analyzed using a fluorescent microscope. In addition, 4-µm serial paraffin sections of the colon and stomach were prepared and stained with hematoxylin-eosin (H&E) for histopathological evaluation, as previously described (Brenner et al., 2004).

**RNA extraction and microarray gene expression analysis**

Total RNA was extracted from sorted cecum MNPs using the RNeasy Mini Kit (QIAGEN, USA). In each experiment, sorted cells were pooled from 3–4 mice. The BioAnalyzer 2100 (Agilent Technologies, USA) was used to determine RNA quality. The RNA from each sample was labeled and hybridized to Affymetrix mouse exon ST 1.0 microarrays, according to the manufacturer’s instructions. Microarrays were scanned using the GeneChip scanner 3000 7G.

Statistical analysis was performed using the Partek® Genomics Suite (Partek Inc., USA) software. The CEL files (raw expression measurements) were imported to Partek GS and the data were preprocessed and normalized using the RMA (robust multichip average) algorithm (Irizarry et al., 2003) with GC correction. To identify differentially expressed genes (DEGs), one-way ANOVA was applied. The lists of DEGs were created by filtering the genes based on an absolute fold change ≥ 1.5, P ≤ 0.05. Log2 gene intensities were used for volcano scatter plots (Partek). The Ingenuity software was used for Gene Ontology (GO) analysis. The GEO accession number is presented in the next section.

### Chromatin immunoprecipitation-sequencing (ChIP-seq) data acquisition and analysis

Two biological replicate ChIP-seq experiments were conducted for the detection of Runx3-bound genomic regions. A total of 30 × 106 cells of the D1 DC cell line were fixed in 1% formaldehyde and sonicated to yield DNA fragments of ~300 bp, according to standard procedures previously described (Pencovich et al., 2011). For immunoprecipitation (IP), 40 µL of anti-Runx3 Ab were added to 15 mL of diluted fragmented chromatin and incubated overnight at 4 °C. Rabbit pre-immune serum was used as the control. Furthermore, DNA was purified using QIAquick spin columns (QIAGEN, USA).

For ChIP-seq analysis, Illumina sequencing of short reads was performed using an Illumina Genome Analyzer. For data analysis, extracted Runx3 IP and control IP sequences were aligned uniquely to the mouse genome (mm9) using the Bowtie software (Langmead et al., 2009). Bound regions were detected using the Model-based Analysis of ChIP-Seq (MACS2) (Feng et al., 2012). The Runx3 bound peaks and coverage data (bigWig files) were uploaded to the UCSC genome browser.

The GREAT algorithm (version 3.0.0) (McLean et al., 2010) was applied to determine genes corresponding to Runx3-bound peaks in D1 cells and splenic CD4+ DCs (Dicken et al., 2013), as well as those corresponding to H3K4me1, H3K27Ac, and ATAC-seq peaks in colonic RMs (Lavin et al., 2014). The Cistrome CEAS platform (Liu et al., 2011) was used to compute enrichment of the genomic sequences. Discovery of TF binding sites was conducted using the Genomatix analysis software (https://www.genomatix.de‬‏). All microarray and ChIP-seq data are available in the GEO public database under the SuperSeries accession number GSE136067.

### RT-qPCR and protein analysis

Total RNA was reverse transcribed using the miScript reverse transcription kit (QIAGEN), according to the manufacturer's instructions. Quantitation of cDNAs was performed by applying sequence-specific primers (see below), the miScript SYBR Green PCR kit (QIAGEN), and the Roche LC480 LightCycler. Target transcript quantification was calculated relative to *Hprt* mRNA. Standard errors were calculated using the Relative expression software tool (REST) (Pfaffl et al., 2002).

qPCR Primers:

Runx3 ex 3–4 F: 5′- GCCGGCAATGATGAGAACT

Runx3 ex 3–4 R: 5′- CACTTGGGTAGGGTTGGT

Hprt-F: 5′- GTTGGATACAGGCCAGACTTTGTTG

Hprt-R: 5′- CCAGTTTCACTAATGACACAAACG

Hpgds-F: 5′- GGAAGAGCCGAAATTATTCGCT

Hpgds-R: 5′- ACCACTGCATCAGCTTGACAT

Ccl24-F: 5′- ACCGAGTGGTTAGCTACCAGTTG

Ccl24-R: 5′- TGGTGATGAAGATGACCCCTG

Fcrls-F: 5′- ACAGGATCTAAGTGGCTGAATGT

Fcrls-R: 5′- CTGGGTCGTTGCCCTATCTG

Cxcl9-F: 5′- TCCTTTTGGGCATCATCTTCC

Cxcl9-R: 5′- TTTGTAGTGGATCGTGCCTCG

Ms4a14-F: 5′- ACCAACAGACCAGCAGTCAGAAGA

Ms4a14-R: 5′- TTGGATGAGCCTGAGCAAGGTGTA

Pd-l2 –F: 5′- CTGCCGATACTGAACCTGAGC

Pd-l2 –R: 5′- GCGGTCAAAATCGCACTCC

For protein analysis, cell proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and analyzed by western blotting using either in-house anti-Runx3 or anti-Runx1 Ab. Furthermore, GAPDH was used as an internal loading control.

### Statistical analysis

Statistical significance was determined using the unpaired, two-tailed Student’s *t*-test.

**RESULTS**

**Loss of Runx3 in non-T cell leukocytes induces colitis**

To establish whether Runx3-/- leukocytes are involved in the development of colitis, we performed a transfer experiment. Because *Runx3-/-* mice on a C57BL/6 background are not viable after birth, we adoptively transferred E13.5 FL cells from either *Runx3-/-* or WT mice into lethally irradiated C57BL/6 mice. Histopathological analysis performed at 2 months after transfer revealed that recipients of the Runx3-/-, but not WT FL cells developed inflammatory bowel disease (IBD) (Figure 1A, B). These findings are consistent with those of Sugai et al. (2011).

To determine whether this phenotype can be attributed to T cells, we crossed *Runx3fl/fl* mice to *Lck*-*Cre* mice to obtain T cell-specific Runx3 conditional knockout (cKO) mice. Unlike the spontaneous colitis seen in *Runx3-/-* mice, *Lck-Runx3****Δ*** mice did not develop spontaneous colitis (Figure 1C, D). As indicated earlier, colitis was not evident in *NKp46-Runx3****Δ*** mice (Ebihara et al., 2015). These results indicate that Runx3 function in T and NK cells, and ILCs is not essential to maintain GIT homeostasis, and suggest that cell-autonomous Runx3 function in other immune cell types, possibly MNPs, is involved.

**Figure 1. Loss of Runx3 in non-T cell leukocytes induces colitis.**

**A–B**, Adoptive transfer of Runx3-/-, but not wild-type (WT) fetal liver (FL) cells induces colitis in lethally irradiated recipient mice. **A**, Number of recipient mice with colitis. **B**, Histological sections of colon stained with hematoxylin and eosin (H&E). **C**, T cell-specific ablation of Runx3 (*Lck-Runx3***Δ**) does not induce colitis, as shown in the table. Upper, schematic representation of *Runx3* gene locus targeted for conditional inactivation. Dark orange boxes represent coding exons; light orange boxes represent untranslated regions (UTRs); exons are indicated by numbers. Green arrowheads represent lox-P sites that flank exon 3, one of the exons comprising the RUNX transcription factor (TF) DNA binding domain. **D**, Western blot of CD8+ T cell extract from WT and *Lck-Runx3***Δ** mice. Note the efficient deletion of Runx3 and up-regulation of Runx1 in *Lck-Runx3***Δ** cells.

**Mice lacking Runx3 in colonic MNPs develop early onset spontaneous colitis**

The MNPs are known for their role in maintaining GIT homeostasis (Joeris et al., 2017). In order to determine whether Runx3 expression in MNPs plays a role in GIT immune tolerance, we generated MNP-specific Runx3-cKO mice by crossing *Runx3fl/fl* mice to two different Cre transgenic strains, *CD11c-Cre* and *Cx3cr1-Cre*, to generate *Runx3***Δ** and *Cx3cr1-Runx3***Δ** mice, respectively. Both CD11c and Cx3cr1 are expressed in DCs and macrophages, but CD11c is expressed at a higher level in DCs, and Cx3cr1 is expressed at a higher level in macrophages.

Strikingly, the majority of ~3-month-old mice of both MNP-specific *Runx3*-cKO mouse models developed spontaneous mild colitis that affected the cecum and proximal colon (Figure 2A, B). Colitis in these *Runx3*-cKO mice displayed similar characteristics to those observed at an earlier age in *Runx3-/-* mice (Brenner et al., 2004), including the accumulation of infiltrating leukocytes associated with pronounced mucosal hyperplasia and the loss of differentiated mucus-secreting goblet cells. The *Runx3***Δ** and *Cx3cr1-Runx3***Δ** mice older than 7 months generally showed exacerbated colon inflammation and about 30% also developed gastropathy (Figure 2C), resembling the phenotype of *Runx3-/-* mice (Brenner et al., 2004). Notably, younger mice aged 6–8 weeks showed low prevalence of colitis with a minimal average disease score.

As colitis developed in the MNP-specific *Runx3*-cKO mice, we determined which subtype of colonic LP MNP cells expresses *Runx3* by employing compound heterozygous mice (Runx3-GFP) (Dicken et al., 2013). Our analyses revealed that within the LP MNP compartment, *Runx3* is expressed in resident macrophages (RM), characterized as CD11cintCD11b+MHCII+F4/80+CD103- (Figure 2D), and at low levels in LP CD11clo monocytes (Figure S1A), but not in circulating monocytes (CD11b+CD115+CD43hiCD11c+Ly6c- and CD11b+CD115+CD43loCD11c-Ly6c+) (Figure S1B). These results are in agreement with the identification of Runx3 as an intestinal RM-specific TF (Lavin et al., 2014), and indicate that Runx3 expression accompanies the differentiation of monocytes to colonic RMs.

In addition to RMs, the MNP compartment contains three DC subsets, commonly characterized as CD11chighMHCII+F4/80low. These subsets include CD103+CD11b- termed conventional DC1 (cDC1), and two cDC2 subsets, CD103-CD11b+ and CD103+CD11b+ DC. We detected the expression of *Runx3* in the majority of CD103-CD11b+ and CD103+CD11b+ DCs, while only a small fraction of cDC1s expressed *Runx3*. Moreover, these *Runx3*-expressing cDC1s had a lower *Runx3* level relative to the two CD11b+ DC subsets (Figure 2E).

Recent studies on the relationship between intestinal DC subsets suggest that CD103+CD11b+ DCs develop from intermediate CD103-CD11b+ DCs in a TGF--dependent manner (Bain et al., 2017). Accordingly, CD103+CD11b+ DCs express higher levels of *Runx3* compared with CD103-CD11b+ DCs (Figure 2E). Taken together, our results imply that Runx3-/- CD11b+ MNPs, including both RMs and CD11b+ DCs, drive the spontaneous development of colitis.

Nevertheless, the contribution of the small fraction of Runx3-expressing CD103+CD11b- DC cannot be ruled out. Using *Runx3-P1AFP/+* or *Runx3-P2GFP/+* reporter mice, we established that in CD103+CD11b+ DCs and in RMs, both promoters mediate *Runx3* expression; however, P1 or P2 were preferentially used in these cells, respectively (Figure S1C). Collectively, these results indicate that the known role of intestinal MNPs in GIT homeostasis (Joeris et al., 2017) is mediated, at least in part, by P1- and P2-driven *Runx3* expression.

**Figure 2. Colitis development in *Runx3-/-* and in two mononuclear phagocyte (MNP)-specific *Runx3*-cKO mice (*Runx3*Δand *Cx3cr1-Runx3*Δ).**

**A**, Graphical summary of postmortem colitis grade in *Runx3***Δ** and *Cx3cr1-Runx3***Δ**(right) relative to wild-type (WT) mice (left). Colitis was scored on a scale of 1 to 4 (1-minimal; 2-mild; 3-moderate; 4-severe). Each dot represents one mouse. (Note the complete absence of pathological signs of colitis in WT mice at 24 weeks of age). **B**, Hematoxylin and eosin (H&E) stained histological sections of colons from WT and three Runx3-deficient mouse strains. **C,** H&Estained histological sections of the stomach of two Runx3-deficient 1-year-old mousestrains compared to the WT. **D**, Flowcytometry analysis of *Runx3* expression in colonic resident macrophages (RMs) of Runx3-GFP+ mice. The RMs were identified by F4/80 expression (red line) and overlaid on F4/80-CD11b+ (blue line) (top and middle panels). Graphical summary of Runx3-GFP+ RMs (bottom, left panel) and mean fluorescence intensity(MFI) relative to the non-GFP (WT) control (bottom, right panel). **E,** Flow cytometry analysis of *Runx3* expression in colonic CD103+CD11b- cDC1 (red), CD103-CD11b+ cDC2 (blue), andCD103+CD11b+ cDC2 (green) (top and middle panels). Orange line represents the F4/80 negative level. Graphical summary of Runx3-GFP+ prevalence among the dendritic cell (DC) subsets (bottom left) and Runx3-GFP+ MFI relative to the non-GFP (WT) control (bottom right).

**Loss of Runx3 in MNP causes an early imbalance of colonic MNP subsets**

As the loss of Runx3 in MNPs triggers spontaneous colitis, we sought to determine whether alterations in the colonic MNP compartment in *Runx3***Δ** mice occur prior to the onset of colitis. Colonic RMs are derived from recruited circulating monocytes, which differentiate to RMs in four stages (S) (SP1 to SP4), described as the “waterfall” differentiation pattern (Bain et al., 2013; Schridde et al., 2017).

Under steady-state conditions in WT mice, the mature anti-inflammatory SP3 and SP4 stages predominated (Figure 3A). In *Runx3***Δ** mice, at 6–8 weeks of age, there was a marked increase in the prevalence of Ly6c+ pro-inflammatory SP2 monocytes with a concomitant decrease in the mature SP3+SP4 fraction (Figure 3A). These results suggest that Runx3 function in MNPs is required for the maturation of colonic monocytes into anti-inflammatory RMs.

The transition from SP3 to fully mature SP4 RMs is characterized by increased expression of Cx3cr1 (Schridde et al., 2017). To evaluate the level of Cx3cr1 expression in Runx3**Δ** versus WT RMs, we generated *CD11c-Runx3***Δ***-Cx3cr1GFP* mice. Colonic sections showed a greater number of cells expressing Cx3cr1GFP signals in *Runx3***Δ** compared with WT RMs. The green fluorescent protein (GFP) signal in the *Runx3***Δ** colon sections was scattered uniformly in the LP, whereas in WT colon sections, these cells were located relatively adjacent to the epithelium (Figure 3B). Moreover, GFP intensity analysis revealed a significant decrease in the number of Cx3cr1GFP-hi SP4 cells and an increased number of the less mature Cx3cr1GFP-int SP3 cells in the *Runx3***Δ** LP (Figure 3C, S2A). These results indicate that Runx3 is involved in the terminal maturation of RMs from the SP3 to the anti-inflammatory SP4 stage.

Analysis of 7–8 week-old *Runx3***Δ** mice revealed that among the three DC subsets, the CD103+CD11b+ subset was decreased and the CD103-CD11b+ DC subset was increased, compared with their WT littermates, while the CD103+CD11b- DC subset was only slightly reduced. Furthermore, quantification of DC subsets displayed a significant reduction in the number of CD103+CD11b+ DCs in *Runx3***Δ** mice (Figure 3D).

After CD24a (another marker of CD103+CD11b+ DCs) was employed, we detected an even more substantial reduction in the prevalence and number of CD103+CD11b+ DCs (Figure 3E). To determine whether the above documented reduction in the abundance of CD103+CD11b+ DCs in *Runx3***Δ** mice compared with WT mice occurs earlier than 7–8 weeks, we examined 5–6-week-old mice. Interestingly, at this younger age, the *Runx3***Δ** mice showed no difference in DC subset distribution and cell numbers compared with the WT mice (Figure S2B). These findings correspond with the pathology data presented in Figure 2A.

However, *Runx3***Δ** CD103+CD11b+ DCs displayed reduced levels of both CD24a and CD103 (Figure S2B and S2C). Thus, these results suggest that the loss of Runx3 in *Runx3***Δ** mice causes an imbalance among colonic MNP subsets and disturbs the development of CD103+CD11b+ DCs, prior to the onset of significant colitis. These changes are associated with the loss of anti-inflammatory, and gain of pro-inflammatory RM and DC populations, as reflected by the increased prevalence of colonic pro-inflammatory SP2 monocytes and CD103-CD11b+ DCs and decrease in the levels of anti-inflammatory SP4 RMs and CD103+CD11b+ DCs.

Overall, these findings support a cell-autonomous Runx3 function in colonic CD11b+ MNPs, emphasizing their importance to intestinal homeostasis. Accordingly, the loss of Runx3 in MNPs causes early impairment of colonic CD11b+ MNPs, making it a sign of early-onset inflammation.

**Figure 3. Loss of Runx3 in MNPs causes an imbalance among colonic MNP subsets. A,** Comparison of wild-type (WT) and *Runx3***Δ** colonic monocyte-macrophage “waterfall.” Representative flow cytometry gating on CD45+CD11b+F4/80+ cells demonstrating SP1, SP2, and SP3+SP4 cells (left). Graphical summary comparing the prevalence and cell numbers of WT and *Runx3****Δ*** colonic SP1, SP2, and SP3+SP4 waterfall cells (right). **B**, Green fluorescent protein (GFP) signal in frozen section of the colon of 6–8-week-old *Runx3***Δ**-*Cx3cr1GFP/+* mice compared with their WT-Cx3cr1GFP/+ littermates. **C,** Analysis of Cx3cr1-GFP expression in WT resident macrophages (RMs) relative to *Runx3***Δ** colonic RMs. Top, representative flow cytometry gating on WT and *Runx3***Δ** CD45+CD11cintMHCII+CD11b+Ly6c- RMs. Bottom, overlaid histogram (left) of Cx3cr1-GFP expression level in WT (blue) and *Runx3***Δ** (red) RMs, and graphical summary (right) of the frequency of Cx3cr1Hi and Cx3cr1Int macrophages in WT and Runx3**Δ** mice. **D,** Dendritic cell (DC) subsets in WT and *Runx3***Δ** colonic lamina propria (LP) of 7–8-week-old mice. Left, a representative flow cytometry of the CD103+CD11b-, CD103+CD11b+, and CD103-CD11b+ DC subsets. Right, graphical summary depicting the prevalence and cell numbers of the three colonic LP DC subsets. **E,** Representative flow cytometry gating on CD45+CD11chiMHCII+CD11b+F4/80lo cells comparing CD24a expression between *Runx3***Δ**and WT at 8 weeks of age (upper). Graphical summary comparing the prevalence and cell numbers of WT and Runx3**Δ** CD103+CD11b+CD24a+ and CD103-CD11b+CD24a+ DCs (lower).

**Transfer of WT BM overcomes the onset of Runx3Δ BM-mediated colitis**

Competitive BM-repopulation assays were conducted to address whether Runx3-sufficient MNPs regulate an anti-inflammatory response, or whether in the presence of WT MNPs, Runx3**Δ** MNPs will still dictate a pro-inflammatory response. Reconstituted mice colons were examined histopathlogically for signs of colitis, and by flow cytometry to assess the replenishment of the colonic MNP compartment. A 1:1 mixture of Runx3**Δ** BM CD45.2 and WT BM CD45.1 cells was transferred into WT CD45.1 recipient mice.

Eleven to fifteen weeks following transfer, colons of experimental mixed chimeric (Runx3**Δ**/WT→WT) mice were compared to those of Runx3**Δ**→WT and WT→WT reconstituted mice. No differences in weight gain were observed between the different recipient mice groups during the course of the transfer experiment (Figure S3A). As predicted, colons of the Runx3**Δ** BM reconstituted mice showed the development of colitis (Figure 4A). Interestingly, however, the mixed chimeric BM reconstituted mice did not develop colitis (Figure 4A). This finding underscores the ability of Runx3-sufficient MNPs to dictate a mucosal anti-inflammatory environment that overrides the pro-inflammatory state of Runx3**Δ** MNPs.

Analysis of the colonic RM waterfall in Runx3**Δ** BM recipients revealed a marked increase in the SP2 fraction and a concomitant decrease in the SP3+SP4 macrophage fraction. These findings are consistent with our observations in *Runx3***Δ** mice and reports on other mouse strains and IBD development (Biswas et al., 2018; Schridde et al., 2017). In accordance with their normal phenotype, the BM mixed chimera replenished mice exhibited a normal RM waterfall resembling that of the WT BM transferred mice (Figure 4B).

Yet, despite the normal histology of the colon in the mixed chimeric recipient mice, flow cytometry analysis comparisons revealed differences in the abundance of WT and Runx3**Δ** LP MNPs in the mixed chimeric mice. While the mixed chimera WT/CD45.1 MNPs consisted of 75% RMs and ~20% CD11b+ DCs, the mixed chimera Runx3**Δ**/CD45.2 MNPs displayed a reduction in the number of RMs to 60%, and a concomitant increased proportion of CD11b+ DCs to 35% (Figure 4C, S3B).

Notably, RM and CD11b+ DC subset distribution in chimeric mice (WT/CD45.1 and *Runx3***Δ**/CD45.2) were similar to those of the corresponding subsets in WT and Runx3**Δ** BM replenished mice, respectively. However, the mixed chimeric Runx3**Δ** MNP mice displayed a reduction in the number of Ly6c+ colonic monocytes, compared to the mice reconstituted only with *Runx3***Δ** BM (Figure S3B).

We then analyzed colonic normalized cell numbers for each subpopulation. Mice reconstituted with Runx3**Δ** BM had a similar RM cell number as mice reconstituted with WT BM (Figure 4D). In contrast, Runx3**Δ** BM reconstituted mice had a substantially increased number of CD11b+ DCs and Ly6c+ monocytes (Figure 4E and F). Importantly, the mixed chimeric *Runx3***Δ**/CD45.2 mice displayed a significant reduction in the number of CD11b+ DCs and Ly6c+ monocytes, compared with Runx3**Δ** BM reconstituted mice (Figure 4E and F).

These results reveal that Runx3-sufficient MNP suppressed the influx and propagation of Runx3**Δ** monocytes and CD11b+ DCs, thereby imposing an anti-inflammatory environment, which prevented the induction of colitis by Runx3Δ MNPs. These phenomena were reflected by the re-established equilibrium within the MNP compartment of mixed chimeric mice.

***Runx3*Δ colonic RM transcriptome displays an anti-inflammatory to pro-inflammatory switch and colonic MNP maturation defect**

The observations presented above revealed that while RMs and CD11b+ DCs in the colon are formed in the absence of Runx3, *Runx3***Δ** micecan stilldevelop colitis. To gain further insight into the Runx3-dependent transcriptional program controlling intestinal MNP homeostatic function, we analyzed the transcriptomes of WT Runx3**Δ** RMs and CD11b+ DCs. The RNA of 6–8-week-old mice was prepared from sorted cecal RMs and CD11b+ DCs (Figure S4A).

Principal component analysis of transcriptome data, including all the indicated samples, revealed two distinct cell populations corresponding to CD11b+ DCs and RMs (Figure S4B). Analysis of differential gene expression between Runx3**Δ** and WT RMs revealed 70 up-regulated and 128 down-regulated genes in Runx3**Δ** (fold change ≥ 1.5, P-value ≤ 0.05) (Figure 5A, Table S1). Several of these Runx3**Δ**RM DEGs

**Figure 4. Colitis and colonic MNP subsets in lethally irradiated mice reconstituted with WT, *Runx3*Δ, or a mixture of WT/*Runx3*Δ BM cells.** **A,** Representative Hematoxylin and eosin (H&E) stained section of the colon of Runx3**Δ** bone marrow (BM) recipient mice [Runx3**Δ** (CD45.2)→WT(CD45.1)], mixed chimera [Runx3**Δ**(CD45.2)/WT (CD45.1)→ WT(CD45.1)], and wild-type (WT) BM recipient mice [WT(CD45.1)→WT (CD45.1)] (top). Colitis was scored on a scale from 1 to 4 (1-minimal; 2-mild; 3-moderate; 4-severe). \*\*\* P<0.001 (bottom). **B,** Comparison of colonic lamina propria (LP) SP1 to SP4 waterfall between WT, Runx3**Δ**, and mixed chimera (WT and Runx3**Δ**) BM recipient mice. Representative waterfall from each group (top) and summary of SP1, SP2, and SP3+SP4 abundance in the three groups (bottom) are shown. \*\*\* P<0.001. **C,** Representative flow cytometry profile of colonic LP mononuclear phagocyte (MNP) subsets in *CD11c-Runx3****Δ***BM recipient mouse (top), mixed chimeric mouse (middle), and WTBM recipient mouse (bottom). **D,** Graphical summary of colonic LP resident macrophage (RM) normalized cell numbers in reconstituted BM mixed chimera mice compared with Runx3**Δ**BM reconstituted mice and WTBM reconstituted mice. **E,** Graphical summary of colonic LP CD11b+ dendritic cell (DC) normalized cell numbers in reconstituted BM mixed chimera mice compared with Runx3**Δ**BM reconstituted mice and WTBM reconstituted mice. \* P<0.05. **F,** Graphical summary of colonic LP Ly6c+ monocytes normalized cell numbers in reconstituted BM mixed chimera mice compared with Runx3**Δ**BM recipient and WTBM recipient mice. \* P<0.05, \*\* P<0.01.

In addition, flow cytometry analysis confirmed the up-regulation of two surface proteins encoded by the Runx3**Δ**RM DEGs, *Pdcd1lg2* and *Clec12a* (Figure S4D, E). Interestingly, GO enrichment analysis of these DEGs using the term “disease and disorder” yielded “experimental colitis” as the top enriched term in the “inflammatory/auto-immune disease” category (Figure 5B). In line with the GO enrichment for the term "colitis", expression of inflammatory genes was affected; 19 out of 21 pro-inflammatory DEGs (85%) were up-regulated, and 14 out of 19 (74%) anti-inflammatory DEGs were down-regulated in Runx3**Δ** RMs (Table 1).

These results indicate that the loss of Runx3 in MNPs, switches colonic RMs from an anti-inflammatory to a pro-inflammatory state. Notably, GO analysis to detect potential upstream regulators of Runx3**Δ** RM DEGs highlighted IL10RA and IFNG as the most significant regulators, with IL10RA having a negative *z*-score, and IFNG, a positive *z*-score (Figure 5C). Conditional deletion of *IL10ra* in MNPs (*Cx3cr1-IL10raΔ*) (Zigmond et al., 2014) and *IL10rb-/-* mice (Redhu et al., 2017; Zigmond et al., 2014) leads to the development of spontaneous colitis, similar to that observed in *Runx3***Δ** mice.

To examine a possible relationship between Runx3 and IL10 signaling in RMs, we cross analyzed Runx3**Δ** DEGs with the transcriptional profile of IL10raΔ and IL10rb-/- RMs. Remarkably, 23 out of the 70 (33%) Runx3**Δ** RM up-regulated genes were also up-regulated in the two other data sets, and 40%–50% of the Runx3**Δ** RM up-regulated genes overlapped with each of the IL10rΔ RM data sets (Figure 5D).

Moreover, the common up-regulated genes in Runx3**Δ**, Cx3cr1-IL10raΔ, and IL10rb*-/-* RMs included 15 of the 21 (71%) pro-inflammatory genes underscored above (Table 1). While MNP-*Runx3***Δ** and MNP-*IL10R***Δ** mice displayed a similar spontaneous colitis phenotype with a significant overlap of RM up-regulated genes, expression of *IL10ra* and *IL10rb* was unaffected in Runx3**Δ** RMs. Together, these observations led us to hypothesize that Runx3 is involved in MNP transcriptional regulation downstream of IL10-induced signaling.

Newly arrived colonic monocytes, termed SP1 monocytes, differentiate through stages SP2 and SP3 until ultimately becoming mature SP4 RMs (Schridde et al., 2017). This process is accompanied by the expression of 613 genes, specifically in SP4, compared with SP1 monocytes (Schridde et al., 2017). As Runx3 is expressed in colonic RMs, and in its absence, the SP4 mature stage is impaired, we hypothesized that Runx3 is involved in the maturation of colon SP4 RMs.

As mentioned above, 128 down-regulated DEGs were identified by cross analysis of Runx3**Δ**/WT RM gene expression profiles (Figure 5A, Table S1). Intersecting these 128 RM Runx3**Δ** down-regulated genes with the 613 SP4-specific genes revealed a marked overlap of 58 genes, comprising 45% of Runx3**Δ** down-regulated genes (Figure 5E). Remarkably, 11 of the 14 (79%) down-regulated anti-inflammatory genes in Runx3**Δ** RMs, including *IL10*, were among these 58 common genes (Table 1, Figure 5E). Additionally, among these 58 down-regulated genes, several were associated with the Notch pathway and cholesterol uptake.

Maturation into the colon SP4 RM stage is dependent on up-regulation of various TGF- signaling genes (Bain et al., 2013; Schridde et al., 2017). As Runx3 has an established role in mediating TGF- signaling (Fainaru et al., 2004), it is reasonable to speculate that the 58 down-regulated genes include Runx3-responsive TGF--regulated genes. Cross analysis of the list of SP4-specific RM genes with those that were down-regulated in *Tgfbr1*Δ*/RAG1-/-* (Schridde et al., 2017) revealed 62 common genes (Table S1). Intersecting these 62 genes with the above mentioned 58 P4-specific genes that were down-regulated in Runx3**Δ** RMs, revealed 9 common genes (Figure 5E, Table S1).

To gain further insight into potential Runx3 directly targeted genes, the list of 198 DEGs in Runx3**Δ** RMs (Table S1) was cross analyzed with lists of genes containing the hallmarks of transcriptionally active regions: ATAC-seq, H3K4me1, and H3K27ac ChIP-seq peaks in colonic RMs (Lavin et al., 2014). Of particular relevance to our findings was the observation that a RUNX motif is highly enriched in enhancer regions, specifically in intestinal macrophages, as compared with macrophages from all other tissues (Lavin et al., 2014).

Interestingly, this analysis revealed that out of the 198 DEGs, 120 (60%) harbored all three peak categories and 28 additional DEGs harbored both H3K4me1 and H3K27ac peaks (Figure S5A), raising the number of peak-bearing DEGs to 148 (75%). Accordingly, only 25 DEGs (13%) contained none of these peaks (Figure S5A). Moreover, 126 of these 148 peak-harboring genes contained at least one region, in which at least two of the three above mentioned peak categories overlapped (in most cases H3K4me1 and H3K27ac).

Manual analysis of the 126 DEGs containing overlapping peaks, including the 40 pro- and anti-inflammatory DEGs (Table 1), revealed that 117 of them (95%) harbored a RUNX motif in the overlapping peak (Table S1 and see *Ass1* and *IL10* as examples in Figure S5B). We defined these Runx3**Δ** RM DEGs as high-confidence Runx3 targets. Interestingly, the human homologs of eight of these RM Runx3 high-confidence target genes, *CFB, IFIH1, IL10, NOS2, PLAU, PRDX5, and TNF*, harbored SNPs that are susceptibility loci for IBD, Crohn’s disease (CD), and/or ulcerative colitis (UC) (Table S1).

Furthermore, we noted that six out of the nine (Figure 5E) Runx3 putative targets regulated by TGF- in the RM SP4 population harbored a RUNX-SMAD module in their Runx3 bound regions (four of these are shown in Figure S5C). Overall, these results suggest that Runx3 is involved in positively regulating TGF--dependent RM maturation and an IL10-driven anti-inflammatory response, while suppressing a pro-inflammatory program.

**Figure 5. Runx3Δ RM transcriptome reveals impaired maturation and up-regulation of pro-**

**inflammatory genes as occurs in IL10rΔ RMs.** **A,** Volcano plot of colonic RM DEGs in 6–8-week-old *Runx3***Δ** and WT control mice. Numerous up- and down-regulated genes in Runx3**Δ** are indicated. **B,** Gene Ontology (GO) analysis categorized by "disease and disorder". **C,** GO analysis categorized by “upstream regulators.” **D**, Venn diagram (lower part) representing the overlap between Runx3**Δ**, Cx3cr1-IL10ra***Δ***, and IL10rb*-/-* RM up-regulated genes. Cutoff for Runx3**Δ** and the two IL10r-deficient RM DEGs was set to 1.5- and 2-fold change, respectively. Heat maps representing the standardized expression values of common genes; asterisks indicate the pro-inflammatory genes. Runx3 high-confidence targets are represented by gray squares in the right column. **E,** Venn diagram representing the overlap between down-regulated genes in *Runx3***Δ** RMs and up-regulated genes in terminally differentiated SP4 RMs vs. their SP1 monocyte precursors (top). Standardized expression values of shared genes are specified in the heat map (bottom). Runx3 high-confidence targets are represented by gray squares in the right column.

**Table 1. Pro- and anti-inflammatory differentially expressed genes (DEGs) in Runx3Δ resident macrophages (RMs) and CD11b+ dendritic cells (DCs) and their up-regulation in IL10rΔ and P4 RMs.** Gene names in red and blue indicate up-regulated and down-regulated genes, respectively.

**Runx3 positively regulates colonic CD11b+ DC differentiation genes**

The colonic CD11b+ cDC2 subsets consist of double positive CD103+CD11b+ DCs and their precursors, CD103-CD11b+ DCs. Both DC subsets express Runx3 (Figure 2E). The increased prevalence of colonic LP CD103-CD11b+ and the decrease in CD103+CD11b+ DC in *Runx3***Δ** compared with WT mice (Figure 3C) raised the possibility that, as in RMs, Runx3 affects CD103+CD11b+ DC differentiation. Because it was hard to isolate sufficient numbers of colonic CD103+CD11b+ DC from *Runx3***Δ** mice, we compared the transcriptome of WT and Runx3**Δ** colonic CD11b+ DCs, including both CD103-CD11b+ and CD103+CD11b+ DCs.

The transcriptome profile revealed 84 up-regulated and 152 down-regulated genes (fold change ≥ 1.5, P-value ≤ 0.05) in Runx3**Δ** vs. WT CD11b+ DCs (Figure 6A, Table S1). Similar to the RMs, GO analysis of DEGs in CD11b+ DCs underscored IL10RA and IFNG as the top significant upstream regulators (Figure 6B). Importantly, we found a subset of 31 Runx3**Δ** DEGs common to both RMs and CD11b+ DCs (Figure 6C), suggesting that some Runx3-regulated functions are shared between these two MNP populations.

Remarkably, 13 of these 31 Runx3**Δ** RM and CD11b+ DC DEGs in common (42%) were inflammation-regulating genes, including 10 up-regulated pro-inflammatory and three down-regulated anti-inflammatory genes (Table 1). Moreover, most of the pro-inflammatory genes up-regulated in Runx3**Δ** CD11b+ DCs were shared by IL10RA and/or IL10RB-deficient RMs (Table 1). These results suggest that Runx3**Δ** CD11b+ DCs display pro-inflammatory properties, similar to monocyte-derived CD11b+ DCs following mild dextran sulfate sodium-induced colitis (Varol et al., 2009). In addition, Runx3**Δ** CD11b+ DCs showed down-regulation of *Ifnb1* and the IFN--regulated genes, *Ifih1* and *Mx2* (Figure 6C, Table S1). Furthermore, the enrichment of genes associated with -catenin and TGF- signaling was noted among Runx3**Δ** CD11b+ DC down-regulated genes (Figure 6D).

The Runx3 ChIP-seq analysis in the D1 DC cell line and splenic CD4+ DCs (Dicken et al., 2013) revealed 13,014 and 15,121 Runx3-bound regions (peaks), respectively, of which 6836 peaks overlapped, corresponding to 6422 genes. Interestingly, GREAT analysis under the “PANTHER Pathway” revealed that the genes corresponding to the overlapping Runx3-bound peaks in D1 cells and splenic CD4+ DCs, as well as the genes corresponding to the peaks in colonic RMs (Lavin et al., 2014), were highly enriched for the term “inflammation mediated by chemokine and cytokine signaling pathway” (Table S1).

To determine the putative CD11b+ DC direct Runx3 target genes, we first cross analyzed the list of DEGs in colonic Runx3**Δ** CD11b+ DCs with the list of common Runx3-bound genes in D1 and splenic CD4+CD11b+ DCs. The results showed that 90 of the 236 DEGs in Runx3**Δ** CD11b+ DCs (38%) harbored Runx3-bound regions (Figure S6A). Moreover, 65 of these 90 Runx3-bound DEGs (71%) contained at least one region with a RUNX motif, suggesting that they were high-confidence Runx3-target genes (Figure 6D, Table S1).

Furthermore, 10 of the 31 common DEGs in Runx3**Δ** CD11b+ DCs and RMs (32%) were also common high-confidence Runx3 target genes (Figure 6C and S6B). Interestingly, three of these 10 common Runx3 target genes in colonic RMs and CD11b+ DCs (*Ifnb1*, *Pdcd1lg2,* and *Stat1*) were either pro- or anti- inflammatory genes (Figure S6C). This suggested that they might contribute substantially to the *Runx3***Δ** mice colitis phenotype.

Notably, the human homologs of four of the CD11b+ DC Runx3 targets, *CD300LF, IFIH1, IRF4,* and *SLC22A5* (mouse *Slc22a21*), harbored SNPs associated with IBD, CD, UC, and/or celiac disease (Table S1), and the two former genes were common Runx3 targets in RMs and CD11b+ DCs. Overall, these results indicated that Runx3 is important for the maturation of CD11b+ DCs into anti-inflammatory and tolerogenic DCs. They also indicated that the loss of Runx3 in CD11b+ DCs affects these anti-inflammatory and tolerogenic properties in a remarkably similar way to that found in Runx3**Δ** RMs and IL10R-deficient RMs.

**Figure 6. A fraction of DEGs between Runx3Δ and WT CD11b+ DCs are shared with Runx3Δ RMs.** **A,** Volcano plot of colonic CD11b+ DC DEGs in 6–8-week-old *Runx3***Δ**and WT control mice. Some up- or down-regulated genes in Runx3**Δ** CD11b+ DCs are indicated. **B,** GO analysis categorized by upstream regulators. Cutoff and *z*-score values were set to 2. **C,** Venn diagrams and heat maps showing common DEGs in colonic Runx3**Δ** CD11b+ DCs and RMs. **D,** Heat map showing standardized expression values of 65 high-confidence Runx3 target genes in CD11b+ DCs.

Loss of Runx3 in MNPs induces tolerogenic to inflammatory CD4 T cell transition

The MNP switch from an anti- to pro-inflammatory response in the absence of Runx3 indicated the impaired activation of T cells. Altered expression of regulatory T cell (Treg)-inducing genes further supported this possibility. Two down-regulated genes in cDC2, *Il33* and *Il6*, encode important cytokines for intestinal Treg generation (Schiering et al., 2014) (Table S1). Furthermore, a previous study has shown that an additional cDC2 down-regulated gene, *IFN-* is important for the intestinal control of Treg differentiation (Nakahashi-Oda et al., 2016).

To verify whether Tregs were affected, we compared the colonic Foxp3+ Tregs of WT and *Runx3***Δ** mice. The prevalence of Tregs was determined by gating on the CD45+CD4+CD45RBlo cells, followed by gating on the CD25+Foxp3+ cells. Remarkably, the Runx3**Δ** colonic LP showed a substantial reduction in the frequency of Foxp3+ Tregs (Figure 7A).

The inducible T cell co-stimulator (ICOS) plays a role in modulating different adaptive immune responses. Hence, we examined whether ICOS expression is affected in CD4+ lymphocytes of *Runx3***Δ** mice. Interestingly, we found that the abundance of ICOS-expressing cells in CD4+ lymphocytes of *Runx3***Δ** mice is significantly increased compared to those of WT mice (Figure 7B), suggesting an increase in the number of activated CD4+ T cells in *Runx3***Δ** mice.

The results of GO analysis suggested that Runx3**Δ** MNPs might also induce T cell activation, as manifested by IFN-γ production. To examine the impact of Runx3 deletion in MNPs on lymphocyte activation, we analyzed IFN-γ expression in LP CD4+ T cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin. The results indicated an increased abundance of IFN-γ expressing activated LP CD4+ T cells in *Runx3***Δ** mice (Figure 7C).

Mucosal immunity depends partly on cross talk between the immune system and the epithelium. For instance, IFN-γ produced by CD4+ T cells plays a role in ameliorating the development of colitis by induction of MHCII expression in the epithelium (Mayer et al., 1991; Thelemann et al., 2014). The induction of MHCII in mucosal epithelial cells has also been reported in patients with IBD (Buning et al., 2006).

Consequently, we addressed whether colonic epithelial MHCII is affected in *Runx3***Δ**mice. Our analysis revealed profound adluminal MHCII expression in Runx3**Δ** LP epithelial cells, whereas MHCII expression in the WT LP was confined to leukocytes (Figure 7D left). Furthermore, we detected MHCII expression in the colonic epithelium of both *Runx3***Δ** and *Cx3cr1-Runx3Δ* mice, as evidenced by co-expression of MHCII and the epithelial marker, EpCAM (Figure 7D, middle and right).

Collectively, these results suggest that Runx3 expression in colonic LP MNPs plays an important role in promoting intestinal immunological tolerance, as reflected by the induction of LP Foxp3+ Tregs and reduction in the number of IFN-γ producing CD4+ T cells. The diversion from regulatory to pro-inflammatory CD4+ T cells in the Runx3**Δ** colon reinforces the impact of the transcriptional transition of Runx3**Δ**MNPs into an immature pro-inflammatory state.

**Figure 7. Runx3-deficient MNPs cause a tolerogenic to inflammatory switch in colonic CD4+ T cells.** **A,** Analysis of Tregs in the *Runx3***Δ** colonic lamina propria (LP). Representative flow cytometry analysis (top) and graphical summary (bottom) of CD25+Foxp3+ Tregs in colonic LP of wild-type (WT) *MNP-Runx3***Δ** mice. \*\*\*P<0.001. **B,** Representative flow cytometry (left) and graphical summary (right) of inducible T cell co-stimulator (ICOS)-expressing WT and Runx3**Δ** colonic LP CD4+CD25- T cells. **C,** Intracellular staining of IFN- in WT and Runx3**Δ** colonic LP CD4+CD25- T cells. **D**, major histocompatibility complex II (MHCII) immunofluorescent (IF) staining in the WT and *Runx3***Δ** colonic epithelia. Frozen sections from WT and *Runx3***Δ**colons stained with anti-MHCII. Magnification ×40; scale bars 50 µm (left). Flow cytometry analysis (middle) and graphical summary (right) of colonic epithelial cells from WT, *Runx3***Δ**, and *Cx3cr1-Runx3Δ*mice stained with anti-EpCAM and anti-MHCII Phycoerythrin (PE). \*\*P<0.01, \*\*\*P<0.001.

**DISCUSSION**

The large surface area of the intestinal mucosa exposed to the external environment poses a great challenge to the mucosal immune system to strike a balance between the defense against pathogens and tolerance to commensal bacteria and food antigens. Striking this balance can lead to IBD (Gross et al., 2015). More than 200 human IBD susceptibility loci have been identified, mostly in genomic regions in the vicinity of genes expressed in various immune cells, including those on chromosome 1p36 in the *RUNX3* locus (Cho, 2001; Cho et al., 1998; Lotem et al., 2017; Peters et al., 2017).

Moreover, many IBD susceptibility loci have been associated with genes that are RUNX3 targets in various immune cells (Lotem et al., 2017). Previously, we reported that Runx3-/- mice develop early onset spontaneous colitis (Brenner et al., 2004). The fact that Runx3 is not expressed in normal colonic epithelium (Brenner et al., 2004; Levanon et al., 2011), suggests that the loss of a leukocytic cell-autonomous Runx3 function is the driving force of colitis development in these mice (Brenner et al., 2004).

Here, we provide direct evidence that the transfer of Runx3-/-, but not WT, FL, or BM cells into lethally irradiated mice induces colitis in the recipient mice. Moreover, the conditional deletion of *Runx3* in MNPs, but not lymphocytes, recapitulated the spontaneous development of colitis observed in *Runx3-/-* mice. These results indicate that Runx3 expression in MNPs is important for their role in GIT homeostasis (Joeris et al., 2017).

The loss of Runx3 in MNPs results in a decreased abundance of colonic SP3-SP4 mature anti-inflammatory RMs and CD103+CD11b+ cDC2, which occurs prior to the onset of significant colitis symptoms. This suggests that these changes are indeed the cause of colitis. This premise is supported by the finding that Runx3**Δ** BM transfer to lethally irradiated mice induced the same changes in MNP populations, as well as colitis, whereas mice transplanted with an equal number of WT and Runx3**Δ** BM cells showed a normal MNP balance and remained healthy. These data are consistent with the conclusion that the presence of WT BM cells overcomes the colitogenic effect of the transplanted Runx3**Δ** BM cells.

Analysis of Runx3**Δ** and WT colonic RM transcriptomes, revealed a prominent up- and down-regulation of pro- and anti-inflammatory genes, respectively, in Runx3**Δ** RMs. These results strongly indicate that the loss of Runx3 in RMs induces an anti- to pro-inflammatory switch in their biological properties. Comparison of RM DEGs in Runx3**Δ** with those in IL10ra**Δ** and IL10rb-/-, which also induce colitis (Redhu et al., 2017; Zigmond et al., 2014), revealed a gain of pro-inflammatory hallmarks in all three models of spontaneous colitis.

The loss of Runx3 in RMs did not affect the expression of *IL10ra* and *IL10rb* in our data, and neither was the expression of *Runx3* significantly affected by the loss of IL10rb (Redhu et al., 2017). Therefore, the similar spontaneous colitis phenotype in these three strains cannot be explained by cross-regulation between Runx3 and signals emanating from the IL10 receptors. However, it is possible that the activation of Stat3 TFs, downstream of IL10 receptor signaling (Biswas et al., 2018), collaborates with Runx3 in the nucleus, which can explain the partially shared effect on gene expression when either of these TFs is deleted. This possibility is supported by the known ability of Stat and RUNX proteins to physically interact with each other (Ogawa et al., 2008), and the spontaneous colitis induced in mice harboring Stat3-deficient MNPs (Kobayashi et al., 2003; Melillo et al., 2010; Takeda et al., 1999).

Notably, the fact that the common DEGs in Runx3- and ILl0 receptor-deficient RMs are mostly confined to up-regulated genes, suggests that the collaboration between Runx3 and Stat3 is employed mainly to suppress their targets, particularly the pro-inflammatory genes. It should also be stressed that while Runx3 and IL10R deficiencies in RMs lead to colitis, each model bears unique features. For example, down-regulation of anti-inflammatory genes occurs in Runx3**Δ** RMs but is not evident in the two IL10R models.

Furthermore, a recent study reported that IL10ra**Δ** RMs show increased expression of IL23, which induces IL22 production in T cells leading to hypertrophy of the colonic epithelium (Bernshtein et al., 2019). In contrast, no change was noted in *IL23* expression in Runx3**Δ** RMs, but expression of *IL22ra2*, encoding a very potent antagonist of IL22 receptor signaling, was down-regulated. Because the balance of IL22 and its antagonist IL22ra2 (IL22RB) is important for intestinal homeostasis (Zenewicz, 2018), it is conceivable that reduced *IL22ra2* expression in Runx3**Δ** RMs increases the response to IL22 itself, and could thus elicit an inflammatory response in the epithelium.

The differentiation of intestinal SP1 monocytes to fully mature anti-inflammatory SP4 RMs is a TGF--dependent process (Schridde et al., 2017) and we have now shown that SP4 RMs express a higher level of Runx3 compared with SP1 monocytes. The loss of Runx3 in MNPs results in defective RM differentiation associated with reduced expression of anti-inflammatory genes. The finding of reduced expression of TGF- and Notch-regulated genes is in line with the defect of Runx3**Δ** RM differentiation. Thus, the function of Runx3 in normal RMs to repress pro-inflammatory genes and induce anti-inflammatory genes, is consistent with its ability to protect against colitis.

Furthermore, ~60% of all DEGs in Runx3**Δ** RMs, including most of the inflammatory genes, are high-confidence Runx3-target genes, as judged by their harboring overlapping ATAC and enhancer chromatin mark peaks containing a RUNX binding motif. Moreover, as with human *RUNX3* itself (Lotem et al., 2017), the human homologs of eight of these high-confidence Runx3 target genes in RMs contain known susceptibility loci for IBD, CD, UC, or celiac GIT diseases.

Runx3 is a key player in cell-lineage fate, including in DCs and exert their effects, to some extent, by mediating the response to TGF-. For example, Runx3 regulates TGF--mediated lung DC functions, facilitates the specification of murine splenic CD11b+Esamhi DCs, and is mandatory to the TGF--dependent development of skin Langerhans cells (Dicken et al., 2013; Fainaru et al., 2004). The TGF-β-dependence of the differentiation of intestinal CD103+CD11b+ DCs has also been demonstrated (Bain et al., 2017; Schridde et al., 2017).

We found that Runx3 is expressed at low levels in ~20% of intestinal cDC1 cells, whereas it is highly expressed in the majority of cDC2 cells. Given that the Runx3**Δ** LP shows reduced abundance of these mature CD103+CD11b+ cDC2 cells, it is conceivable that Runx3 participates in TGF-β signaling in cDC2 subsets. Mice lacking colonic cDC1 can still establish tolerance, presumably by their CD11b+ cDC2 subsets (Veenbergen et al., 2016). Thus, the fact that colonic CD11b+ cDC2 cells are affected in *Runx3***Δ** mice may imply that these cells contribute to the development of colitis.

Analysis of cDC2 transcriptomes of Runx3**Δ** vs. WT mice revealed that merely 31 of the 236 DEGs in Runx3**Δ** cDC2 cells were similar to those of Runx3**Δ** RMs. Remarkably, ~40% of these 31 common DEGs were inflammation-regulating genes, including 10 commonly up-regulated pro-inflammatory genes and three commonly down-regulated anti-inflammatory genes. Most of these common pro-inflammatory genes that are up-regulated in Runx3**Δ** RMs and cDC2 cells, are also up-regulated in IL10ra and/or IL10rb-deficient RMs (Table 1). These results imply that like their Runx3**Δ** RM counterparts, LP Runx3**Δ** cDC2 cells contribute to the colitis phenotype by acquiring a pro-inflammatory state.

Cross analysis of Runx3**Δ** cDC2 DEGs with genes that harbored overlapping Runx3-bound peaks in ChIP-seq assays revealed that 65 of these genes contained a RUNX motif, marking them as high-confidence Runx3 target genes. Ten of these cDC2 high-confidence Runx3 targets were common to those in RMs, and the human homologs of four, *CD300LF, IFIH1, IRF4,* and *SLC22A5* (mouse *Slc22a21*) harbored SNPs associated with IBD, CD, UC, and/or celiac disease. Of particular interest is the known importance of Irf4 for the survival of intestinal CD103+CD11b+ DCs (Persson et al., 2013), which can explain the reduced abundance of these cells in the Runx3**Δ** colon.

Intestinal DCs participate in immune tolerance and barrier protection by driving the differentiation of Tregcells and Th17 cells, respectively (Zhou and Sonnenberg, 2018). Interestingly, some of the DEGs in Runx3**Δ** cDC2 cells suggest an impairment of the β-catenin signaling pathway, which is important in the induction of tolerogenic DCs (Bain et al., 2013; Schridde et al., 2017).

Another critical element in the tolerance breach in Runx3**Δ** mice is the substantial reduction in the number of Foxp3+ regulatory T cells, a crucial component in the induction of GIT mucosal tolerance. This reduction can be attributed to a defective ability of Runx3**Δ** cDC2 to generate Treg cells, as essential Treg-inducing factor genes, such as *Aldh1a2*, *Il33,* and *Ifnβ* (Bakdash et al., 2015; Nakahashi-Oda et al., 2016; Schiering et al., 2014) are down-regulated in Runx3**Δ** cDC2 cells. The decreased *Ifnβ* expression that is also evident in Runx3**Δ** RMs suggests that the loss of Runx3 expression in RMs participates in the failure of Runx3**Δ** mice to generate and/or maintain GIT Tregs. In addition, the induced surface expression of Pd-l2 in Runx3**Δ**MNPs exemplifies the pro-inflammatory state acquired by Runx3**Δ**RMs, which can explain the increased production of IFN by Runx3**Δ** CD4+ T cells.

Besides the similarity in inflammatory DEGs between Runx3**Δ** RMs and cDC2 cells, other DEGs unique to each MNP subset may also contribute to the colitis phenotype. The RMs are normally maintained in a state of inflammatory anergy through the acquisition of a non-inflammatory gene expression profile (Bain et al., 2013; Rivollier et al., 2012; Weber et al., 2011), yet they retain their bactericidal capacity (Niess et al., 2005). Interestingly, the anti-bacterial autophagy gene, *Clec12a*, which is reportedly associated with an increased risk for CD (Begun et al., 2015), and *Slc7a11*, a potential blocking target for the treatment of IBD (Bridges et al., 2012), were up-regulated specifically in Runx3**Δ** RMs, but not in Runx3**Δ** cDC2 cells.

While the increased and decreased expression of pro-inflammatory and anti-inflammatory genes, respectively, in Runx3**Δ** MNPs can contribute to the development of colitis, it may be insufficient, as in the context of the BM chimera replenishment assays, the mixed WT/Runx3**Δ** MNP chimeric mice were protected from colitis. This finding implies that WT Runx3-sufficient MNPs confer an immune-suppressive GIT condition, by maintaining a proper balance of pro- and anti-inflammatory gene expression in MNPs themselves, together with an indirect effect that prevents the loss of Tregs.

To summarize, all of our results point toward one major conclusion: MNP Runx3 maintains colon homeostasis by directing proper colon MNP specification into mature anti-inflammatory MNP and concomitantly repressing expression of a harmful pro-inflammatory program, similar to that which occurs in IL10 receptor-deficient MNPs.

Another layer of MNP Runx3 contribution to intestinal homeostasis is its impact on maintaining colonic Tregs. These results imply that human MNP RUNX3 plays an important role in preventing the development of inflammatory GIT diseases, including IBD, CD, UC, and celiac disease in humans. This premise is strongly supported by the presence of susceptibility loci for these diseases in the RUNX3 gene and in 10 other genes that are high-confidence RUNX3 targets in RMs and/or cDC2 cells.

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**AUTHOR CONTRIBUTIONS**

SH, VN, OB, DG, and JD performed the experiments. SH, DeL, JL, DiL, OB, and YG designed the experiments and analyzed the data. SH, JL, DL, and YG wrote the paper.

The authors declare that they have no conflicts of interest.