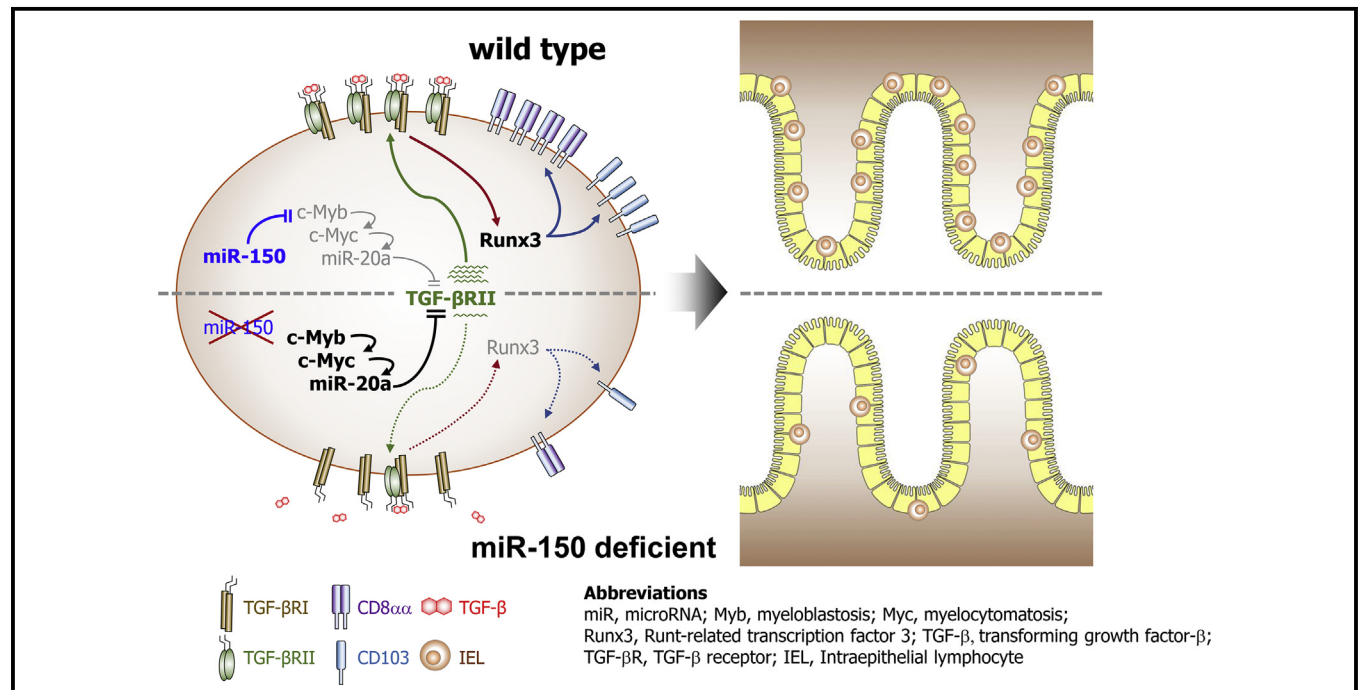


MicroRNA-150 controls differentiation of intraepithelial lymphocytes through TGF- β receptor II regulation

Sang-Hwan Seo, PhD,^{a,*} Min Seong Jang, DVM, PhD,^{b,*} Doo-Jin Kim, PhD,^{b,*} Seok-Min Kim, BS,^{a,c} Se-Chan Oh, MS,^{a,c} Cho-Rok Jung, PhD,^d Yunji Park, PhD,^e Sang-Jun Ha, PhD,^f Haiyoung Jung, PhD,^{a,c} Young-Jun Park, PhD,^{a,c} Suk Ran Yoon, PhD,^{a,c} Inpyo Choi, PhD,^{a,c,†} and Tae-Don Kim, PhD^{a,c,‡} Daejeon, Pohang, and Seoul, Korea

GRAPHICAL ABSTRACT



Background: Intraepithelial lymphocytes (IELs) in the intestines play pivotal roles in maintaining the integrity of the mucosa, regulating immune cells, and protecting against pathogenic invasion. Although several extrinsic factors, such as TGF- β , have been identified to contribute to IEL generation, intrinsic regulatory factors have not been determined fully. **Objective:** Here we investigated the regulation of IEL differentiation and the underlying mechanisms in mice. **Methods:** We analyzed IELs and the expression of molecules associated with IEL differentiation in wild-type control and

microRNA (miRNA)-150 knockout mice. Methotrexate was administered to mice lacking miR-150 and control mice. **Results:** miR-150 deficiency reduced the IEL population in the small intestine and increased susceptibility to methotrexate-induced mucositis. Evaluation of expression of IEL differentiation-associated molecules showed that miR-150-deficient IELs exhibited decreased expression of TGF- β receptor (TGF- β R) II, CD103, CD8 $\alpha\alpha$, and Runt-related transcription factor 3 in all the IEL subpopulations. The reduced expression of TGF- β R II in miR-150-deficient IELs was

From ^athe Immunotherapy Convergence Research Center, ^bthe Infectious Disease Research Center, and ^dthe Stem Cell Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon; ^cthe Department of Functional Genomics, KRIBB School of Bioscience, Korea University of Science and Technology (UST), Daejeon; ^ethe Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology; and ^fthe Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul.

*These authors contributed equally to this work.

†These authors contributed equally to this work.

Supported by the KRIBB Research Initiative Program, a National Research Foundation of Korea (NRF) grant (NRF-2016R1A2B4014230), and a National Research Council

of Science & Technology (NST) grant (CRC-15-02-KRIBB) from the Korean government (MSIP).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication January 11, 2017; revised July 3, 2017; accepted for publication July 12, 2017.

Corresponding author: Inpyo Choi, PhD, or Tae-Don Kim, PhD, KRIBB, Immunotherapy Research Center, 125 Gwahakro Yuseong-gu, Daejeon, 34141 Korea.

E-mail: ipchoi@kribb.re.kr. Or: tdkim@kribb.re.kr.

0091-6749/\$36.00

© 2017 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2017.07.019>

caused by increased expression of c-Myb/miR-20a. Restoration of miR-150 or inhibition of miR-20a recovered the TGF- β RII expression.

Conclusion: miR-150 is an intrinsic regulator of IEL differentiation through TGF- β RII regulation. miR-150-mediated IEL generation is crucial for maintaining intestinal integrity against anticancer drug-induced mucositis. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: MicroRNA, intraepithelial lymphocytes, TGF- β , CD103, Runt-related transcription factor 3, intestinal epithelium, mucositis, methotrexate

The intestinal epithelium, which is responsible for nutrient uptake, is continuously exposed to foreign materials that can disrupt intestinal homeostasis. Epithelial cells interact with neighboring immune cells, such as intraepithelial lymphocytes (IELs), to maintain integrity, prevent pathogenic infections, and induce tolerance to dietary and commensal bacterial antigens. IELs comprise heterogeneous cell populations and are classified into 2 major subgroups based on their generation sites and characteristics: natural IELs and induced IELs.¹ Natural IELs are generated in the thymus; they are divided into T-cell receptor (TCR) $\gamma\delta$ -expressing IELs ($\gamma\delta$ IELs) and TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs (CD8 $\alpha\alpha$ IELs), protecting the intestinal epithelium by secreting regulatory cytokines or suppressing activated T cells.²⁻⁵ Induced IELs are generated in the mesenteric lymph nodes (mLNs) or Peyer patches. They comprise CD8 $\alpha\beta$ ⁺ TCR $\alpha\beta$ ⁺ IELs (CD8 $\alpha\beta$ IELs) and CD4⁺ TCR $\alpha\beta$ ⁺ IELs (CD4 IELs) and protect against viral and parasitic infections.⁶⁻⁹ Thus IELs are crucial for maintaining intestinal homeostasis in both steady-state and infected conditions.

TGF- β is a major factor influencing IELs from development to retention in the intestinal epithelium. In the thymus TGF- β inhibits apoptosis of double-negative (DN) IEL precursors by regulating antiapoptotic and proapoptotic molecules.¹⁰ TGF- β also induces expression of CD8 $\alpha\alpha$, a well-known IEL marker, in DN IEL precursors by suppressing T_H cell-inducing POZ-Kruppel factor expression. During differentiation in the periphery, TGF- β induces CD8 $\alpha\alpha$ expression by promoting Runt-related transcription factor 3 (Runx3) and suppressing POZ-Kruppel factor expression¹⁰⁻¹² and also induces CD103, another IEL marker.¹²⁻¹⁴ Therefore regulation of TGF- β receptor (TGF- β R) is crucial for IEL differentiation. However, the molecular mechanisms of and regulators involved in TGF- β R expression during IEL differentiation are largely unknown. Because IELs are involved in intestinal homeostasis, elucidating the regulatory molecular pathways underlying IEL differentiation would contribute to the development of therapeutic and preventive approaches against intestinal diseases.

MicroRNAs (miRs) are noncoding RNAs of approximately 22 nucleotides that regulate gene expression through translational repression or degradation of target mRNAs by directly binding to their 3' untranslated region (UTR).¹⁵ Because miRs can regulate diverse target molecules as long as the target contains the appropriate miR-binding sequence in its 3'-UTR, miRs fine-tune the development, biological function, and survival of diverse cells, including immune cells. In particular, miR-150 controls the development of B,^{16,17} natural killer (NK), and NK T cells by suppressing the expression of myeloblastosis viral oncogene homolog (Myb)¹⁸ and regulates T-cell development in the thymus

Abbreviations used

AANAT:	Arylalkylamine N-acetyltransferase
DC:	Dendritic cell
DN:	Double negative
IEL:	Intraepithelial lymphocyte
IL-15R:	IL-15 receptor
KRIBB:	Korea Research Institute of Bioscience and Biotechnology
LP:	Lamina propria
MED12:	Mediator complex subunit 12
miR:	MicroRNA
mLN:	Mesenteric lymph node
MTX:	Methotrexate
Myb:	Myeloblastosis viral oncogene homolog
NK:	Natural killer
RA:	Retinoic acid
Runx3:	Runt-related transcription factor 3
TCR:	T-cell receptor
TGF- β R:	TGF- β receptor
TLA:	Thymic leukemia antigen
UTR:	Untranslated region
WT:	Wild-type

by suppressing neurogenic locus notch homolog protein 3.¹⁹ miR-150 also affects regulatory T-cell differentiation and NK cell activity by repressing mammalian target of rapamycin and regulating perforin expression,^{20,21} respectively. Although miRs, including miR-150, exert diverse effects on various cells, the roles of miRs in IEL regulation are still not clear.

In this study we demonstrated that miR-150 is critical for small intestinal IEL differentiation. miR-150 increases TGF- β RII expression by suppressing the c-Myb/miR-20a pathway. Increased TGF- β RII expression in turn increases CD103 and CD8 $\alpha\alpha$ expression by inducing Runx3. These results reveal an unappreciated role of miR-150 in modulating IEL differentiation and provide insight into the maintenance of intestinal homeostasis.

METHODS

Mice

C57BL/6 (B6) and *mir-150*^{-/-} mice on a B6 background were purchased from the Jackson Laboratory (Bar Harbor, Me) and kept in a specific pathogen-free facility at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). All animals were treated in accordance with guidelines established by the Institutional Animal Use and Care Committee of KRIBB.

Bone marrow transfer model

Bone marrow cells (1×10^6) from B6 or *mir-150*^{-/-} mice were transferred intravenously into lethally irradiated (900 rad) recipient mice. At 16 weeks after bone marrow transfer, the mice were killed for additional analysis.

Experimental mucositis model

Mice were injected with methotrexate (MTX; Sigma-Aldrich, St Louis, Mo) twice intraperitoneally with a 1-day interval (first injection: 120 mg/kg, second injection: 60 mg/kg), and body weight was monitored for 2 weeks. In IEL transfer experiments IELs (3.5×10^5 cells per mouse) were transferred intravenously at 2 days before MTX injection.

In vitro IEL differentiation

Naive CD8⁺ T cells (2×10^5 cells per well) were cultured in 96-well plates in the presence of anti-CD3/CD28 beads (4 μ L per well; Invitrogen, Carlsbad,

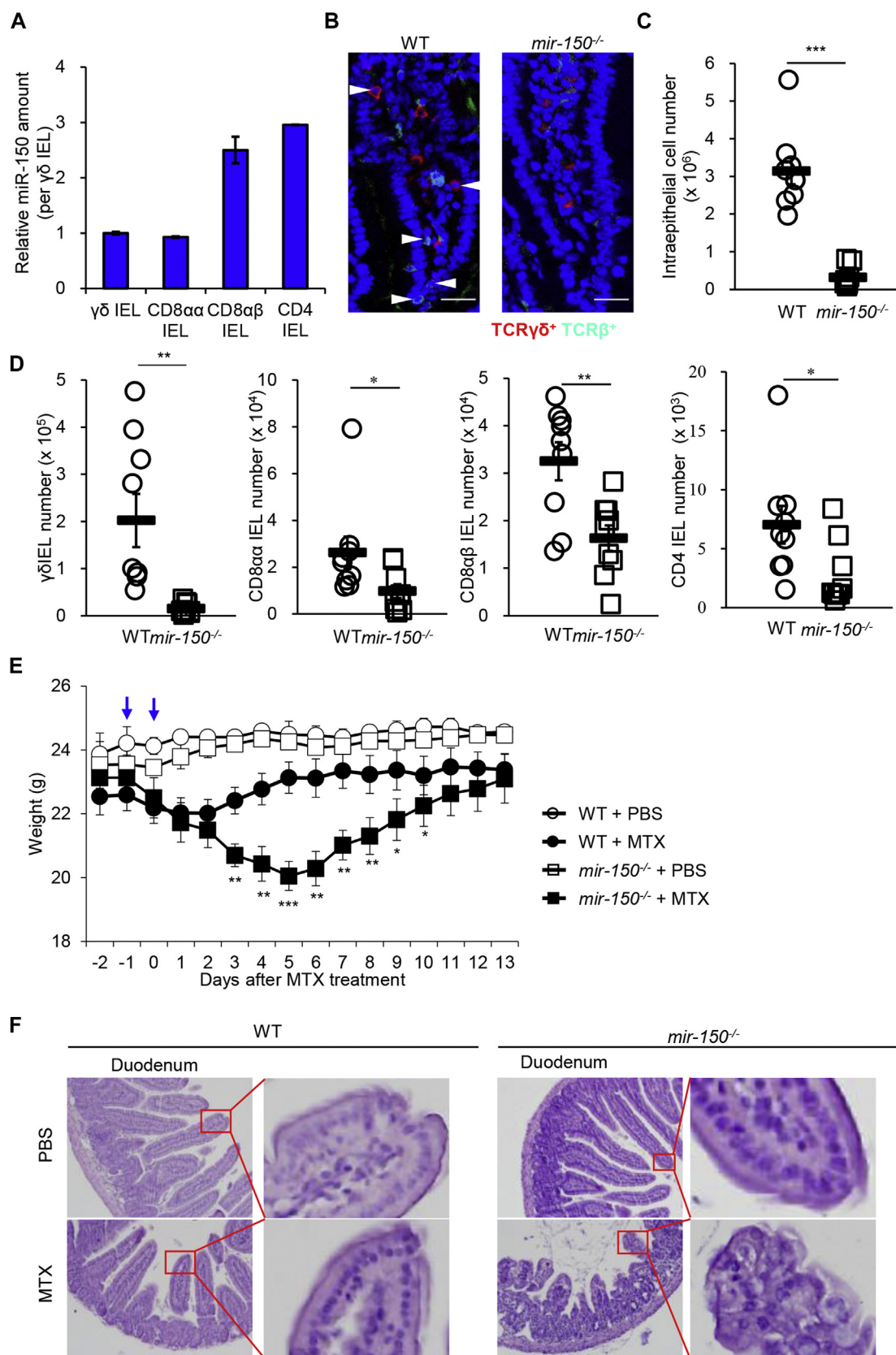


FIG 1. miR-150 deficiency reduced IEL subpopulations and increased intestinal damage by MTX treatment. **A**, Relative miR-150 expression in IELs ($n = 5$ mice). **B**, TCR β ⁺ and TCR $\gamma\delta$ ⁺ IELs in the small intestine. **C** and **D**, Absolute numbers of IELs (Fig 1, C) and IEL subpopulations (Fig 1, D; $n = 8$ mice per genotype). **E** and **F**, Changes in body weight change (Fig 1, E) and small intestine (Fig 1, F) after MTX treatment ($n = 7$ mice per group). Data (means $\pm$ SEMs) are representative of at least 2 independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$.

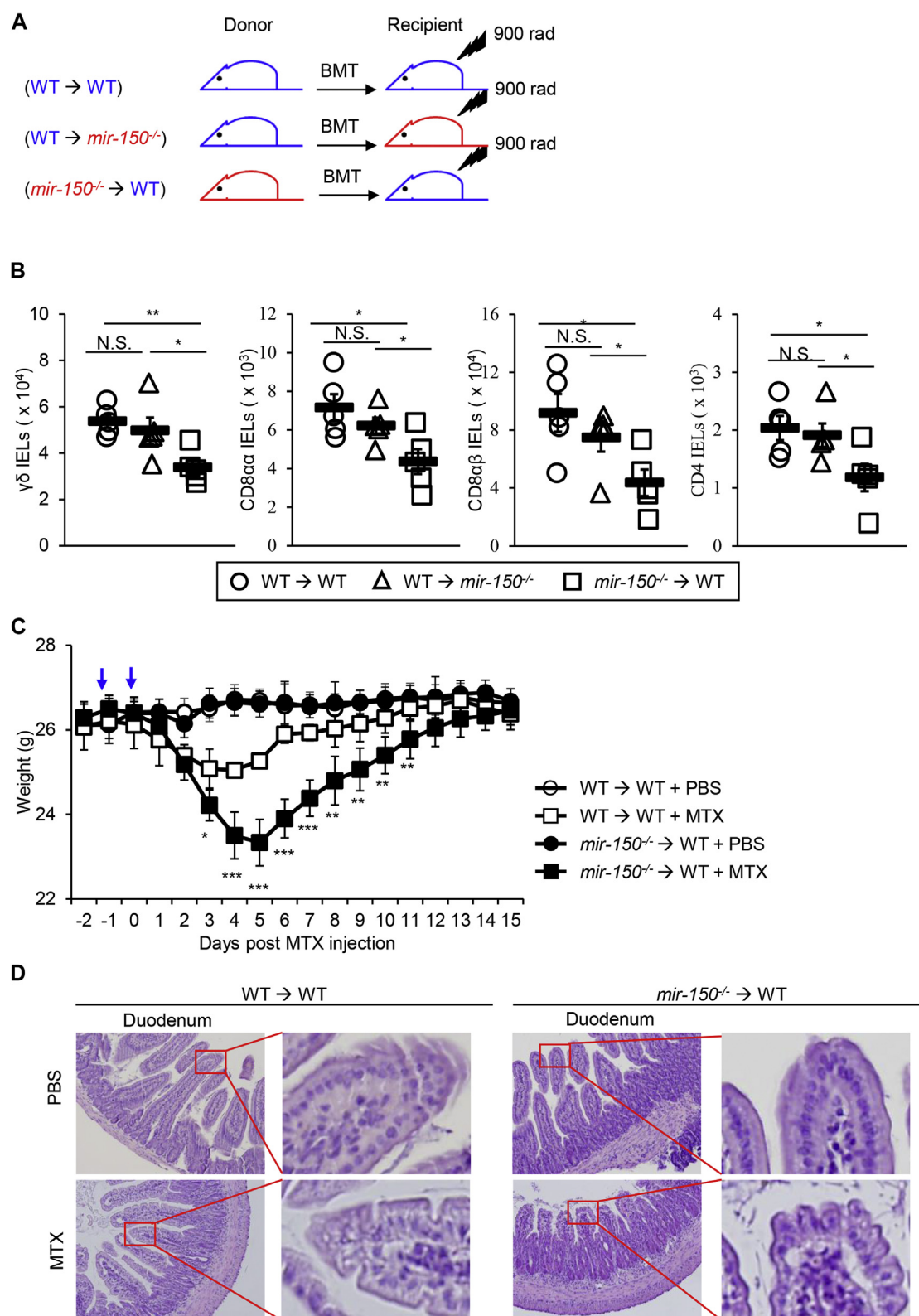


FIG 2. Small intestinal epithelial cells are not associated with reduction of the IEL population through lack of miR-150. **A**, Schematics for bone marrow transfer (BMT). **B**, Absolute cell numbers of IEL subpopulations in bone marrow-transferred mice. **C** and **D**, Body weight changes (Fig 2, C) and histologic analysis (Fig 2, D) in bone marrow-transferred mice after MTX treatment (n = 7 mice per group). Data (means ± SEMs) are representative of 2 independent experiments. **P* < .05, ***P* < .01, and ****P* < .001. N.S., Not significant.

Calif), TGF- β 1 (5 ng/mL; R&D Systems, Minneapolis, Minn), and all-trans retinoic acid (RA; 1 nmol/L; Sigma-Aldrich) for 3 days. Transfection of miR-150 mimic or miR-20a inhibitor was performed with an Amaxa Nucleofector (Lonza, Basel, Switzerland), according to the manufacturer's specifications.

Statistics

Statistical differences among groups were assessed by using an unpaired 2-tailed Student *t* test with Prism software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant.

A detailed description of the methods is presented in Table E1 and the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

miR-150 deficiency limits IELs in the small intestine

To assess the role of miR-150 in IELs, we investigated miR-150 expression in IELs (Fig 1, A) and the IEL population in the small intestine of wild-type (WT) and miR-150-deficient (*mir-150*^{-/-}) mice. *mir-150*^{-/-} mice had significantly fewer intestinal TCR $\alpha\beta$ ⁺ and TCR $\gamma\delta$ ⁺ IELs than WT mice (Fig 1, B and C). Each IEL subpopulation ($\gamma\delta$, CD8 $\alpha\alpha$, CD8 $\alpha\beta$, and CD4 IELs) was significantly reduced in *mir-150*^{-/-} mice compared with WT mice, indicating the importance of miR-150 in IEL regulation (Fig 1, D, and see Fig E1 in this article's Online Repository at www.jacionline.org).

IELs are known to protect the intestinal barrier and repair damaged epithelial tissues by producing cytokines.^{5,22-25} Given that IEL populations in the small intestine were significantly reduced by miR-150 deficiency, we hypothesized that *mir-150*^{-/-} mice would be more susceptible to intestinal epithelial damage than WT mice. To verify this, MTX was administered to mice to damage the intestinal epithelium.²⁶ MTX-administered *mir-150*^{-/-} mice lost more body weight and required more recovery time than WT mice (Fig 1, E). Histologic analysis also revealed that *mir-150*^{-/-} mice exhibited increased villus degeneration with epithelial lining destruction, mucosal ulceration, and infiltration of inflammatory cells in all parts of the small intestine (Fig 1, F; and see Fig E2, A, in this article's Online Repository at www.jacionline.org). Intriguingly, IEL transfer into *mir-150*^{-/-} mice significantly ameliorated MTX-induced mucositis, suggesting that *mir-150*^{-/-} mice showed increased susceptibility to MTX-induced physiologic and pathologic changes because of decreased intestinal IELs (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus miR-150 protects and/or repairs the damaged epithelial barrier by influencing the IEL populations.

Reduced IEL populations in *mir-150*^{-/-} mice are not associated with miR-150 deficiency in intestinal epithelial cells

Given that miR-150 influences various cell types, IEL populations in *mir-150*^{-/-} mice might be reduced by changes in IEL-interacting intestinal epithelial cells. To verify this, we transferred bone marrow cells from WT mice into *mir-150*^{-/-} mice and *vice versa* (WT $\rightarrow$ *mir-150*^{-/-} and *mir-150*^{-/-} $\rightarrow$ WT) and analyzed IEL populations (Fig 2, A). All IEL subpopulations ($\gamma\delta$, CD8 $\alpha\alpha$, CD8 $\alpha\beta$, and CD4 IELs) were significantly reduced in *mir-150*^{-/-} $\rightarrow$ WT mice compared with WT $\rightarrow$ WT control mice, whereas the number of IELs in WT $\rightarrow$ *mir-150*^{-/-} and

control mice was comparable (Fig 2, B). When bone marrow-transferred mice were treated with MTX, *mir-150*^{-/-} $\rightarrow$ WT mice lost more body weight (Fig 2, C) and had more damaged intestinal epithelium than WT $\rightarrow$ WT control mice (Fig 2, D; and see Fig E2, B). These data indicate that reduced IEL populations and increased vulnerability to MTX-induced mucositis in *mir-150*^{-/-} mice are independent of miR-150 deficiency in intestinal epithelial cells.

miR-150 does not affect the number or function of IEL-generating CD103⁺ dendritic cells

Gut-residential CD103⁺ dendritic cells (DCs) induce IEL differentiation by producing RA.²⁷⁻²⁹ Considering the pivotal role of CD103⁺ DCs in IEL differentiation, miR-150 deficiency might affect IEL populations by altering CD103⁺ DCs. To verify this, we assessed the CD103⁺ DC population and activity in the small intestine. The number of CD103⁺ DCs in Peyer patches, mLNs, and lamina propria (LP; Fig 3, A) and the RA-generating activities of CD103⁺ DCs from the mLNs and Peyer patches (Fig 3, B) were similar in WT and *mir-150*^{-/-} mice. Transpresentation of IL-15 by IL-15 receptor (IL-15R) α on intestinal epithelial cells is known to be crucial for IEL differentiation.³⁰ If miR-150 deficiency alters IL-15R α expression in CD103⁺ DCs, it could potentially influence IELs. However, IL-15R α expression on CD103⁺ DCs was not affected by miR-150 deficiency (Fig 3, C). These data suggest that the reduced IEL populations in *mir-150*^{-/-} mice are not associated with changes in the number or function of CD103⁺ DCs.

Generation of IEL precursors is miR-150 independent

We next investigated the number of IEL precursors and thymic CD4/CD8⁺ T-cell progenitors that further differentiate into IELs in the periphery. The total number of thymocytes and subpopulations (DN, CD8, and CD4 single positive) was similar in WT and *mir-150*^{-/-} mice and was comparable in bone marrow-transferred mice (Fig 4, A and B, and see Fig E4, A and B, in this article's Online Repository at www.jacionline.org). The TCR $\gamma\delta$ ⁺ IEL (DN TCR $\gamma\delta$ ⁺)³¹ and CD8 $\alpha\alpha$ IEL (DN TCR β ⁺NK1.1⁻)³² precursors were also unaffected by miR-150 deficiency (Fig 4, C and D, and see Fig E4, C and D). Because induced IELs (CD4 and CD8 $\alpha\beta$) originate from naive CD4⁺ and CD8⁺ T cells,¹⁰ numbers of these T cells in the spleens and mLNs of WT and *mir-150*^{-/-} mice were assessed. Total numbers of splenocytes, MLN cells, and naive CD4⁺ and CD8⁺ T cells were similar in WT and *mir-150*^{-/-} mice (Fig 4, E-H). The number of induced IEL precursors was also similar in bone marrow-transferred mice (*mir-150*^{-/-} $\rightarrow$ WT and WT $\rightarrow$ *mir-150*^{-/-} mice; see Fig E4, E-H). These data indicate that miR-150 deficiency does not affect IEL precursor generation from the developmental stage in the thymus to the mature stage in the secondary lymphoid organs.

TGF- β RII expression is reduced in miR-150-deficient IELs

During the final stage of IEL differentiation, TGF- β plays an important role in expression of the characteristic IEL molecules CD103 and CD8 $\alpha\alpha$ by inducing Runx3 expression.^{10,12} Thus we

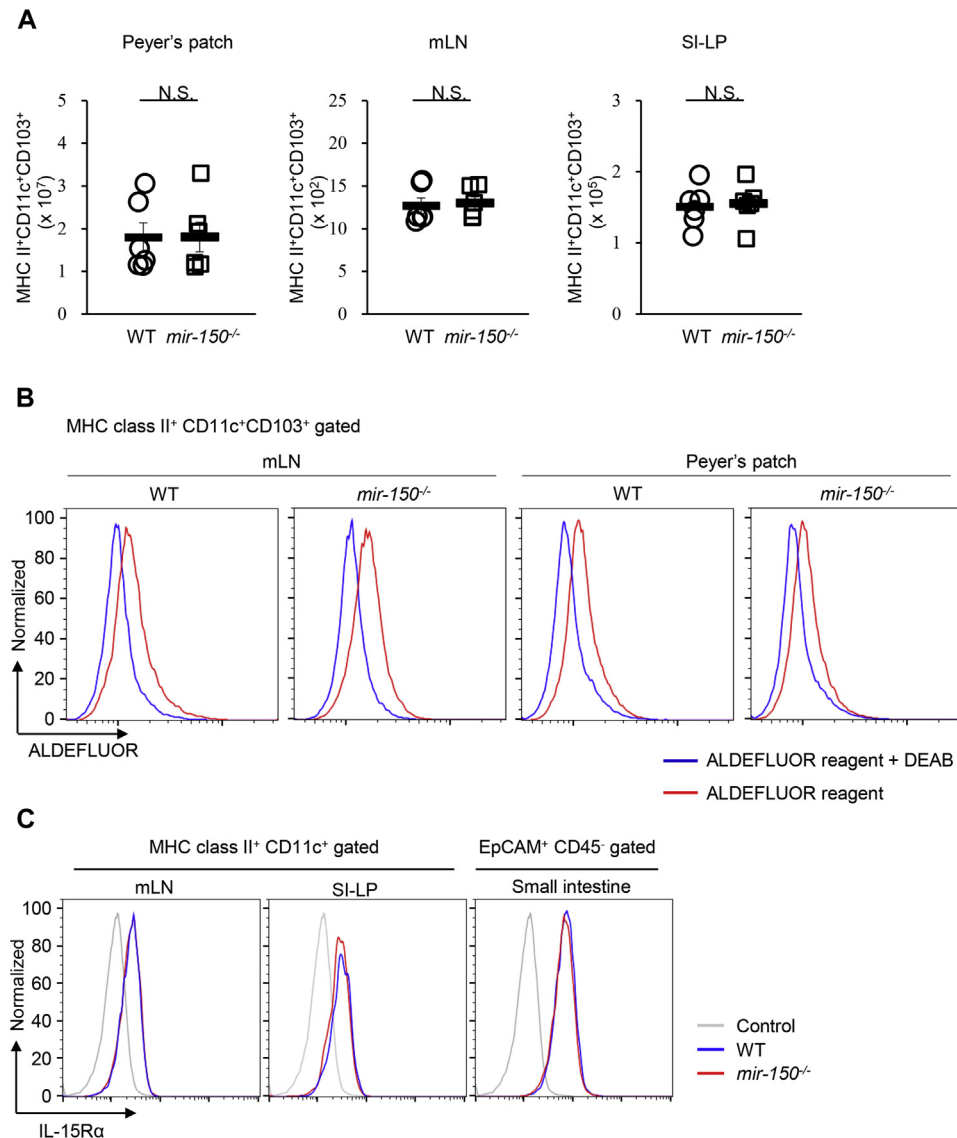


FIG 3. miR-150 does not affect the population and function of IEL-generating CD103⁺ DCs. **A**, Absolute DC numbers in Peyer patches, mLNs, and small intestinal lamina propria (SI-LP) from WT and *mir-150*^{-/-} mice. (n = 5 mice per genotype). N.S., Not significant. **B**, RA-producing activity in DCs from WT and *mir-150*^{-/-} mice (n = 5 mice per group). **C**, Expression levels of IL-15Rα on DCs in mLNs and SI-LP from WT and *mir-150*^{-/-} mice (n = 5 mice per group). Data are representative from at least 2 independent experiments.

compared expression levels of TGF-βRs on IELs of WT and *mir-150*^{-/-} mice. Expression of TGF-βRI on IEL subpopulations was not affected by miR-150 deficiency (see Fig E5, A, in this article's Online Repository at www.jacionline.org). However, TGF-βRII levels were significantly reduced in the IELs of *mir-150*^{-/-} mice but not in IEL precursors in the thymus and mLNs of *mir-150*^{-/-} mice (Fig 5, A and B, and see Fig E5, B and C). In addition, expression levels of Runx3 (mRNA) and CD103 (mRNA and protein) were significantly reduced in *mir-150*^{-/-} IELs (Fig 5, C-E). Expression of CD8αα, another Runx3-induced IEL marker, was also significantly reduced in *mir-150*^{-/-} IELs (see Fig E6 in this article's Online Repository at www.jacionline.org).

Because signals through IL-7, IL-15, aryl hydrocarbon, and RA receptors are also involved in IEL differentiation and survival,^{27,30,33-35} we investigated the influence of miR-150

deficiency on the expression of these receptors. IELs from WT and *mir-150*^{-/-} mice expressed comparable levels of IL-7 receptor (CD127) and IL-15Rs (CD122 and CD132; see Fig E5, D-F). Expression levels of the aryl hydrocarbon and RA receptors were also similar in IELs from WT and *mir-150*^{-/-} mice (see Fig E5, G, and Fig E7, A-C, in this article's Online Repository at www.jacionline.org). These data indicate that reduced IEL populations in *mir-150*^{-/-} mice are associated with decreased TGF-βRII expression on IELs.

miR-150 deficiency downregulates TGF-βRII expression by suppressing the c-Myb/miR-20a pathway

After the discovery that TGF-βRII expression is reduced in *mir-150*^{-/-} IELs, the mechanism underlying the regulation of

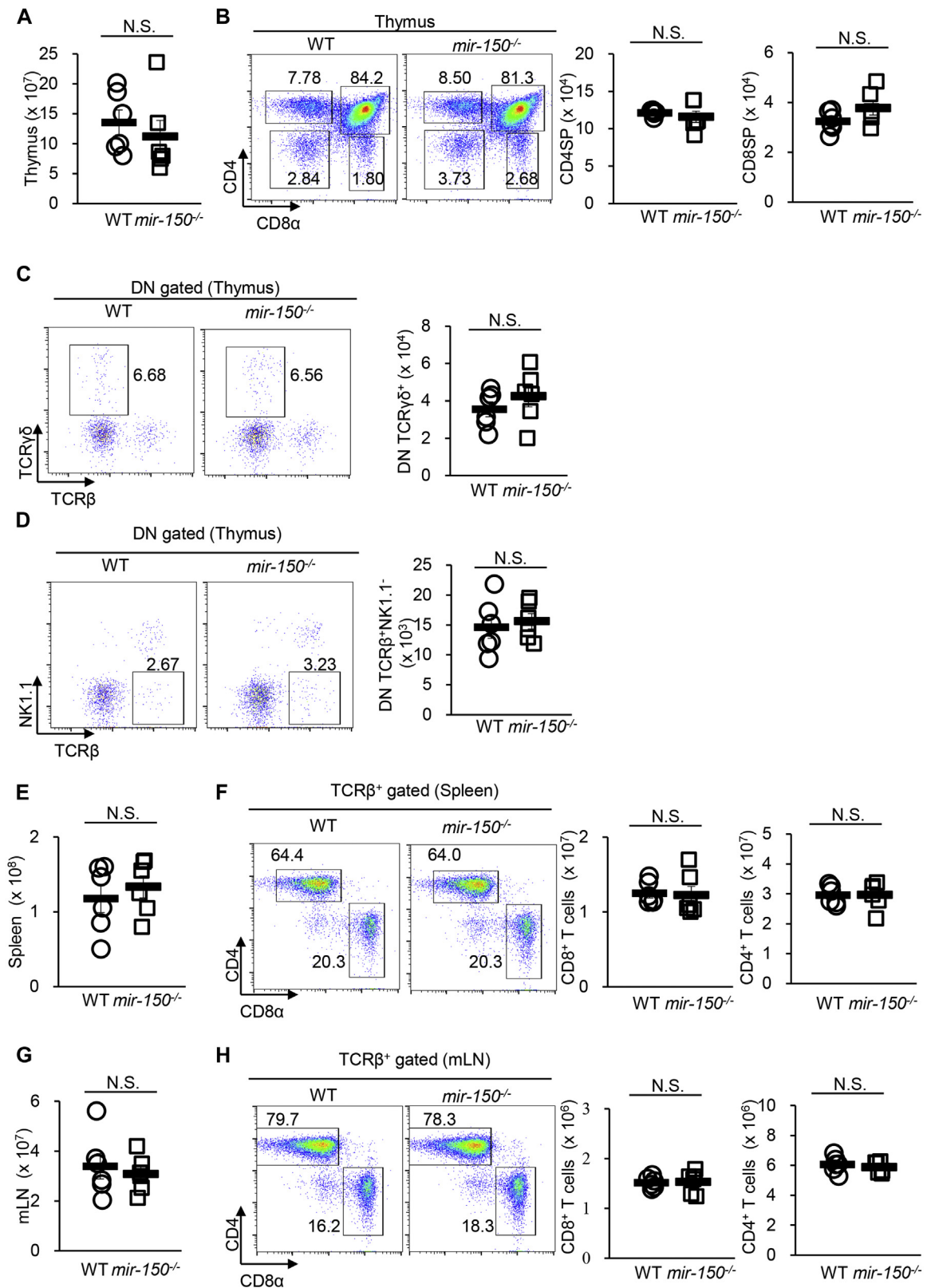


FIG 4. Generation of IEL precursors is not associated with miR-150. **A-D**, Absolute numbers of IEL precursors in the thymus ($n = 6$ mice per genotype). **E** and **F**, Absolute numbers of splenocytes and CD4⁺ and CD8⁺ T cells in WT and *mir-150*^{-/-} mice ($n = 6$ mice per genotype). **G** and **H**, Calculated numbers of total lymphocytes and CD4⁺ and CD8⁺ T cells in mLN from WT and *mir-150*^{-/-} mice ($n = 6$ mice per genotype). Data indicate means $\pm$ SEMs and are representative of 2 independent experiments. N.S., Not significant.

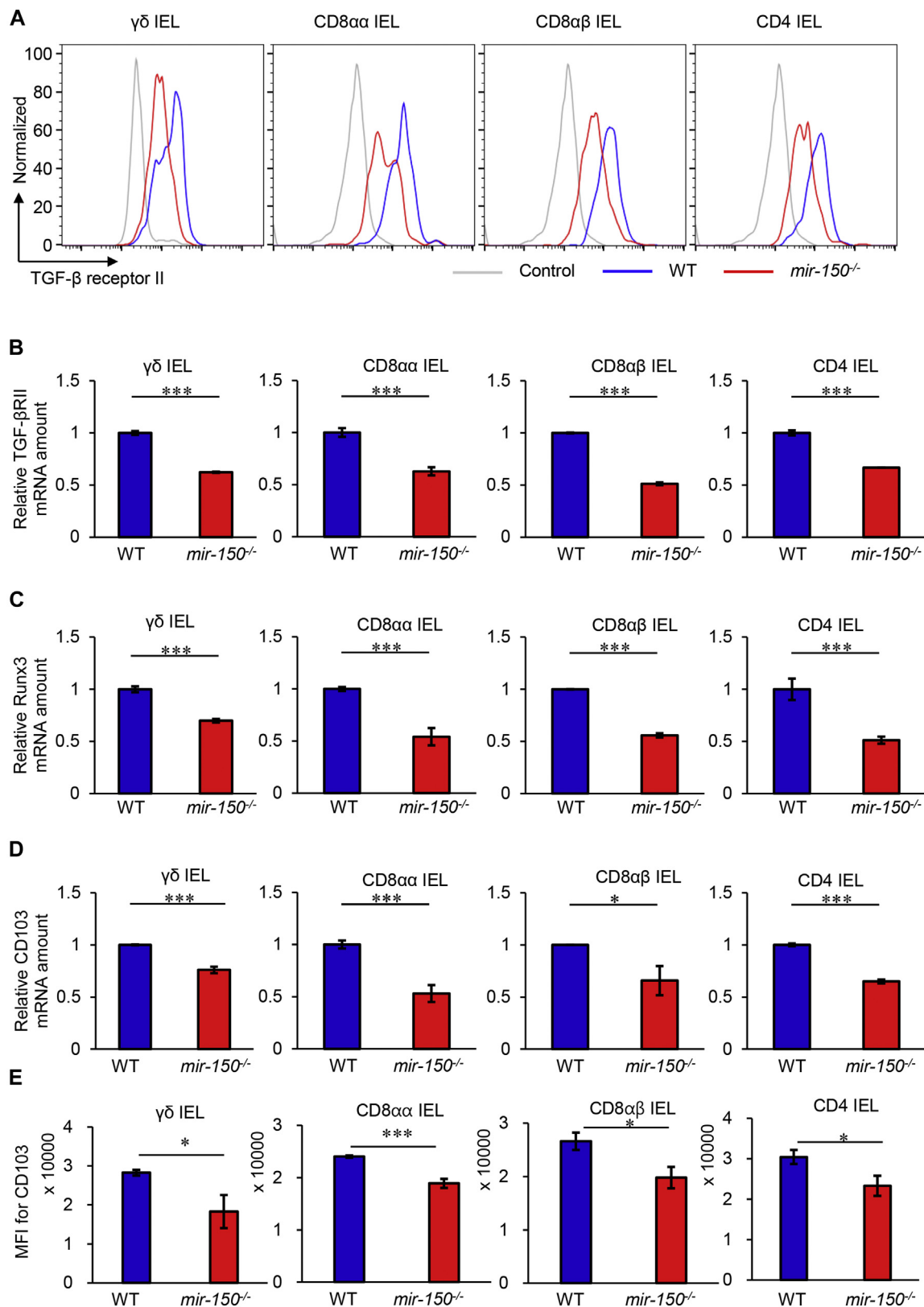


FIG 5. miR-150 deficiency reduces TGF- β RII expression in IELs. **A**, Expression levels of TGF- β RII in IEL subpopulations from WT and *mir-150*^{-/-} mice (n = 5 mice per genotype). **B-E**, mRNA levels of TGF- β RII, Runx3, and CD103 (fluorescence-activated cell sorting analysis; Fig 4, E) in IEL subpopulations (n = 5 mice per genotype). Data are representative (Fig 4, A) and presented as means $\pm$ SEMs. **P* < .05 and ****P* < .001. MFI, Mean fluorescence intensity.

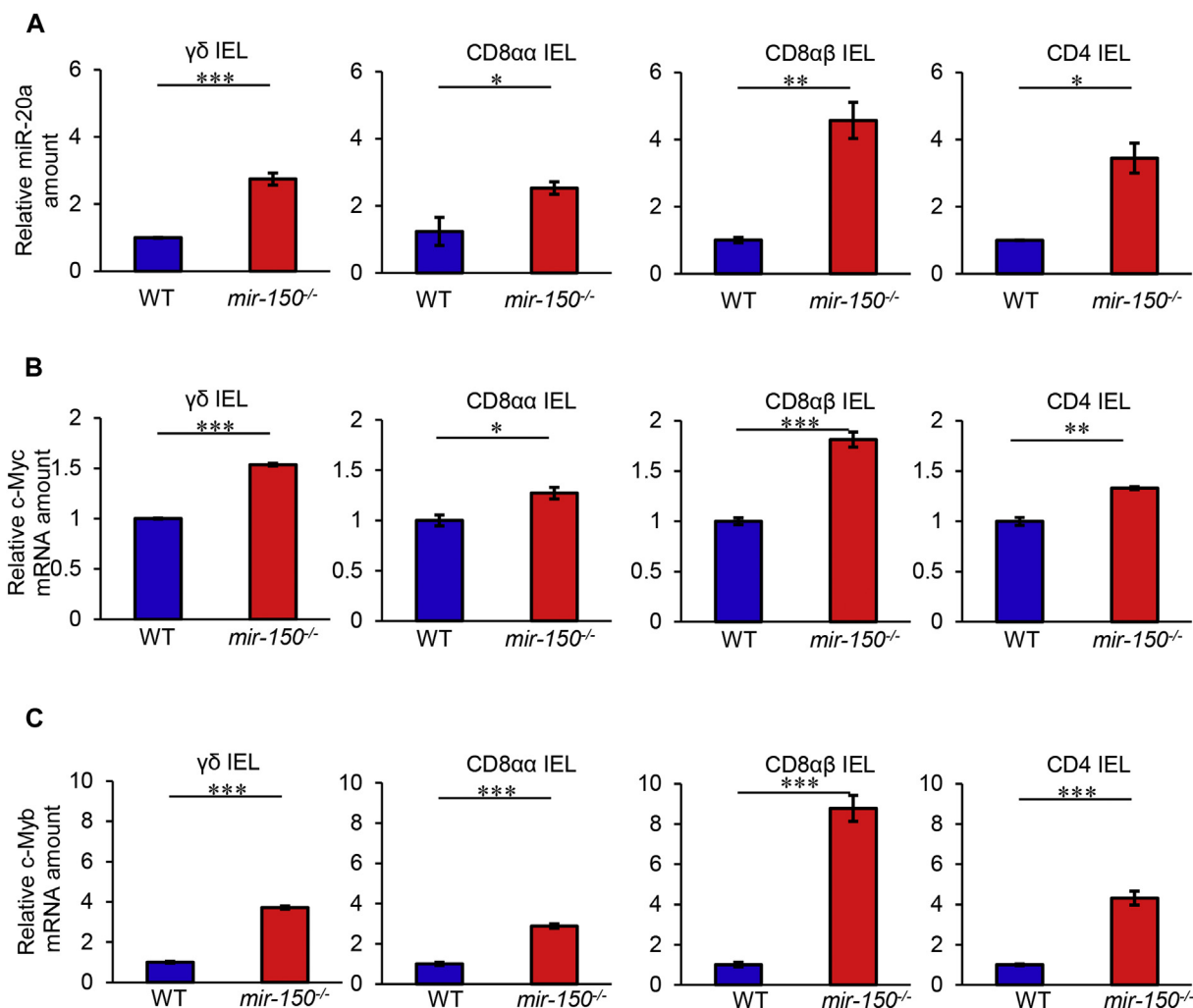


FIG 6. miR-150 deficiency increases expression of Myb, Myc, and miR-20a in IELs. Expression levels of miR-20a (A), c-Myc (B), and c-Myb (C) in IEL subpopulations are shown (n = 4 mice per genotype). Data are representative of 2 independent experiments and indicate means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$.

TGF- β RII expression by miR-150 was elucidated. Based on previous studies that showed suppression of TGF- β RII by mediator complex subunit 12 (MED12) and miR-20a,^{36,37} we investigated whether miR-150 could modulate *MED12* mRNA and miR-20a expression in IELs. Although *MED12* mRNA expression was not affected by miR-150 deficiency (see Fig E7, D), miR-20a expression was significantly higher in *mir-150*^{-/-} IELs than in WT IELs (Fig 6, A). A previous report showed that miR-20a expression is induced by c-Myc.³⁷ Consistently, c-Myc expression was increased in *mir-150*^{-/-} IELs (Fig 6, B). According to miR target prediction algorithms, c-Myc has no putative binding sequences for miR-150 in its 3'-UTR (data not shown). Instead, c-Myc expression can be induced by c-Myb, a well-known target molecule for miR-150.^{16,38,39} Thus we investigated whether miR-20a and c-Myc expression are increased by increased c-Myb expression in *mir-150*^{-/-} IELs. c-Myb expression was significantly greater in *mir-150*^{-/-} IELs than in WT IELs (Fig 6, C), indicating that increased

miR-20a and c-Myc expression was caused by enhanced c-Myb expression in *mir-150*^{-/-} IELs. Inhibition of c-Myb and TGF- β RII expression by miR-150 and miR-20a, respectively, was confirmed by using an arylalkylamine N-acetyltransferase (AANAT) reporter assay, as previously described.²¹ Cotransfection of an miR-150 mimic reduced AANAT expression from the *AANAT-c-Myb* 3'-UTR construct in a dose-dependent manner, whereas cotransfection of an miR-150 mutant lacking the binding sequence for c-Myb 3'-UTR did not (see Fig E8, A, in this article's Online Repository at www.jacionline.org). Cotransfection of an miR-20a mimic also decreased AANAT expression from the *AANAT-Tgfbrii* 3'-UTR construct (see Fig E8, B), indicating that miR-150 and miR-20a specifically inhibit c-Myb and TGF- β RII expression, respectively, as reported previously.^{16,37} These data show that reduced TGF- β RII expression in *mir-150*^{-/-} IELs is caused by an increase in negative regulatory molecules, c-Myb, c-Myc, and miR-20a.

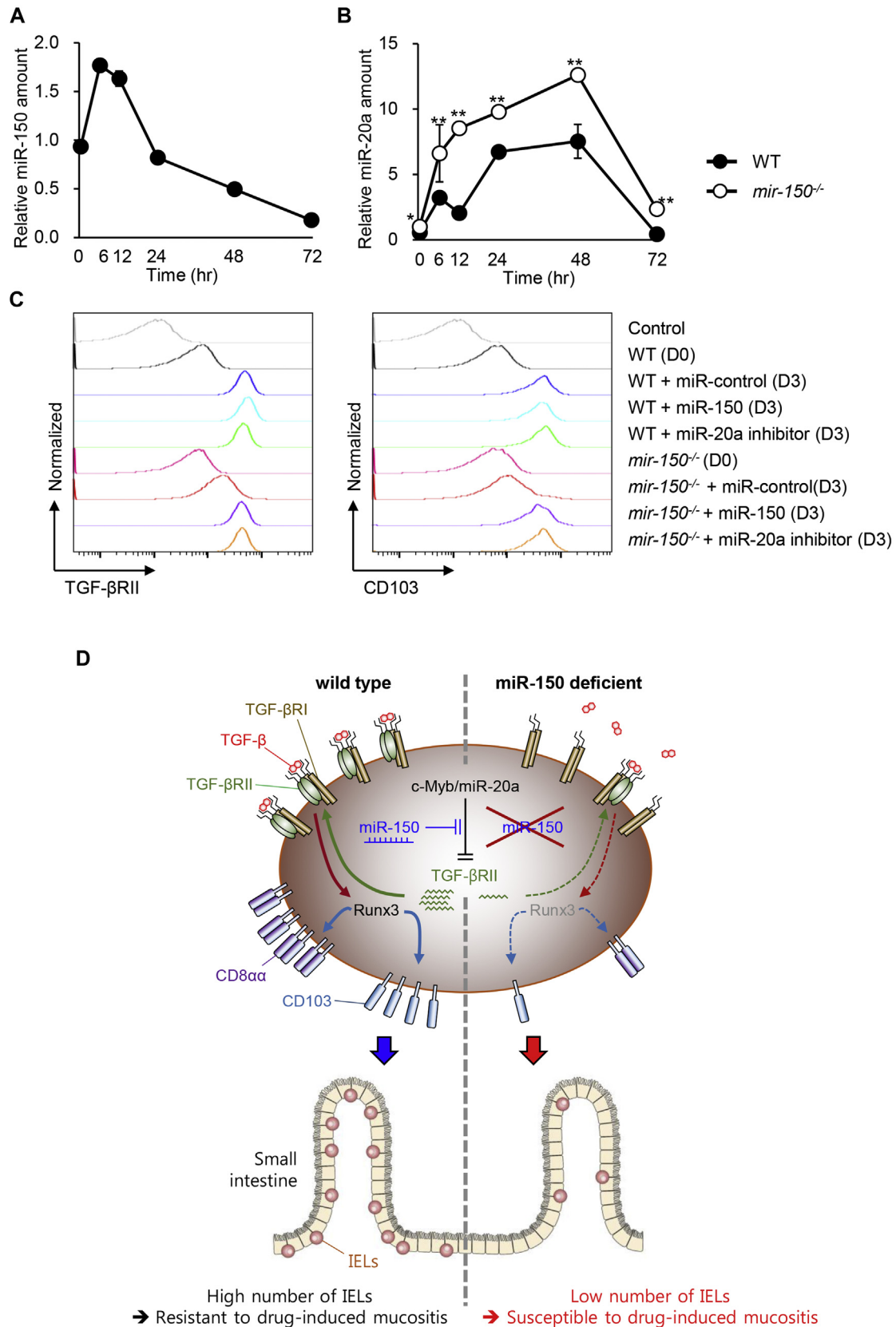


FIG 7. miR-150 add-back or miR-20a inhibition restores TGF-βRII expression during IEL generation. **A** and **B**, Expression kinetics of miR-150 (Fig 7, A) and miR-20a (Fig 7, B) in WT CD8⁺ T cells under IEL-generating culture conditions. **C**, Expression levels of TGF-βRII and CD103 in miR-150 mimic, miR-20a inhibitor, or miR-control transferred WT or *mir-150*^{-/-} CD8⁺ T cells at 3 days after culture under the IEL-generating conditions. **D**, Schematic cartoon for the role of miR-150 in TGF-βRII expression during IEL generation. Data (means ± SEMs) are representative of 2 independent experiments. **P* < .05 and ***P* < .01.

miR-150 restoration or miR-20a inhibition recovers TGF- β RII and CD103 expression levels in *mir-150*^{-/-} IELs

Given that miR-150 controls TGF- β RII expression by regulating miR-20a, we investigated the expression kinetics of miR-150 and miR-20a during IEL differentiation. miR-150 expression first increased for 6 hours and then decreased (Fig 7, A), whereas the change in miR-20a expression was relatively delayed (Fig 7, B). In addition, miR-20a expression was significantly increased by miR-150 deficiency during IEL differentiation.

Next, we investigated whether miR-150 restoration or miR-20a inhibition recovered the TGF- β RII and CD103 expression in *mir-150*^{-/-} IELs. Because naive CD8⁺ T cells underwent differentiation into IELs *in vitro*, WT cells expressed TGF- β RII and CD103 (Fig 7, C, WT + miR-control). However, *mir-150*^{-/-} cells showed significantly decreased expression of these molecules (Fig 7, C, *mir-150*^{-/-} + miR-control), which is consistent with our *in vivo* observations of *mir-150*^{-/-} IELs (Fig 5). Notably, forced expression of miR-150 or inhibition of miR-20a (see Fig E9 in this article's Online Repository at www.jacionline.org) significantly increased TGF- β RII and CD103 expression in *mir-150*^{-/-} cells (Fig 7, C, *mir-150*^{-/-} + miR-150 and *mir-150*^{-/-} + miR-20a inhibitor, respectively). Collectively, these data indicate that miR-150-induced miR-20a suppression is critical for the acquisition of IEL phenotypes through promotion of TGF- β RII expression (Fig 7, D) and that miR-150 regulates IEL differentiation by providing a molecular environment favorable for TGF- β RII expression.

DISCUSSION

TGF- β signaling is known to be critical for IEL differentiation.^{10,13} However, the regulatory mechanisms behind TGF- β R expression in IELs are still unknown. In this study we showed that miR-150 is a key regulator of TGF- β RII expression during IEL differentiation. miR-150 regulates TGF- β RII by suppressing a negative regulatory pathway (Myb/Myc/miR-20a), thus inducing expression of TGF- β RII and molecules of IEL phenotypes, such as CD103 and CD8 α , during IEL differentiation.

During IEL differentiation, TGF- β -induced Runx3 upregulates expression of CD103 and CD8 α , which play important roles in IELs. CD103 is known to be important for gut tropism. Although CD103 has not been shown to be directly required for IEL migration into intraepithelial sites, it has been shown to be essential for IELs to reside in intraepithelial sites by binding to E-cadherin on intestinal epithelial cells.^{1,40} CD8 α enables IELs to reside in intraepithelial sites by binding to the thymic leukemia antigen (TLA) on intestinal epithelial cells.¹ Consequently, decreased CD103 and CD8 α expression in *mir-150*^{-/-} IELs might negatively influence the localization of IELs after differentiation, resulting in reduced IEL populations at the intraepithelial site. In addition to gut residency, CD8 α also contributes to the survival of IELs in epithelial sites. Interaction between CD8 α and TLA is known to increase the survival of activated T cells by attenuating TCR signaling.^{1,41} Given that IELs are a partially activated population exposed to an intestinal environment with continuous high-pressure antigenic stimulation, interaction between CD8 α and TLA might support IEL survival.⁴² Collectively, reduced expression

of CD103 and CD8 α could result in IEL deficiency in *mir-150*^{-/-} mice.

We found that miR-150 provides a favorable molecular milieu for TGF- β RII expression during IEL differentiation. In *mir-150*^{-/-} mice, TGF- β RII, Runx3, and CD103 expression levels were significantly decreased in all IEL populations. In addition, *mir-150*^{-/-} IELs showed reduced TGF- β RII and CD103 levels during *in vitro* IEL differentiation. These reduced expression levels were restored by forced expression of miR-150, indicating that miR-150 is an inducer of TGF- β RII. Considering the significant increase in miR-20a expression in *mir-150*^{-/-} IELs both *in vivo* and *in vitro* (Figs 6, A, and 7, B), miR-150 might induce TGF- β RII through miR-20a expression. The increased miR-20a expression under *miR-150*-deficient conditions can be explained by the increased expression of c-Myc, a transcription factor for miR-20a, and c-Myb, a c-Myc-inducing protein.^{16,37-39} Moreover, miR-150 is known to act as a direct suppressor of c-Myb.¹⁶ miR-150-mediated inhibition of c-Myb and c-Myc caused the reduced miR-20a expression. Hence miR-150 promotes TGF- β RII expression by suppressing a negative regulatory pathway (Myb/Myc/miR-20a) during IEL differentiation.

miR-150 expression is controlled by Wnt/ β -catenin signaling and glucocorticoid treatment in colorectal cancer cells and multiple myeloma cells, respectively.^{43,44} However, the signal that induces miR-150 expression in T cells, including IELs, has not been identified. Studies showed that naive T cells in the spleen and lymph nodes had relatively high miR-150 levels, which were decreased by TCR stimulation with a costimulatory signal.⁴⁵ Consistent with these previous reports, miR-150 levels transiently increased and then gradually decreased during IEL differentiation, suggesting that TCR ligation/activation was not required for induction of the miR-150-TGF- β RII-CD103 axis in IELs. Rather, TCR ligation appeared to be required for CD8 α expression during IEL differentiation. TCR signaling in the presence of TGF- β -induced Runx3 epigenetically modulates the CD8 α promoter region and facilitates CD8 α expression.^{11,46} This TCR ligation is probably provided by the CD103⁺ DCs that process intestinal bacteria and food antigens in the intestinal lumen.⁴⁷ Further studies are required to understand the underlying mechanisms controlling miR-150 expression in IELs and the role of TCR ligation in IEL differentiation.

In *mir-150*^{-/-} mice all IEL subpopulations, including TCR $\gamma\delta$ ⁺ IELs, were reduced in the small intestine. Given that *Tgfb*^{-/-} and *Tgfb*^{+/+} mice had a similar number of TCR $\gamma\delta$ ⁺ IELs and that TGF- β signaling is not essential for TCR $\gamma\delta$ ⁺ IEL generation,^{10,12,40} a reduced population of TCR $\gamma\delta$ ⁺ IELs in *mir-150*^{-/-} mice cannot be completely attributed to reduced TGF- β RII expression and subsequently decreased CD103 levels. TCR $\gamma\delta$ ⁺ IEL migration was reported to be differentially regulated by occludin, another migration-associated molecule.⁴⁸ Apart from TGF- β RII and CD103, other migration-associated and/or retention-related molecules, such as occludin, might be involved in the reduction of TCR $\gamma\delta$ ⁺ IEL numbers in the setting of miR-150 deficiency. This possibility needs to be investigated further.

Gut-residential CD103⁺ DCs play a critical role in the development of IELs by generating RA, but there were no significant differences in numbers and functions of CD103⁺ DCs between WT and *mir-150*^{-/-} mice (Fig 3), indicating that miR-150 did not affect CD103⁺ DCs. In addition, no potential

targets of miR-150 were found among the genes required for CD8 α ⁺ or CD103⁺ DC development, such as inhibitor of DNA binding 2, interferon regulatory factor 8, and basic leucine zipper ATF-like 3 transcription factor (data not shown).⁴⁹⁻⁵¹ Although the level of miR-150 in CD103⁺ DCs was not determined, it is plausible that miR-150 does not affect the development of CD103⁺ DCs.

Although we showed that *mir-150*^{-/-} mice were more susceptible than WT mice, presumably because of reduced IEL populations (Fig 1, E and F), we did not determine the IEL subpopulation that majorly contributed to the resistance to MTX-induced enteropathy. Many studies have addressed the protective role of IELs in intestinal inflammation. MTX-induced mucositis was more severe in IL-10 knockout mice than in WT mice, indicating that the IL-10-producing IEL population might be involved in alleviating pathogenesis.²⁶ In a CD4⁺ T-cell transfer model of inflammatory bowel disease, transfer of CD8 α IELs before CD4⁺ T-cell transfer prevented colitis development in an IL-10-dependent manner, implying that CD8 α IELs could prevent MTX-induced mucositis during early enteropathy by suppressing production of inflammatory cytokines and protecting intestinal epithelial cells from apoptosis.²⁴ $\gamma\delta$ IELs also prevented MTX-induced intestinal damage in a keratinocyte growth factor-dependent manner, indicating that $\gamma\delta$ IELs also have an important role in intestinal tissue repair.²² Although CD4 IELs are a minor subset of IEL subpopulations, a previous study showed that they might have a protective function in bowel inflammation.⁵² In a CD4⁺ T-cell transfer model of colitis, TGF- β RII-deficient CD4⁺ T cells had colitis because of defective differentiation of CD4 IELs.

In addition to not specifying the IEL subpopulation that played a preservative role, our study has another limitation that the role of IELs were assessed only at a single dose of MTX. The exact function of each IEL subpopulation need to be further investigated at various doses of MTX.

Our study clarified the role of miRs in IEL regulation. We found that miR-150 regulates IEL differentiation by promoting TGF- β RII expression. Given that TGF- β signaling is associated with the fate of T cells, defining regulatory factors for TGF- β signaling, such as miR-150, provides a better understanding of the fate-determining mechanisms of T cells and a basis for therapeutic and preventive treatment of T cell-associated intestinal diseases.

We thank Dr Dong Oh Kim (KRIBB) for technical support in bone marrow transfer experiments and Dr Hilde Cheroutre (La Jolla Institute for Allergy & Immunology, San Diego, Calif) for kindly providing TL-monomer. We also thank DVMs/Drs Chul-Ho Lee and Yong-Hoon Kim (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) for pathologic analysis.

Key messages

- miR-150 is a critical regulator of IEL differentiation but not precursor generation.
- miR-150 promotes TGF- β RII expression by inhibiting the c-Myb/miR-20a pathway.
- Loss of miR-150 results in fewer IEL numbers and increases susceptibility to MTX-induced mucositis.

REFERENCES

- Cheroutre H, Lambolez F, Mucida D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol* 2011;11:445-56.
- Shires J, Theodoridis E, Hayday AC. Biological insights into TCR γ delta+ and TCR α beta+ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE). *Immunity* 2001;15:419-34.
- Bhagat G, Naiyer AJ, Shah JG, Harper J, Jabri B, Wang TC, et al. Small intestinal CD8+TCR γ delta+KNG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J Clin Invest* 2008;118:281-93.
- Boismenu R, Havran WL. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science* 1994;266:1253-5.
- Yang H, Antony PA, Wildhaber BE, Teitelbaum DH. Intestinal intraepithelial lymphocyte gamma delta-T cell-derived keratinocyte growth factor modulates epithelial growth in the mouse. *J Immunol* 2004;172:4151-8.
- Muller S, Buhler-Jungo M, Mueller C. Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. *J Immunol* 2000;164:1986-94.
- Lepage AC, Buzoni-Gatel D, Bout DT, Kasper LH. Gut-derived intraepithelial lymphocytes induce long term immunity against *Toxoplasma gondii*. *J Immunol* 1998;161:4902-8.
- Hershberg RM, Cho DH, Youkum A, Bradley MB, Lee JS, Framson PE, et al. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *J Clin Invest* 1998;102:792-803.
- Khanna R, Burrows SR, Thomson SA, Moss DJ, Cresswell P, Poulsen LM, et al. Class I processing-defective Burkitt's lymphoma cells are recognized efficiently by CD4+ EBV-specific CTLs. *J Immunol* 1997;158:3619-25.
- Konkel JE, Maruyama T, Carpenter AC, Xiong Y, Zamarron BF, Hall BE, et al. Control of the development of CD8 α beta+ intestinal intraepithelial lymphocytes by TGF-beta. *Nat Immunol* 2011;12:312-9.
- Huang Y, Park Y, Wang-Zhu Y, Larange A, Arens R, Bernardo I, et al. Mucosal memory CD8(+) T cells are selected in the periphery by an MHC class I molecule. *Nat Immunol* 2011;12:1086-95.
- Reis BS, Hoytema van Konijnenburg DP, Grivennikov SI, Mucida D. Transcription factor T-bet regulates intraepithelial lymphocyte functional maturation. *Immunity* 2014;41:244-56.
- Suzuki R, Nakao A, Kanamaru Y, Okumura K, Ogawa H, Ra C. Localization of intestinal intraepithelial T lymphocytes involves regulation of alphaEbeta7 expression by transforming growth factor-beta. *Int Immunol* 2002;14:339-45.
- Schon MP, Arya A, Murphy EA, Adams CM, Strauch UG, Agace WW, et al. Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol* 1999;162:6641-9.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215-33.
- Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* 2007;131:146-59.
- Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci U S A* 2007;104:7080-5.
- Beznan NA, Chakraborty T, Bender T, Lanier LL. miR-150 regulates the development of NK and iNKT cells. *J Exp Med* 2011;208:2717-31.
- Ghisi M, Corradin A, Basso K, Frasson C, Serafin V, Mukherjee S, et al. Modulation of microRNA expression in human T-cell development: targeting of NOTCH3 by miR-150. *Blood* 2011;117:7053-62.
- Warth SC, Hoefig KP, Hiekel A, Schallenberg S, Jovanovic K, Klein L, et al. Induced miR-99a expression represses mTOR cooperatively with miR-150 to promote regulatory T-cell differentiation. *EMBO J* 2015;34:1195-213.
- Kim N, Kim M, Yun S, Doh J, Greenberg PD, Kim TD, et al. MicroRNA-150 regulates the cytotoxicity of natural killers by targeting perforin-1. *J Allergy Clin Immunol* 2014;134:195-203.
- Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* 2002;99:14338-43.
- Meehan TF, Witherden DA, Kim CH, Sendaydiego K, Ye I, Garijo O, et al. Protection against colitis by CD100-dependent modulation of intraepithelial gamma delta T lymphocyte function. *Mucosal Immunol* 2014;7:134-42.
- Poussier P, Ning T, Banerjee D, Julius M. A unique subset of self-specific intraintestinal T cells maintains gut integrity. *J Exp Med* 2002;195:1491-7.
- Denning TL, Granger SW, Mucida D, Graddy R, Leclercq G, Zhang W, et al. Mouse TCR α beta+CD8 α beta+ intraepithelial lymphocytes express genes that down-regulate their antigen reactivity and suppress immune responses. *J Immunol* 2007;178:4230-9.

26. de Koning BA, van Dieren JM, Lindenbergh-Kortleve DJ, van der Sluis M, Matsumoto T, Yamaguchi K, et al. Contributions of mucosal immune cells to methotrexate-induced mucositis. *Int Immunol* 2006;18:941-9.
27. Eksteen B, Mora JR, Haughton EL, Henderson NC, Lee-Turner L, Villablanca EJ, et al. Gut homing receptors on CD8 T cells are retinoic acid dependent and not maintained by liver dendritic or stellate cells. *Gastroenterology* 2009;137:320-9.
28. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Roseblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 2003;424:88-93.
29. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 2004;21:527-38.
30. Ma LJ, Acero LF, Zal T, Schluns KS. Trans-presentation of IL-15 by intestinal epithelial cells drives development of CD8alphaalpha IELs. *J Immunol* 2009;183:1044-54.
31. De Geus B, Van den Enden M, Coolen C, Nagelkerken L, Van der Heijden P, Rozing J. Phenotype of intraepithelial lymphocytes in euthymic and athymic mice: implications for differentiation of cells bearing a CD3-associated gamma delta T cell receptor. *Eur J Immunol* 1990;20:291-8.
32. Gangadharan D, Lambolez F, Attinger A, Wang-Zhu Y, Sullivan BA, Cheroutre H. Identification of pre- and postselection TCRalpha beta+ intraepithelial lymphocyte precursors in the thymus. *Immunity* 2006;25:631-41.
33. Klose CS, Blatz K, d'Hargues Y, Hernandez PP, Kofoed-Nielsen M, Ripka JF, et al. The transcription factor T-bet is induced by IL-15 and thymic agonist selection and controls CD8alphaalpha(+) intraepithelial lymphocyte development. *Immunity* 2014;41:230-43.
34. Yang H, Spencer AU, Teitelbaum DH. Interleukin-7 administration alters intestinal intraepithelial lymphocyte phenotype and function in vivo. *Cytokine* 2005;31:419-28.
35. Li Y, Innocentin S, Withers DR, Roberts NA, Gallagher AR, Grigorieva EF, et al. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell* 2011;147:629-40.
36. Huang S, Holzel M, Knijnenburg T, Schlicker A, Roepman P, McDermott U, et al. MED12 controls the response to multiple cancer drugs through regulation of TGF-beta receptor signaling. *Cell* 2012;151:937-50.
37. Yang S, Cho YJ, Jin L, Yuan G, Datta A, Buckhaults P, et al. An epigenetic auto-feedback loop regulates TGF-beta type II receptor expression and function in NSCLC. *Oncotarget* 2015;6:33237-52.
38. Evans JL, Moore TL, Kuehl WM, Bender T, Ting JP. Functional analysis of c-Myb protein in T-lymphocytic cell lines shows that it trans-activates the c-myc promoter. *Mol Cell Biol* 1990;10:5747-52.
39. Lin YC, Kuo MW, Yu J, Kuo HH, Lin RJ, Lo WL, et al. c-Myb is an evolutionary conserved miR-150 target and miR-150/c-Myb interaction is important for embryonic development. *Mol Biol Evol* 2008;25:2189-98.
40. Zhang N, Bevan MJ. Transforming growth factor-beta signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. *Immunity* 2013;39:687-96.
41. Madakamutil LT, Christen U, Lena CJ, Wang-Zhu Y, Attinger A, Sundararajan M, et al. CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors. *Science* 2004;304:590-3.
42. Olivares-Villagomez D, Van Kaer L. TL and CD8alpha: enigmatic partners in mucosal immunity. *Immunol Lett* 2010;134:1-6.
43. Guo YH, Wang LQ, Li B, Xu H, Yang JH, Zheng LS, et al. Wnt/beta-catenin pathway transactivates microRNA-150 that promotes EMT of colorectal cancer cells by suppressing CREB signaling. *Oncotarget* 2016;7:42513-26.
44. Palagani A, Op de Beeck K, Naulaerts S, Diddens J, Sekhar Chirumamilla C, Van Camp G, et al. Ectopic microRNA-150-5p transcription sensitizes glucocorticoid therapy response in MM1S multiple myeloma cells but fails to overcome hormone therapy resistance in MM1R cells. *PLoS One* 2014;9:e113842.
45. Trifari S, Pipkin ME, Bandukwala HS, Aijo T, Bassein J, Chen R, et al. MicroRNA-directed program of cytotoxic CD8+ T-cell differentiation. *Proc Natl Acad Sci U S A* 2013;110:18608-13.
46. Hassan H, Sakaguchi S, Tenno M, Kopf A, Boucheron N, Carpenter AC, et al. Cd8 enhancer E81 and Runx factors regulate CD8alpha expression in activated CD8+ T cells. *Proc Natl Acad Sci U S A* 2011;108:18330-5.
47. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* 2008;8:435-46.
48. Edelblum KL, Shen L, Weber CR, Marchiando AM, Clay BS, Wang Y, et al. Dynamic migration of gammadelta intraepithelial lymphocytes requires occludin. *Proc Natl Acad Sci U S A* 2012;109:7097-102.
49. del Rio ML, Bernhardt G, Rodriguez-Barbosa JJ, Forster R. Development and functional specialization of CD103+ dendritic cells. *Immunol Rev* 2010;234:268-81.
50. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 2008;322:1097-100.
51. Mildner A, Jung S. Development and function of dendritic cell subsets. *Immunity* 2014;40:642-56.
52. Reis BS, Rogoz A, Costa-Pinto FA, Taniuchi I, Mucida D. Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4(+) T cell immunity. *Nat Immunol* 2013;14:271-80.

METHODS

Preparation of cells

IELs were isolated by using a previously reported method.^{E1} Briefly, the small intestines were removed, separated from Peyer patches, opened longitudinally, and cut into 0.5-cm segments. Intestinal tissues were transferred to 50-mL conical tubes containing PBS supplemented with 10 mmol/L EDTA and 1% (vol/vol) FBS, which were then shaken at 250 rpm for 30 minutes at 37°C by using a shaking incubator (Hanil, Gimpoo, Korea). The tissue suspension was passed through a 70- μ m cell strainer (BD Biosciences, San Diego, Calif), and the cells were collected by means of centrifugation at 1500 rpm for 10 minutes. Cell pellets were resuspended in HBSS and layered over a discontinuous 40% to 70% (vol/vol) Percoll (GE Healthcare, Marlborough, Mass) gradient for 30 minutes at 2500 rpm. Then IELs were isolated from the Percoll gradient interface, washed, and resuspended in complete RPMI media.

To isolate LP cells, intestinal fragments with IELs removed were minced and digested in RPMI containing 3% FBS, 20 mmol/L HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acids, 400 U/mL collagenase D (Roche, Basel, Switzerland), and 100 μ g/mL DNase I (Roche) at 37°C for 45 minutes with continuous stirring. After adding 10 mmol/L EDTA and incubating for 5 minutes at 37°C, the cell suspension was filtered with a cell strainer and pelleted by means of centrifugation for 5 minutes at 2000 rpm. LP cells were purified with Percoll, as described above.

The thymus, spleen, mLNs, and Peyer patches were also removed, mashed through a cell strainer, and washed. Single-cell suspensions were obtained after erythrocyte lysis. For preparation of DCs from Peyer patches and mLNs cell suspensions were incubated with RPMI containing collagenase D and DNase I, as described above.

Histology

The 3 parts (duodenum, jejunum, and ileum) of the small intestine were removed, washed in ice-cold PBS, and fixed in 4% paraformaldehyde on ice for 2 hours. Next, tissues were washed 3 times in ice-cold PBS and incubated in 30% (wt/vol) sucrose/phosphate buffer overnight. The tissue samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, Calif) and snap-frozen by using liquid nitrogen. Ten-micrometer-thick cryostat sections were collected and dried at 25°C for 30 minutes. The slide-embedded samples were incubated under MeOH at -20°C for 10 minutes, dried at room temperature for 10 minutes, washed with PBS, and blocked with anti-CD16/CD32 antibody (BD Biosciences). Then the slides were incubated with antibodies against TCR $\gamma\delta$ and TCR β and counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride. To assess pathology in MTX-treated mice, 4% paraformaldehyde-fixed samples were embedded with paraffin, sectioned to a thickness of 4 μ m, and stained with hematoxylin and eosin.

ALDEFLUOR assay

The ALDEFLUOR assay was performed according to the manufacturer's instructions (STEMCELL Technologies, Vancouver, British Columbia, Canada). Briefly, 1×10^6 cells freshly isolated from mLNs, Peyer patches, or small intestinal LPs were suspended in ALDEFLUOR assay buffer (5 μ L of ALDH substrate per 1×10^6 cells) and incubated for 1 hour at 37°C. As a negative control, corresponding cells were treated with 5 μ L dimethylaminobenzaldehyde, an ALDH inhibitor. The incubated samples were blocked with anti-CD16/CD32 to prevent nonspecific binding of antibodies and stained with antibodies against CD11c, MHC class II (MHC II), and CD103. Cells were then analyzed by using flow cytometry.

Reverse transcription and quantitative PCR

Total RNA was extracted with TRIzol (Invitrogen), and the samples were reverse transcribed with Moloney murine leukemia virus reverse transcriptase and oligo-dTs (Thermo Fisher Scientific, Waltham, Mass), according to the manufacturer's instructions. For real-time quantitative PCR, cDNA was

amplified with ViiA 7 (Thermo Fisher Scientific) with specific primers, as described in the Online Repository. mRNA expression levels were calculated relative to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. Quantitative real-time PCR for mature miRs was performed with the TaqMan MicroRNA Assay Kit (Applied Biosystems, Carlsbad, Calif), according to the manufacturer's instructions. miR expression was calculated relative to that of U6 small nuclear RNA.

Flow cytometry

The prepared single-cell suspensions were incubated with anti-CD16/CD32 blocking antibody to prevent nonspecific binding of antibodies and fluorescence-conjugated antibodies sequentially. The antibodies were purchased from BD Pharmingen (anti-CD4, 550954; anti-CD8 α , 552877; anti-CD103, 557495; anti-CD122, 553361; anti-CD127, 552543; and anti-CD132, 554457) or eBioscience (San Diego, Calif; anti-CD8 β , 47-0083-82; anti-CD11b, 48-0112-82; anti-CD103, 11-1031-82; anti-CD127, 25-1271-82; anti-IL-15R α , 12-7149-82; anti-MHC II, 47-5321; anti-TCR β , 17-5961-83; and anti-TCR $\gamma\delta$, 12-5711-82). Cell suspensions were incubated with rabbit anti-TGF- β RI (Abcam, GR243828-1) or anti-TGF- β RII (Abcam, GR83562-24) and anti-rabbit IgG Fab2 (Cell Signaling, Danvers, Mass; 44125) sequentially after blocking Fc receptor with anti-CD16/CD32, as described above, to assess expression levels of TGF- β RI and TGF- β RII. Flow cytometric data were acquired with a FACSCanto II or Beckman Coulter Gallios and analyzed with FlowJo software (TreeStar, Ashland, Ore). For measurement of CD8 $\alpha\alpha$ expression, phycoerythrin-conjugated TL-tetramer was used, as described previously.^{E2}

Plasmids and reporter assay

Standard PCR techniques were used to amplify the desired sequences. The 3'-UTR of *c-Myb* and *Tgfbrii* were amplified with specific primers (*c-Myb* forward: 5'-AAGATATCGACATTTCCAGAAAAGCA-3', *c-Myb* reverse: 5'-AACTCGAGGGGAAGTATGTAAAATAAGA-3', *Tgfbrii* forward: 5'-AAGAATTCCTTTTCTGGGCAGGCTGGG-3', and *Tgfbrii* reverse: 5'-AACTCGAGGGATTTATTTGATCTACTAG-3') and confirmed by means of sequencing to construct the reporter plasmids pcAANAT-*c-Myb* 3'-UTR and pcAANAT-*Tgfbrii* 3'-UTR. The PCR-amplified *c-Myb* 3'-UTR and *Tgfbrii* 3'-UTR were cut with *EcoRV/XhoI* or *EcoRI/XhoI* and then cloned into the *EcoRV/XhoI* or *EcoRI/XhoI* site of the reporter pcAANAT,^{E3} respectively. For the AANAT reporter assay, HEK293FT cells were seeded at 2×10^5 cells in 12-well plates and grown overnight. The cells were cotransfected with 1 μ g of AANAT plasmids containing 3'-UTR of *c-Myb* or *Tgfbrii* and miR-150 either miR-150 mimic or miR-150 mutant (for pcAANAT-*c-Myb* 3'-UTR) and miR-20a mimic or miR-20a mutant (for pcAANAT-*Tgfbrii* 3'-UTR) at indicated concentrations, respectively, by using TransIT-X2 (Mirus Bio, Madison, Wis), according to the manufacturer's protocol. As a negative control, a nontargeting control (Bioneer, Daejeon, Korea) was also transfected in another cell. Transfected cells were grown for a further 24 hours before harvesting, and immunoblotting and quantitative PCR were performed.

Immunoblotting

Cytoplasmic lysates of the transfected cells were extracted with PBS containing 1% NP-40 and complete protease inhibitor (Roche). The lysates were incubated overnight at 4°C with rabbit anti-AANAT (Abcam, ab3506) and mouse anti- β -actin (Santa Cruz Biotechnology, Dallas, Tex; sc-47778) as primary antibodies. After washing with PBS containing 0.5% Tween-20 (Sigma-Aldrich), the samples were incubated for 1 hour at room temperature with anti-rabbit IgG (Thermo Fisher Scientific, NCI1460KR) or anti-mouse IgG (Thermo Fisher Scientific, NCI1430KR), respectively, and visualized with an Immobilon Western kit (Millipore, Temecula, Calif).

REFERENCES

- E1. Huang Y, Park Y, Wang-Zhu Y, Larange A, Arens R, Bernardo I, et al. Mucosal memory CD8(+) T cells are selected in the periphery by an MHC class I molecule. *Nat Immunol* 2011;12:1086-95.

- E2. Huang Y, Park Y, Wang-Zhu Y, Larange A, Arens R, Bernardo I, et al. Mucosal memory CD8(+) T cells are selected in the periphery by an MHC class I molecule. *Nat Immunol* 2011;12:1086-95.
- E3. Kim N, Kim M, Yun S, Doh J, Greenberg PD, Kim TD, et al. MicroRNA-150 regulates the cytotoxicity of natural killers by targeting perforin-1. *J Allergy Clin Immunol* 2014;134:195-203.

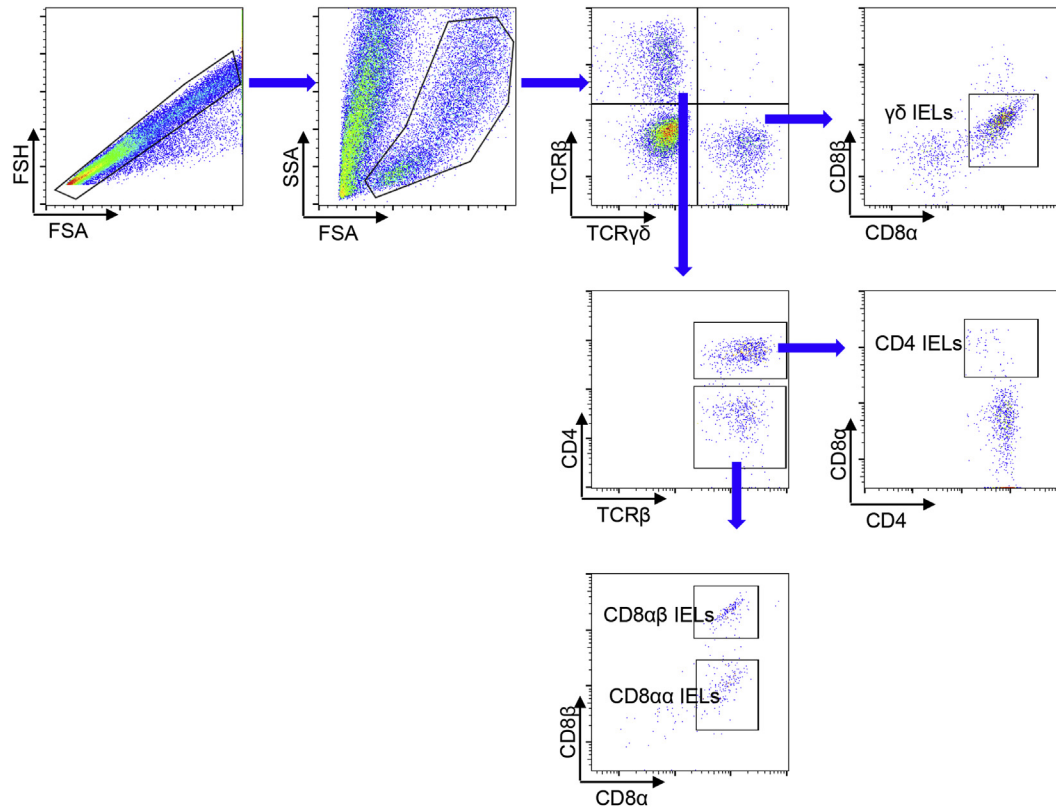


FIG E1. Gating strategy of flow cytometric analysis for IELs. Stepwise gating process to analyze IEL subpopulations by using flow cytometry. *FSA*, Forward scatter-area; *FSH*, forward scatter-height; *SSA*, side scatter-area.

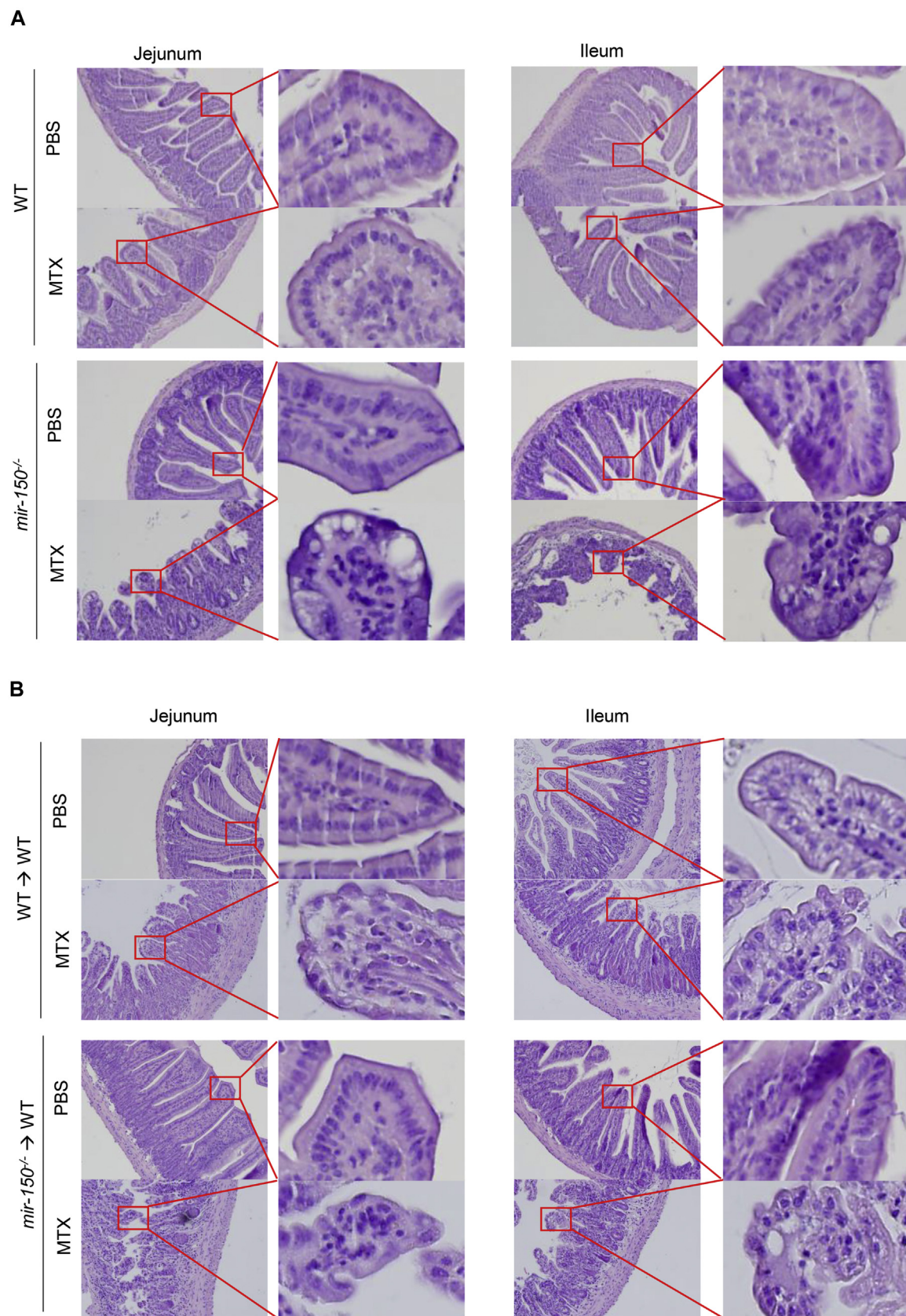


FIG E2. Changes in small intestines after MTX treatment (n = 7 mice PERSONAL group). Histologic changes in small intestines of WT and *mir-150^{-/-}* (**A**) and WT mice that received bone marrow from WT or *mir-150^{-/-}* mice (**B**).

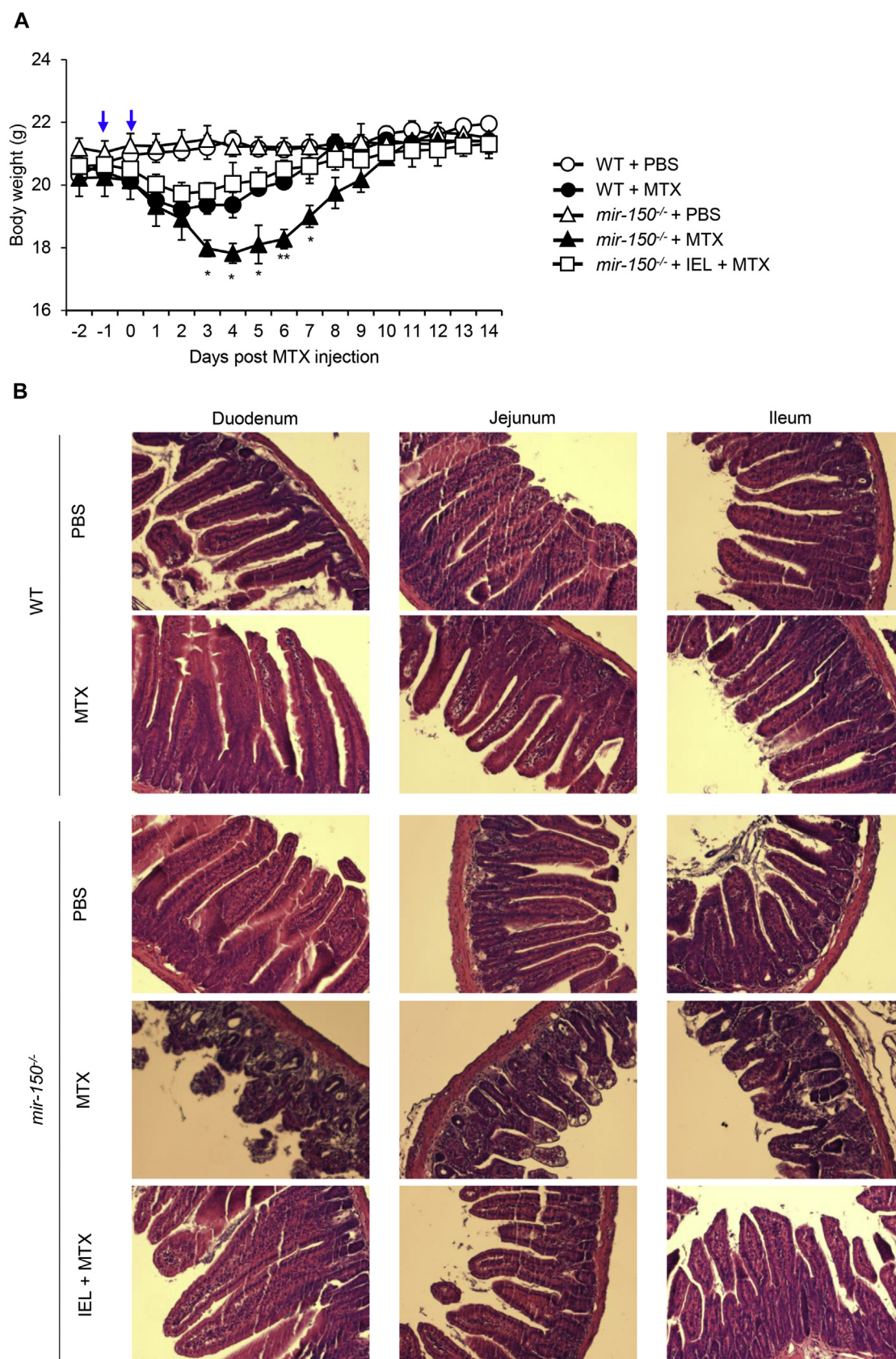


FIG E3. IEL replenishment ameliorates MTX-induced mucositis in *mir-150*^{-/-} mice. Changes in body weight (**A**) and histology (**B**) in small intestines after MTX treatment (n = 7 mice per group). IEL + MTX, IEL-transferred *mir-150*^{-/-} mice that received MTX. **P* < .05 and ***P* < .01.

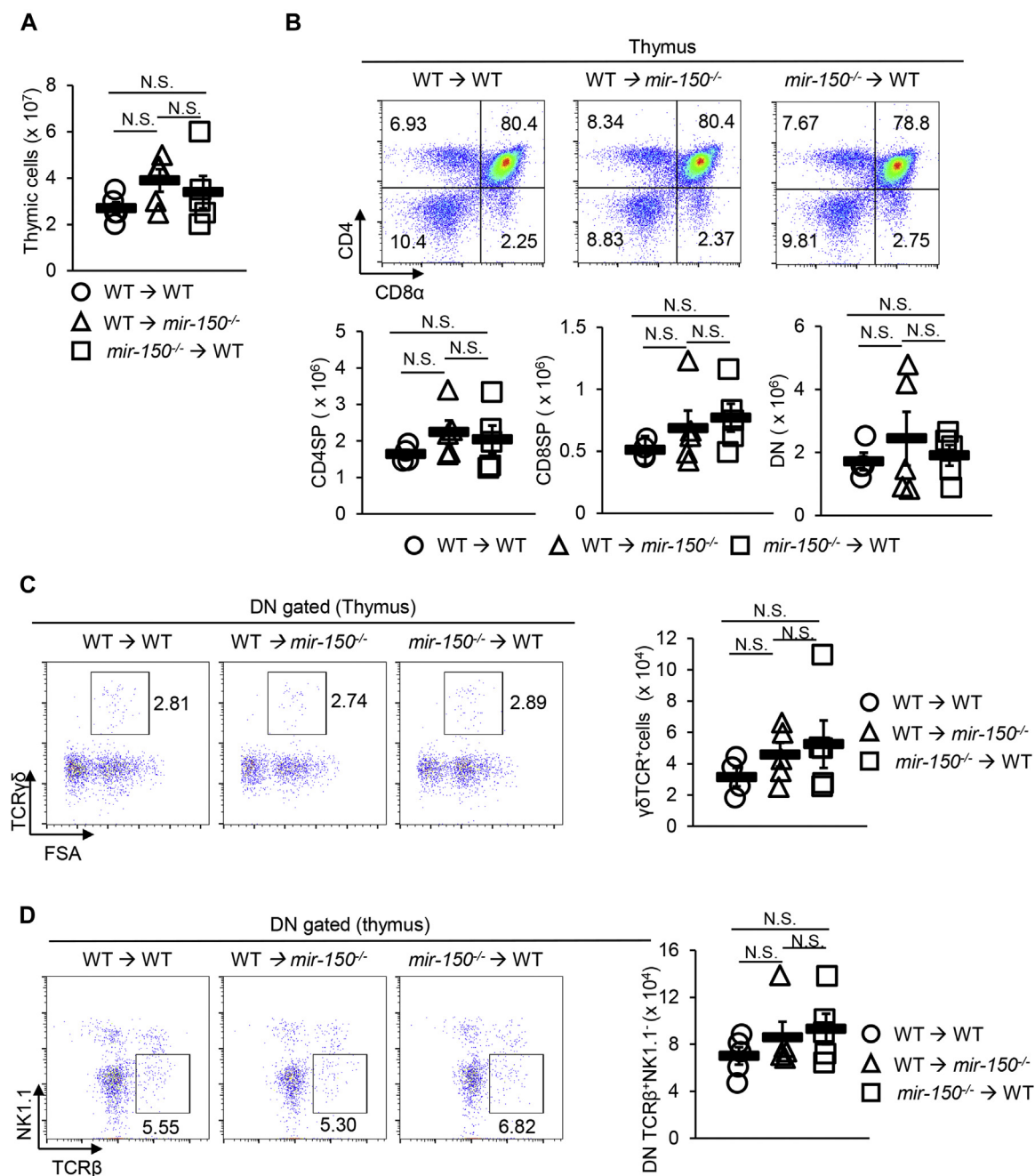


FIG E4. Generation of IEL precursors is independent of miR-150. **A-D**, Absolute numbers and flow cytometric analysis for indicated IEL precursors in the thymus after bone marrow transfer ($n = 5$ mice per group). **E-H**, CD4⁺ and CD8⁺ T-cell numbers in spleens and mLN from bone marrow-transferred mice, as described above ($n = 5$ mice per group). Results are presented as means $\pm$ SEMs. Similar data are obtained from 2 independent experiments. *N.S.*, Not significant.

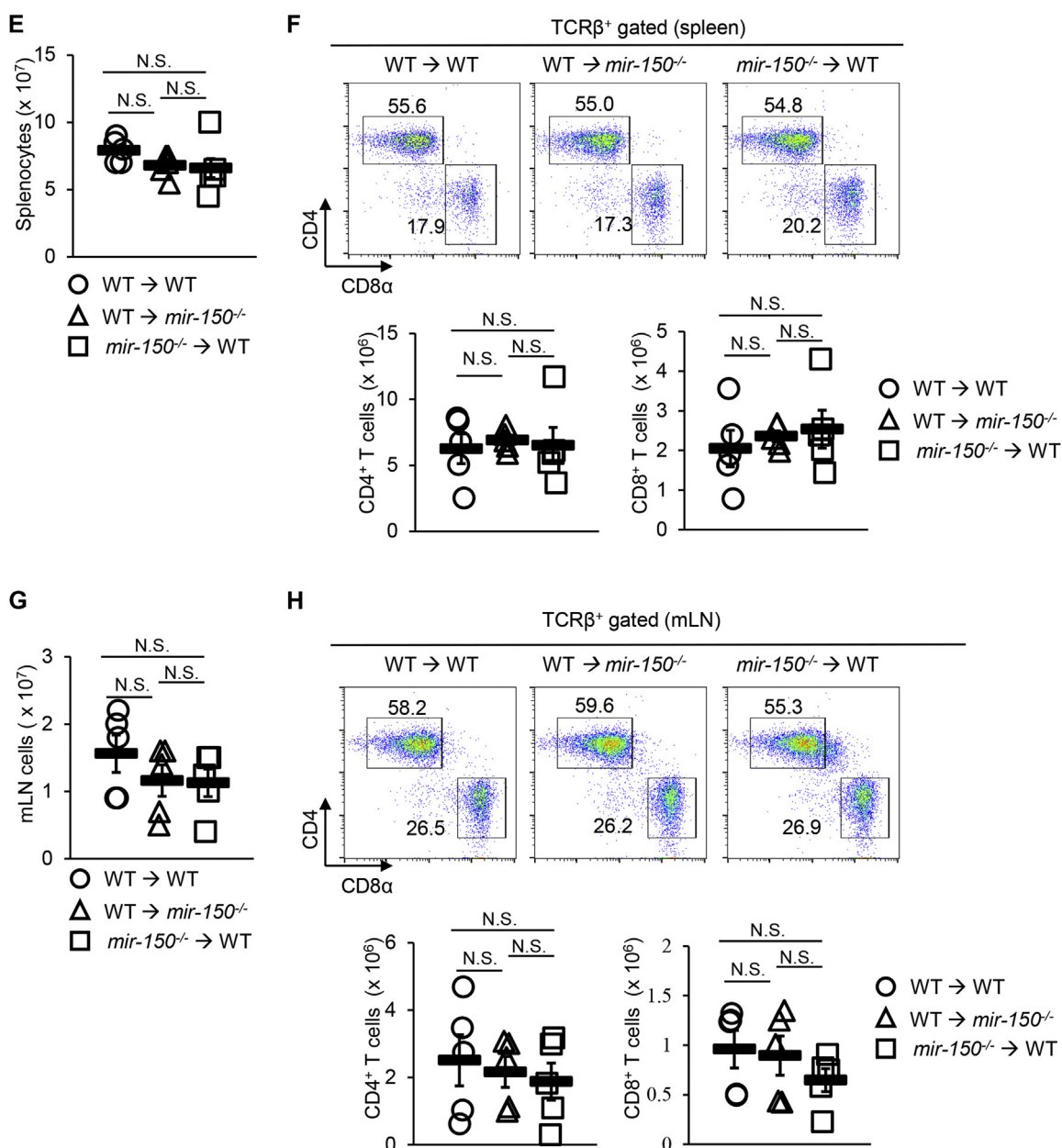


FIG E4. (Continued).

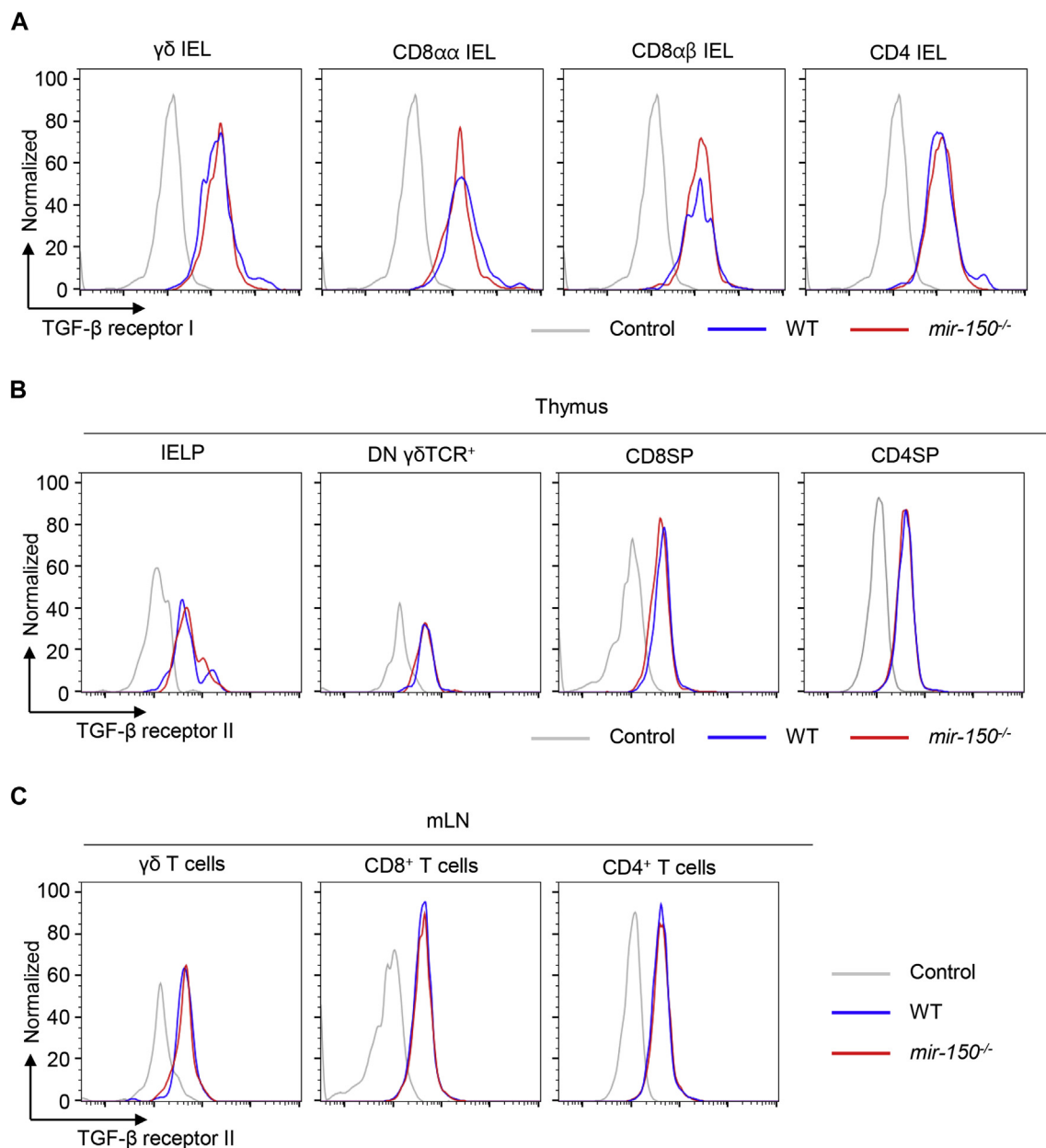


FIG E5. Expression levels of TGFβ-RI, IL-15R, IL-7 receptor, and aryl hydrocarbon receptor (*AHR*) in IELs and TGFβ-RII in IEL precursors are independent of miR-150. **A**, TGFβ-RI expression on IELs from WT and *mir-150*^{-/-} mice (*n* = 5 mice per genotype). **B** and **C**, TGFβ-RII expression on IEL precursors in thymus and mLNs (*n* = 5 mice per genotype). **D-G**, Expression levels of CD122 (Fig E5, *D*), CD132 (Fig E5, *E*), CD127 (Fig E5, *F*), and AHR (Fig E5, *G*) on IELs from WT and *mir-150*^{-/-} mice (*n* = 5 mice per genotype). Data are representative from 2 independent experiments.

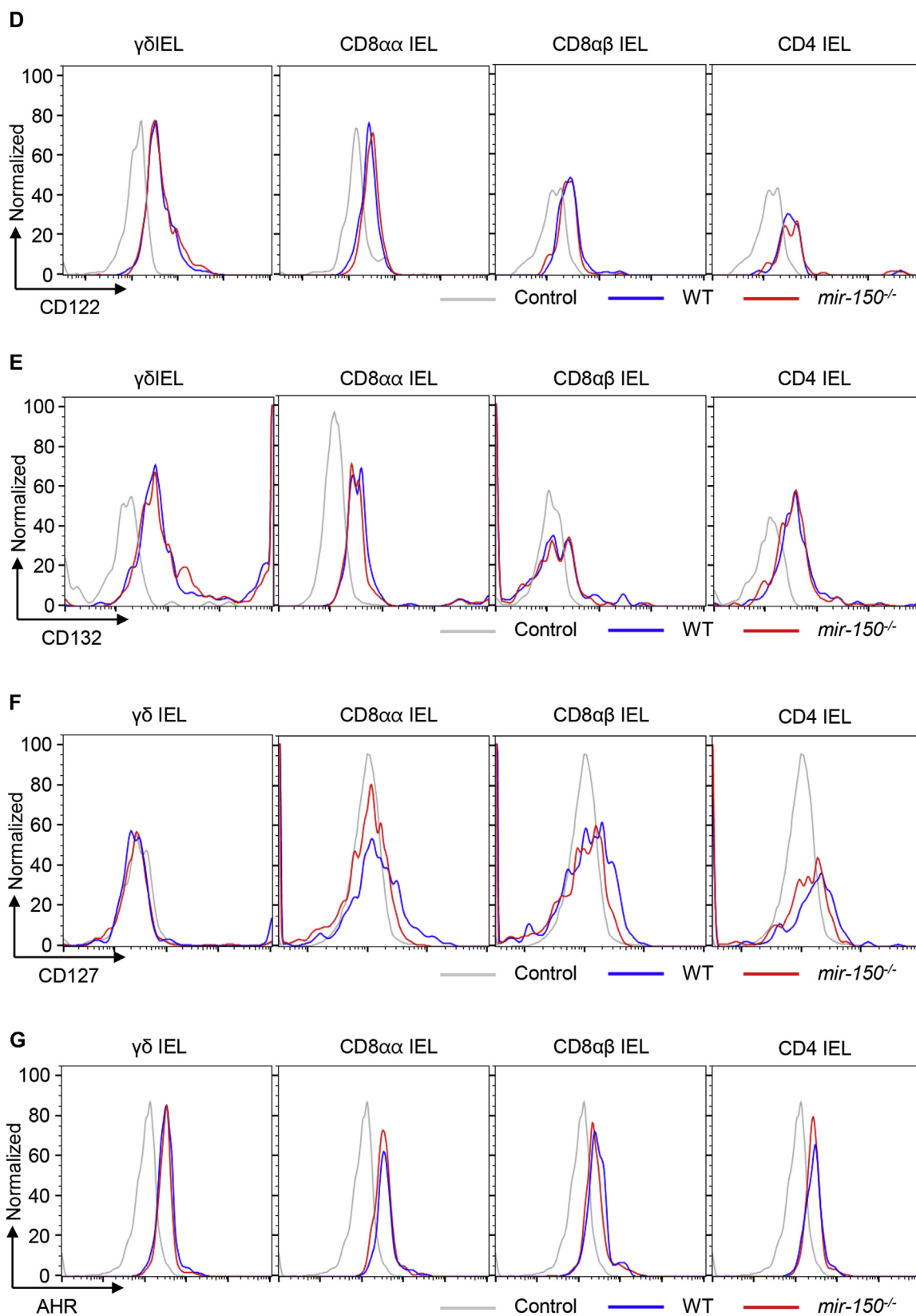


FIG E5. (Continued).

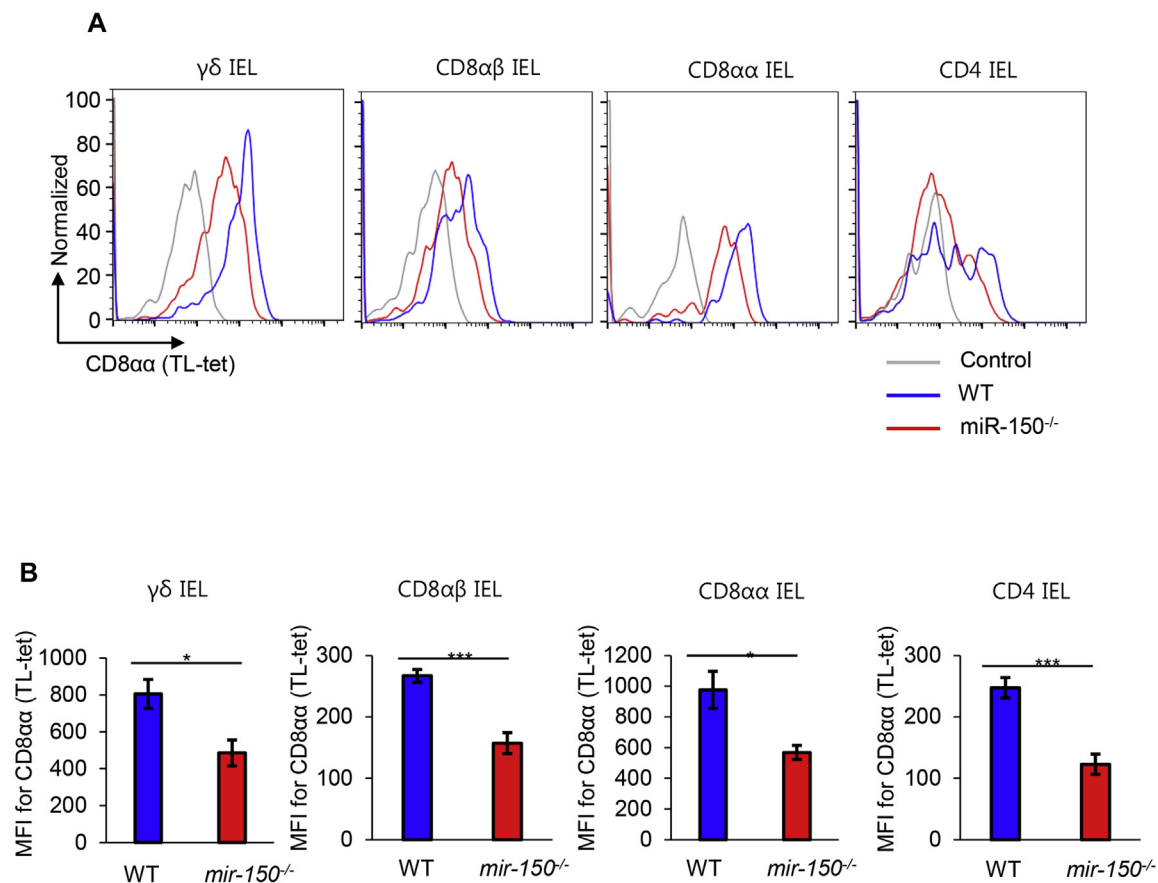


FIG E6. Reduced expression of CD8αα in *mir-150*^{-/-} IELs. CD8αα expression as measured by using TL-tetramer and analyzed by using fluorescence-activated cell sorting (**A**), and mean fluorescence intensity (MFI) was calculated for CD8αα (**B**). **P* < .05 and ****P* < .001.

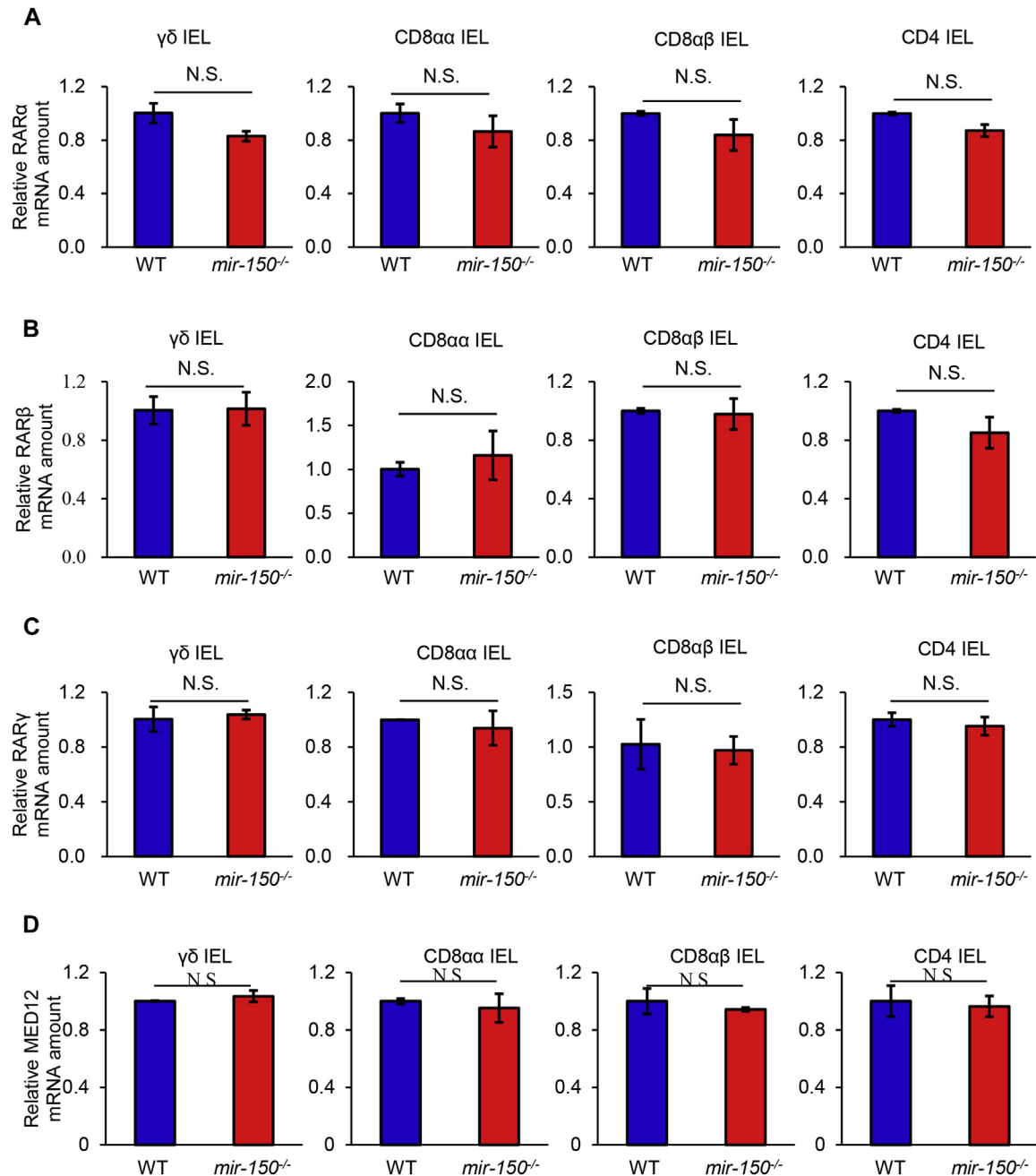


FIG E7. Expression levels of RA receptors and MED12 in IELs are not affected by miR-150 deficiency. Relative amounts of RA receptors α (A), β (B), and γ (C) and MED12 (D) in IELs from WT and *mir-150*^{-/-} mice (n = 5 mice per genotype) are shown. Data are representative of 2 independent experiments. N.S., Not significant.

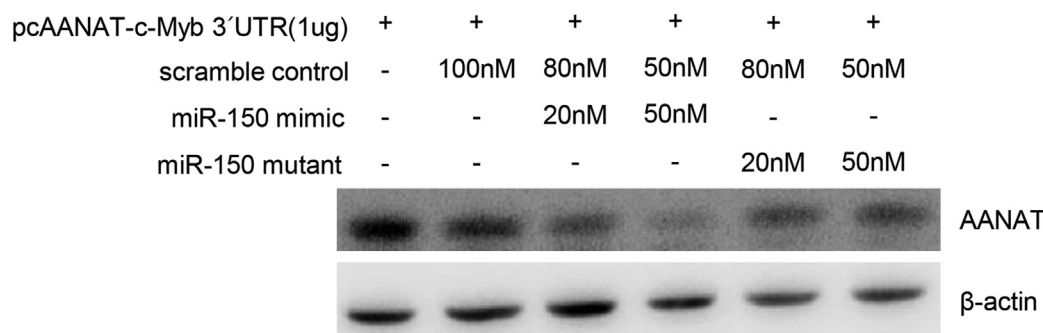
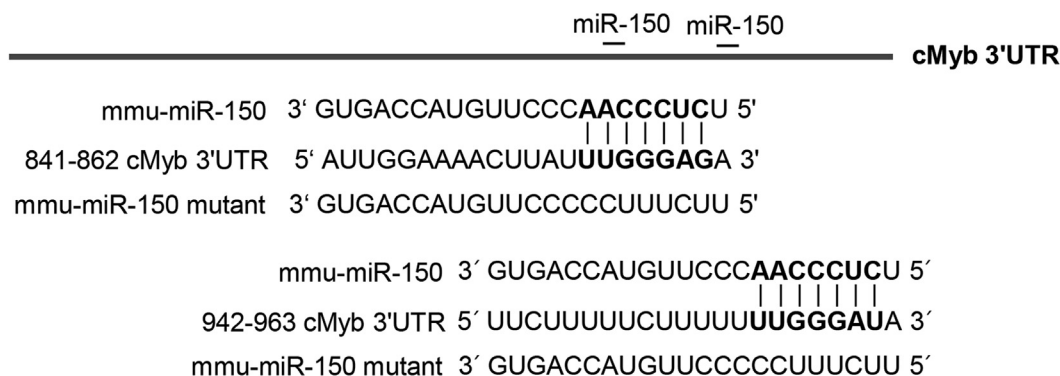
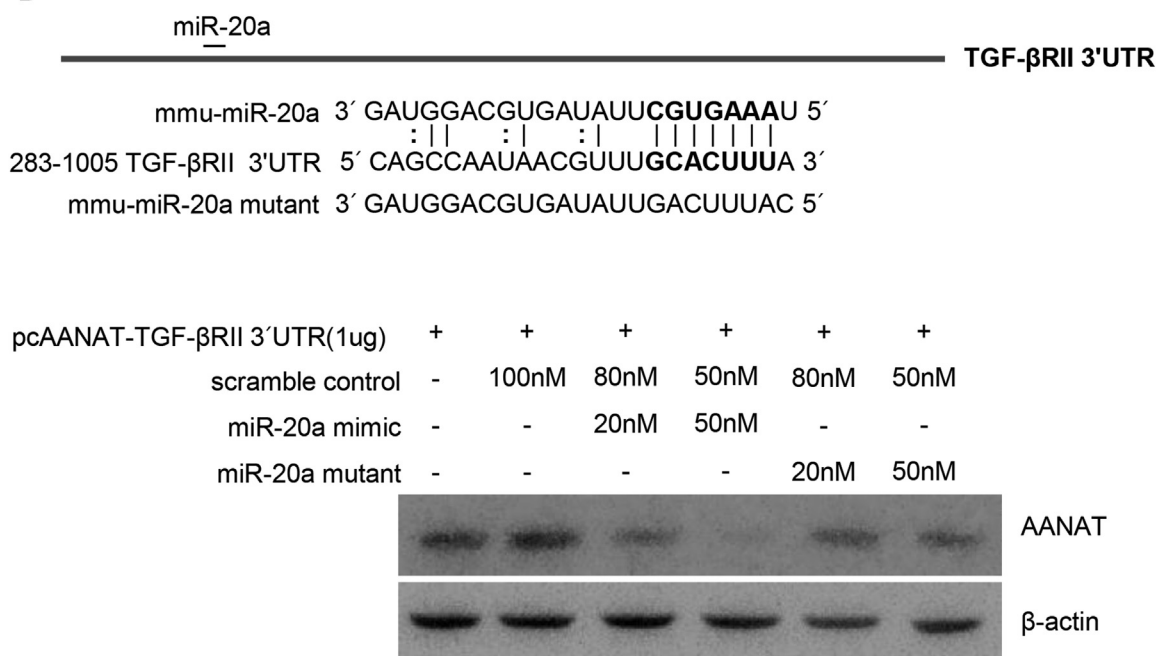
A**B**

FIG E8. miR-150 and miR-20a inhibit expression of cMyb and TGF-βRII in a 3'-UTR-dependent manner. **A**, Schematics for binding of miR-150 on the 3'-UTR of *c-Myb* (top). The level of AANAT protein (bottom) in HEK293FT cells cotransfected with plasmid-expressing AANAT reporter containing *c-Myb* 3'-UTR and miR-150 mimic or miR-150 mutant is shown. **B**, Schematics for binding of miR-20a on 3'-UTR of *Tgfbll* (top). Expression levels of AANAT protein (bottom) in HEK293FT cells cotransfected with plasmid-expressing AANAT reporter containing *Tgfbll* 3'-UTR and miR-20a mimic or miR-20a mutant are shown.

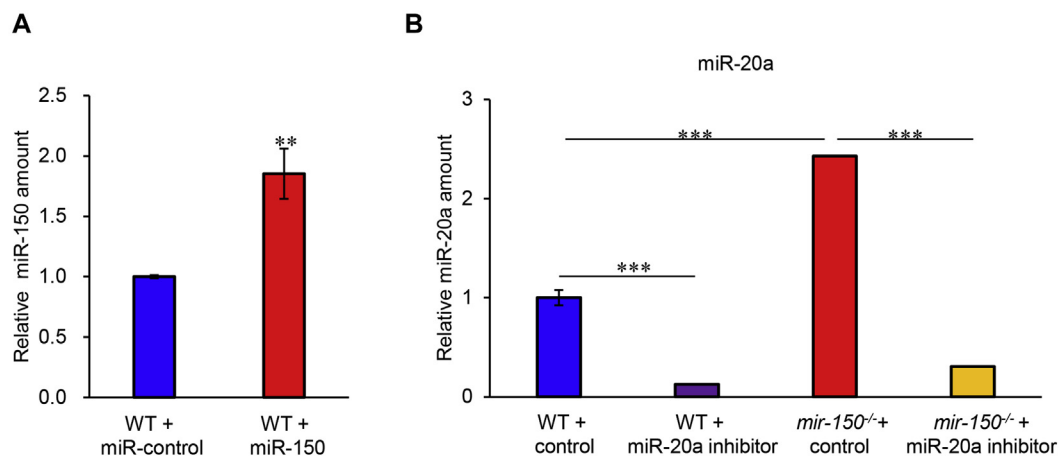


FIG E9. Expression levels of miR-150 and miR-20a after transfer of miR-150mimic and miR-20a inhibitor from WT and *mir-150*^{-/-} CD8⁺ T cells. **A**, Expression levels of miR-150 in control or miR-150 mimic transferred WT CD8⁺ T cells. **B**, Relative expression of miR-20a mimic in miR-20a inhibitor or control transferred WT and *mir-150*^{-/-} CD8⁺ T cells. Data are presented as means $\pm$ SEMs. ** $P < .01$ and *** $P < .001$.

TABLE E1. Primer sequences that were used for real-time PCR

Gene name	Direction	Sequence
<i>AANAT</i>	Forward	5'-CATCTGCCTCTTGGGACCT-3'
	Reverse	5'-AGCTCTGGACACAGGGTGAG-3'
Mouse <i>CD103</i>	Forward	5'-CAGGAGACGCCGGACAGT-3'
	Reverse	5'-CAGGGCAAAGTTGCACTCAA-3'
Mouse <i>c-Myb</i>	Forward	5'-CTCCCTCTTTGCTGACGTCA-3'
	Reverse	5'-TTGATAGCTGGAGTTCTAAA-3'
Mouse <i>c-Myc</i>	Forward	5'-TCTCCACTCACCAGCACAA-3'
	Reverse	5'-GTTCTCTCTGACGTTCCA-3'
Mouse <i>Gadph</i>	Forward	5'-ATCACTGCCACCCAGAAGAC-3'
	Reverse	5'-AGATCCACGACGGACACATT-3'
Mouse <i>MED12</i>	Forward	5'-TCAGGCAGTGGGATTACAATGA-3'
	Reverse	5'-TCCAGGGCGTATTTTCTCAAAAC-3'
Mouse <i>RARα</i>	Forward	5'-CTGGACTGCTCAGTGCCATCT-3'
	Reverse	5'-TGCAGCATGTCCACCTTGTC-3'
Mouse <i>RARβ</i>	Forward	5'-GGGTAAATACACCACGAATTCCA-3'
	Reverse	5'-TGTCCCAGAGGCCCAAGTC-3'
Mouse <i>RARγ</i>	Forward	5'-CCAGGAGACTTTTCCCTCACTCT-3'
	Reverse	5'-GCTGCACCCGGTGATCTG-3'
Mouse <i>Runx3</i>	Forward	5'-AGGGAAGAGTTTCACGCTCA-3'
	Reverse	5'-AGGCCTTGGTCTGGTCTTCT-3'
Mouse <i>TgfbβII</i>	Forward	5'-AGCATCACGGCCATCTGTG-3'
	Reverse	5'-TGGCAAACCGTCTCCAGAGT-3'

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *RAR*, retinoic acid receptor.