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miR-183-96-182 Cluster Is Involved in Invariant NKT Cell Development, Maturation, and Effector Function

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The development, differentiation and function of invariant NKT (iNKT) cells require a well-defined set of transcription factors, but how these factors are integrated to each other and the detailed signaling networks remain poorly understood. Using a Dicer-deletion mouse model, our previous studies have demonstrated the critical involvement of microRNAs (miRNAs) in iNKT cell development and function, but the role played by individual miRNAs in iNKT cell development and function is still not clear. In this study, we show the dynamic changes of miRNA 183 cluster (miR-183C) expression during iNKT cell development. Mice with miR-183C deletion showed a defective iNKT cell development, sublineage differentiation, and cytokine secretion function. miRNA target identification assays indicate the involvement of multiple target molecules. Our study not only confirmed the role of miR-183C in iNKT cell development and function but also demonstrated that miR-183C achieved the regulation of iNKT cells through integrated targeting of multiple signaling molecules and pathways. ***The Journal of Immunology*****, 2019, 203: 3256–3267.**

Invariant NKT (iNKT) cells represent a rare and distinct subset of T lymphocytes that coexpress TCR and various NK cell–related surface markers. Upon activation, iNKT cells rapidly secrete large quantities of cytokines that regulate immune responses associated with a variety of diseases ranging from transplantation and tumors to various forms of autoimmunity and infections. Unlike conventional T lymphocytes, which express a diverse repertoire of TCRα and TCRβ, iNKT cells combine the Vα14i TCR α-chain (Vα24-Jα18 in human) with a restricted repertoire of TCR β-chains that contain either Vβ8, Vβ7, or Vβ2 segments (Vβ11 in humans) in mice. After positive selection by $CD4^+CD8^+$ double-positive (DP) thymocytes (stage 0, $CD24^+CD44^{lo}$ $NK1.1^-$), iNKT cells downregulate CD24, proliferate, and proceed through a three-stage maturation process from immature $CD24^-CD44^{lo}NK1.1^-$ (stage 1) to semimature $CD24^-CD44^{hi}NK1.1^-$ (stage 2) and then to mature $CD44^{hi}NK1.1^+$ (stage 3) iNKT cells. Recent studies have led to a new iNKT subsets model emphasizing their functional heterogeneity based on T helper categories: NKT1, NKT2, and NKT17 (1, 2). These iNKT cell functional subsets can be recognized by expression patterns of specific transcription factors. The development and function of iNKT cells is under the control of a complex network, including both transcriptional and posttranscriptional mechanisms. Recent studies have revealed the critical involvement of microRNAs (miRNAs) as a posttranscriptional mechanism in the development and function of iNKT cells (3, 4). Nevertheless, the role of individual miRNAs in iNKT regulation and their position in the molecular network of iNKT regulation remain not completely understood.

The miRNA 183 cluster (miR-183C), composed of miRs 183, 96, and 182, is a miRNA family of which the sequence homology and genomic organization is highly evolutionarily conserved (5). It has been shown that miR-183C is highly expressed in sensory organs and is required for the maturation of these organs (6–8). Meanwhile, miR-183C is frequently highly expressed in a variety of nonsensory organ diseases, including cancer and autoimmune disorders (5). In macrophages and neutrophils, the miR-183C modulate their phagocytosis and intracellular bacterial-killing capacity (9) and production of proinflammatory cytokines (10). In T cells, the miR-183C members regulate several proinflammatory cytokine pathways, which is vital to the immune cell function (11, 12). However, the role of miR-183C in iNKT cell development and function is completely unknown.

In the current study, we found that miR-183C was highly expressed and dynamically regulated during iNKT cell development and maturation. Deletion of miR-183C results in defective iNKT cell development, sublineage differentiation, and effector function. Multiple target genes potentially work in concert to mediate miR-183C iNKT cell regulation. Collectively, our results demonstrate the role of miR-183C in iNKT cell developmental and functional regulation.

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L.Z. and Q.-S.M. conceived and designed the study; J.W., G.L., X.W., and Q. L. performed experiments; J.W., L.Z., and G.L. analyzed data; C.Y. performed mouse mating and genotyping; S.L.B. performed mouse irradiation for bone marrow transfer; S.X. originally made and supplied the miR-183C knockout mouse strain; L.Z., J.W., and Q.-S.M. wrote the manuscript, which was commented on by all authors.

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Abbreviations used in this article: BM, bone marrow; DP, double-positive; 5F, 5′ forward; Foxo1 KO, Csf1r-$Cre^+Foxo1^{fl/fl}$; iNKT, invariant NKT; KO, knockout; LN, lymph node; miR-183C, miRNA 183 cluster; miRNA, microRNA; P/I, PMA and ionomycin; qRT-PCR, quantitative RT-PCR; WT, wild-type.

Materials and Methods

Mice

The miR-183C knockout (KO) mouse strain was derived from a gene-trap embryonic stem cell clone and described previously (8). Mice carrying a conditional floxed allele of Foxo1 (Foxo1$^{fl/fl}$ from The Jackson Laboratory) were mated to mice carrying the Csf1r-Cre allele to generate Csf1r-Cre^{+}Foxo1$^{fl/fl}$ (Foxo1 KO) mice. The littermate Csf1r Cre^{-}Foxo1$^{fl/fl}$ mice served as wild-type (WT) control. Csf1r-Cre–mediated gene mutation occurs in most innate and adaptive immune cells, including iNKT cells (13). Experiments were conducted on 6–8-wk-of-age– and gender-matched KO and WT littermate controls. Mice were housed in a specific pathogen–free barrier unit. Handling of mice and experimental procedures were in accordance with requirements of the Institutional Animal Care and Use Committee.

Genotyping

miR-183C KO mice were genotyped using the following PCR primer pairs, as describe previously (8): 5′ forward (5F) intron l: 5′-GAA CGT GCT TGT GCT GTG CAC-3′; 3′ reverse intron l: 5′-CTA CAT CCT CTG CCA GGT CTC-3′; 353: 5′-CAG GGT TTT CCC AGT CAC GAC-3′. The 5F intron l/353 amplifies the KO (miR-183C KO) allele with a 1.2-kb PCR product, whereas the 5F intron l/3′ reverse intron 1 detect a 523-bp product of the WT allele.

Flow cytometry analyses

Single-cell suspensions were washed twice with staining buffer (PBS, 2% FBS) and incubated with Fc block (clone 2.4G2). Cells were stained with CD1d tetramers and/or the following conjugated mAbs were used: TCRβ (H57-597), CD24 (30-F1), CD44 (IM7), CD122 (5H4), CD69 (H1.2F3), NK1.1 (PK136), Ki-67 (SolA15), Bcl-2 (10C4), PLZF (9E12), RORγt (B2D), T-bet (eBio17B7), IL-4 (11B11), IFN-γ (XMG1.2), IL-17 (eBio17B7), TNF-α (MP6-XP22), CD45.1 (A20), and CD45.2 (104). All mAbs were purchased from eBioscience or Tonbo Bioscience. Data were analyzed using FlowJo 10.0 software. Apoptosis assays were carried out by staining with Annexin V (eBiosciences), according to the manufacturer's instructions.

iNKT cell enrichment and sorting

Thymic iNKT cells were enriched from total thymocytes by depletion of CD8^{+} T cells using MagniSort (eBioscience) with biotin-conjugated anti-mouse CD8 Ab and anti-biotin magnetic beads (eBioscience). Negatively selected CD8^{-} cells were then stained with anti-mouse TCRβ, CD1d tetramer, anti-mouse NK1.1, and anti-mouse CD44 Abs. Thymic iNKT cells of whole population or of different developmental stages were then sorted by BD FACSAria II.

In vitro PMA and ionomycin activation assay

Cells from WT and miR-183C KO mice were cultured in T cell culture medium (RPMI 1640 with 10% FBS, HEPES, penicillin and streptomycin, pyruvate, nonessential amino acids, L-glutamine, and 2-ME) in the presence of PMA (50 ng/ml) and ionomycin (1 μM) for a total of 4 h, and brefeldin A was added for the last 2.5 h before harvesting at a final concentration of 1 μM. The harvested cells were intracellularly stained with the following anti-mouse Abs: IFN-γ, IL-4, IL-17, and TNF-α before flow cytometry analysis.

Mixed bone marrow transfer experiments

To generate bone marrow (BM) chimeras, 7–8-wk-old C57BL/6. SJL (B6.SJL) recipient mice were lethally irradiated, initially with 9.5 Gy with a dosage rate of 2.5 Gy per minute. Quality assurance of the radiation exposure was performed using multiple dosimetry endpoints, including an electrometer, micro-thermoluminescent dosimeter and Gafchromic film. Donor BM was harvested from age- and sex-matched SJL (CD45.1^{+}) and miR-183C KO or WT control mice (CD45.2^{+}). After erythrocyte lysis, mature T cells (CD3^{+}) were depleted by biotin-conjugated anti-mouse CD3 (BD Biosciences) mAbs and anti-biotin magnetic beads (BD Biosciences) from BMs of each donor, using MagniSort. Over 90% of mature T cell depletion was confirmed by flow cytometry. CD45.1^{+} SJL and CD45.2^{+} miR-183C KO or WT littermate control BMs were mixed at a 1:1 ratio, and 1×10^{7} cells per mouse (in a volume of 100 μl) were then injected into the irradiated recipients by tail vein. The chimeras were analyzed 8 wk after reconstitution.

Quantitative RT-PCR analysis

Total RNA was isolated by TRIzol reagent (Sigma-Aldrich) and was quantified by a NanoDrop ND-1000 spectrophotometer. The A_{260}/A_{280} ratio was >1.9 for all the samples. First-strand cDNA was prepared by using a cDNA Synthesis Kit (Sigma-Aldrich) following the manufacturer's instructions. The PCR amplification was carried out on the Applied Biosystem 7900 Real-time PCR System; relative quantification using the ΔCT values in the cells from miR-183C KO versus WT control mice was carried out, and fold changes were calculated.

Statistical analysis

Data were analyzed using GaphPad Prism 8, and a two-tailed Student *t* test was used. A p value <0.05 was set as the threshold to determine statistical significance.

Results

miR-183C expression is dynamically regulated during iNKT cell development and maturation

To investigate the possible regulatory role of miR-183C in iNKT cell development and differentiation, we first assessed the expression of individual members of the miR-183C in the developing iNKT cells. WT thymus CD4^{+}CD8^{+} DP T cells and iNKT cells at different developmental stages were first sorted out, based on CD44 and NK1.1 expression as shown previously (14). The expression of miR-183C members was then evaluated and compared in DP T and iNKT cells at different developmental stages. As shown in Fig. 1A, the overall expression levels of miR-183C were relatively higher in iNKT cells than DP T cells. In different iNKT populations, miR-182 and miR-183 were relatively more abundant than that of miR-96. Furthermore, the expression of miR-183 and miR-182 were both gradually downregulated during the development and maturation of iNKT cells and reached the lowest level at the final maturation stage 3 (CD44^{hi} NK1.1^{+}). Nevertheless, miR-96 remained at a similarly low expression in all stages of iNKT development (Fig. 1A). These results showed the predominant expression of miR-183C in iNKT cells, especially miR-183/miR-182, compared with T cell progenitors, and the dynamic regulation of miR-182/miR-183 during the development and maturation of iNKT cells. These results strongly suggest the potential regulatory role of miR-183C in the development and differentiation of iNKT cells.

miR-183C deficiency interferes with overall iNKT cell development

To determine the role of miR-183C in iNKT cells development, iNKT cells from thymus and different peripheral immune organs were evaluated and compared between miR-183C KO and WT littermate controls. Based on the surface expression of TCRβ^{+} and CD1d tetramer, we detected a 2-fold reduction in thymus iNKT cell frequencies from miR-183C KO mice compared with WT controls (Fig. 1B, 1C), and the absolute number of thymus iNKT cells was reduced to a similar extent (Fig. 1C). In peripheral immune organs, splenic iNKT cells from miR-183C KO showed a 2-fold reduction in frequency and number; however, iNKT cell frequencies and numbers from lymph nodes (LN), liver, and lung remained comparable between miR-183C KO and WT controls (Fig. 1B, 1C). To further evaluate the potential role of miR-183C in conventional αβ T cell development, the frequencies of thymic DP T, CD4^{+}CD8^{-}, or CD4^{-}CD8^{+} single-positive T cells and CD4^{-}CD8^{-} double-negative T cells T were compared between miR-183C KO and WT controls. CD4 and CD8 single-positive T cells and double-negative T cell frequencies were comparable, but a mild decrease of DP T cell frequencies was identified in miR-183C

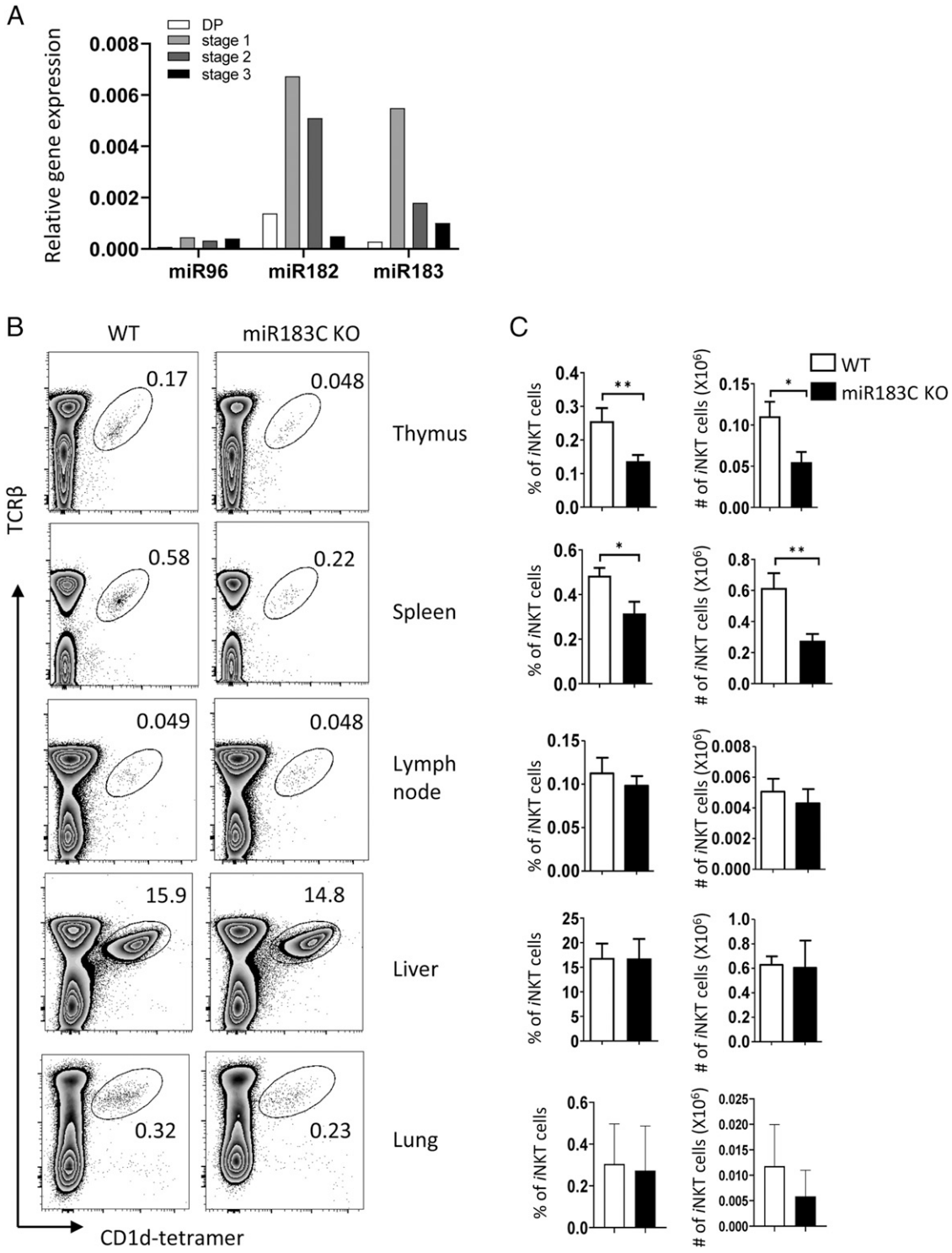

FIGURE 1. miR-183C deficiency interferes with iNKT cell development. (**A**) Individual expression of miR-183C in WT and miR-183C KO mice. qRT-PCR analysis of individual miR-183C in $CD4^+CD8^+$ DP thymocytes and different developmental stages of purified iNKT cell populations in the thymus, presented relative to results obtained for the small nuclear RNA U6 (endogenic control). Data are from one experiment representative of triplicates. (**B**) Defective iNKT cell development in miR-183C KO mice. Representative flow cytometric plots showing the percentages of iNKT cells ($TCR\beta^+CD1d$ $tetramer^+$) in the thymus, spleen, LNs, liver, and lung of WT and miR-183C KO mice. Numbers adjacent to outlined areas indicate percentage of indicated populations. (**C**) The frequencies (left) and the absolute numbers (right) of $TCR\beta^+CD1d$ $tetramer^+$ iNKT cells in the indicated organs of miR-183C KO mice. Data are from two to four independent experiments. $*p < 0.05$, $**p < 0.01$ compared with WT controls.

KO compared with WT controls (Supplemental Fig. 1). Furthermore, no significant difference on $CD4^+$ and $CD8^+$ T cell frequencies in peripheral immune organs, including spleen, LN, and liver, were identified when comparing miR-183C KO with WT controls (Supplemental Fig. 1). No difference was observed in thymus γδT cell and regulatory T cell frequencies between miR-183C KO and WT controls (Supplemental Fig. 1).

Taken together, these data indicated that deletion of miR-183C interrupted overall iNKT cell development without substantial impact on the development of other T cell types.

miR-183C deletion interferes with iNKT cell maturation and lineage differentiation

iNKT progenitor cells originate from DP thymocytes and then undergo four developmental stages based on cell surface expression of CD24, CD44, and NK1.1 (15). In this study, we found that frequencies and numbers of stage 0 ($CD24^+$ $CD44^{lo}$ $NK1.1^-$) and stage 1 ($CD24^-$ $CD44^{lo}$ $NK1.1^-$) thymic iNKT cells were comparable between miR-183C KO and WT controls. However, increased stage 2 ($CD44^{hi}$ $NK1.1^-$) but decreased stage 3 ($CD44^{hi}$ $NK1.1^+$) thymic iNKT frequencies were observed in miR-183C KO mice as compared with WT controls. Consistent with the frequency changes, the absolute number of stage 3 thymic iNKT cells reduced dramatically, whereas stage 2 iNKT cells remained comparable because of the decreased total thymic iNKT cell number (Fig. 2A, 2B). These results indicated the requirement of miR-183C in iNKT cell terminal maturation. In addition to NK1.1 and CD44, CD122 and CD69 are important markers reflecting the final maturation of iNKT cells. Interestingly, there were no alterations in the expressions of CD69 and CD122, although NK1.1 expression was downregulated dramatically in thymic iNKT cells from miR-183C KO mice (Fig. 2C, 2D). Further analysis showed the equivalent expression of CD69 and CD122 in the subsets of thymic iNKT cells, based on NK1.1 expression in miR-183C KO mice compared with WT controls (Fig. 2C, 2D). In addition, similar downregulated NK1.1 and comparable CD69 and CD122 expression were identified in splenic iNKT cells (Fig. 2E). These results indicated that miR-183C plays a role in iNKT cell final maturation and NK1.1 expression but is independent of other maturation marker CD69 and CD122 expression, suggesting the discordant role of miR-183C in different iNKT cell maturation marker expressions.

Recently a new iNKT classification system categorizes iNKT cells into NKT1, NKT2, and NKT17, based on their transcription factors and cytokine expression profiles (1, 2). The new classification of iNKT cells, an alternative to the shared developmental stages, favors functional heterogeneity and clear lineage separation. NKT1 cells are phenotypically $PLZF^{lo}$ T-bet^{hi} (mainly in stage 3) and mainly produce IFN-γ; NKT2 cells are phenotypically $PLZF^{hi}$ T-bet^{lo} $ROR\gamma t^-$(mainly in stage 2) and mainly produce IL-4; NKT17 cells are defined as $PLZF^{int}$ $ROR\gamma t^+$ (mainly in stage 2), and mainly produce IL-17. To evaluate the potential role of miR-183C in iNKT functional lineage differentiation, iNKT cells from thymus, spleen, LN, and lung were analyzed for related transcription factor expression and compared with miR-183C KO and WT controls. In accordance with the defective maturation, thymic iNKT cells showed reduced NKT1, accompanied by upregulated NKT2 but unchanged NKT17 in miR-183C KO compared with WT controls (Fig. 3A). Nevertheless, the thymic bias toward NKT2 differentiation in miR-183C KO mice was absent in peripheral spleen (Fig. 3B), lung (Fig. 3C), and LN iNKT cells (Fig.3D, Supplemental Fig. 1D), presumably resulting from the differential migration and expansion of individual iNKT effector lineages after emigration from the thymus. Thus, these results indicate the role of miR-183C in iNKT cell final maturation and functional sublineage differentiation, especially in thymus, proposing the role of other mechanisms in peripheral iNKT cells.

The role of miR-183C in iNKT cell homeostasis

To assess whether the impairment of iNKT cell development in miR-183C KO mice is related to iNKT cell homeostasis, the apoptosis and proliferation capacity of iNKT cells were evaluated. As shown in Fig. 3E, a comparable thymic iNKT cell Annexin V binding frequency was detected between miR-183C KO and WT control mice. Consistent with unchanged cell apoptosis, similar expression of anti-apoptotic protein Bcl-2 was observed in thymic iNKT cells in miR-183C KO mice compared with WT controls (Fig. 3E). Meanwhile, the similar unchanged apoptosis and Bcl-2 expression was also observed in the splenic iNKT cells of miR-183C KO mice and WT controls (Fig. 3F). To detect the proliferation capacity of iNKT cells, Ki-67 expression was evaluated by flow cytometry. As shown in Fig. 3E, increased frequency of Ki-67–expressing cells were observed in thymic iNKT cells in miR-183C KO mice compared with WT controls. Meanwhile, a trend of increased Ki-67 was also observed in splenic iNKT cells in miR-183C KO mice, albeit the level of statistical significance was not reached (Fig. 3F). To dissect whether increased overall thymic iNKT cell Ki-67 expression in miR-183C KO mice is related to their defective maturation phenotype, we further analyzed the Ki-67 expression in separated $NK1.1^+$ and $NK1.1^-$ iNKT subsets. As shown in Fig. 3G and 3H, we found that Ki-67 expression is comparable between miR-183C KO and WT controls within the same iNKT subsets in both thymus and spleen. As immature thymic iNKT cells ($NK1.1^-$) have relatively higher proliferation capacity, the increased overall thymic iNKT cell proliferation observed from miR-183C KO mice should be related to their immature phenotype compared with WT controls. In addition, we observed comparable Annexin V, Bcl-2, and Ki-67 expression in both thymic and splenic conventional T cells in miR-183C KO mice compared with WT controls (Supplemental Fig. 2). Taken together, from our evidence, we concluded that miR-183C has no significant impact on conventional T cell and iNKT cell homeostasis.

miR-183C regulates NKT17 effector function

As miR-183C plays a role in iNKT cell maturation and lineage differentiation (Figs. 2, 3), in this study, we further assess whether miR-183C is involved in iNKT cell effector function. To investigate the role of miR-183C in the function of iNKT cells, we in vitro stimulated both thymocytes and splenocytes with PMA and ionomycin (P/I) for 4 h, and iNKT cell activation capacity and cytokine secretion function were evaluated via flow cytometry. After stimulation, thymic and splenic iNKT cells from miR-183C KO mice showed a similar magnitude of CD69 upregulation compared with that from WT controls (Fig. 4A, 4B), suggesting the comparable iNKT cell activation capacity in miR-183C KO mice and WT controls. Although thymic iNKT cell lineage differentiation deflected from NKT1 toward NKT2 in miR-183C KO mice (Fig. 2A), comparable frequencies of IFN-γ– and TNF-α–producing thymic iNKT cells were detected in miR-183C KO mice compared with WT controls (Fig. 4A). However, thymic iNKT cells from miR-183C KO mice showed a trend of increased IL-4 secretion, although it did not reach the statistical significance, supporting the upregulation of thymic NKT2 in miR-183C KO mice. Meanwhile, comparable frequencies of splenic iNKT cells producing either IL-4 or IFN-γ and TNF-α after P/I stimulation were identified in miR-183C KO versus WT control mice (Fig. 4B), which is consistent with the unchanged functional lineage in splenic iNKT cells in miR-183C KO and WT controls. Interestingly, IL-17 production of thymic, splenic, and similarly stimulated LN and lung iNKT cells from miR-183C KO mice concurrently decreased (Fig. 4), although no significant downregulation of NKT17 differentiation was observed in miR-183C KO compared with WT controls (Fig. 3A–D). Similar functional phenotype changes were also found in CD4 T cells (Supplemental Fig. 3), indicating that miR-183C promotes the effector function of NKT17 and Th17 cells in a similar pattern.

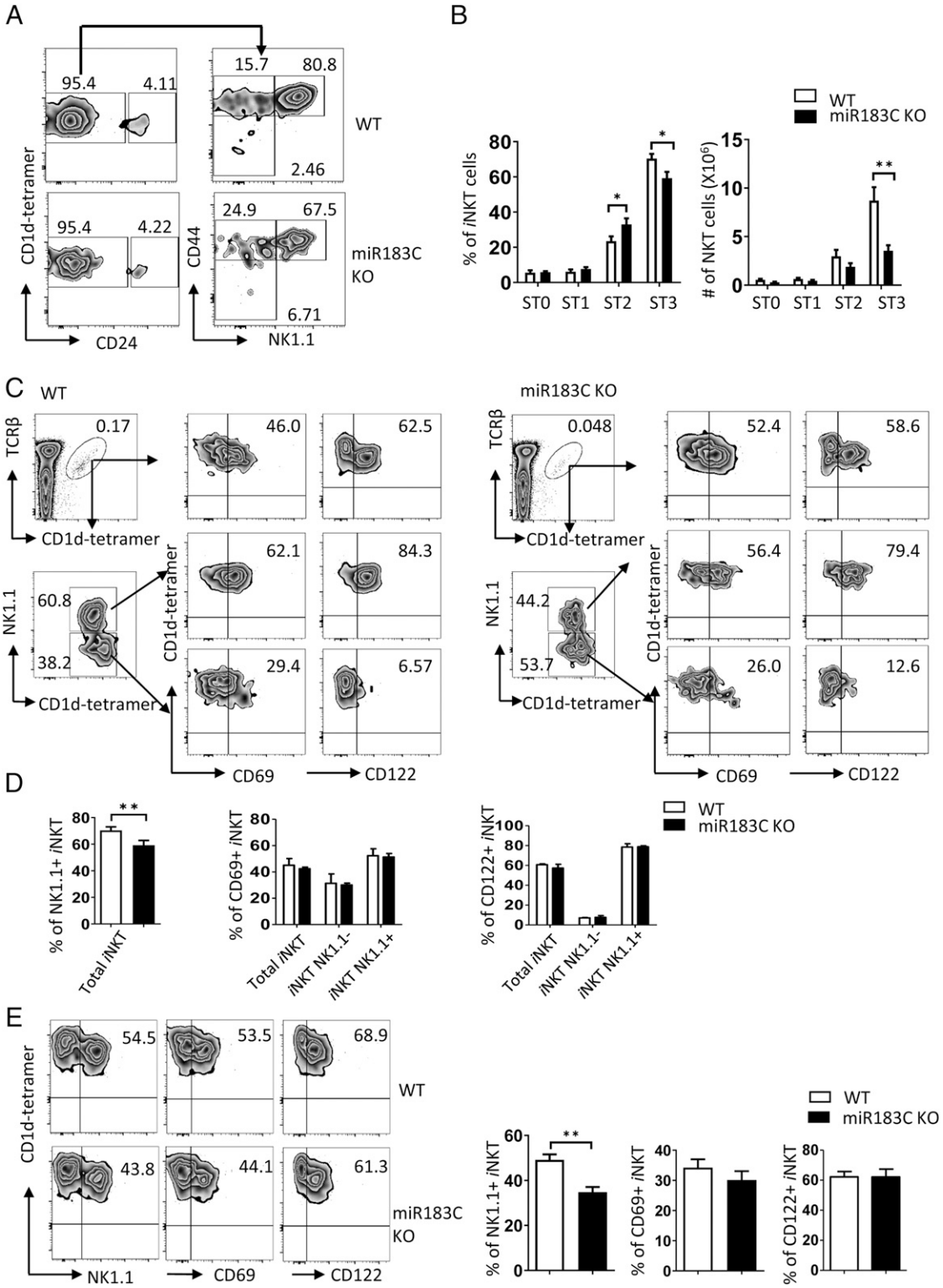

FIGURE 2. Deletion of miR-183C interferes with iNKT cell maturation. (**A**) The developmental stages of thymic iNKT cells was assessed by examining the surface levels of CD24, CD44, and NK1.1 expression on gated iNKT cells. Identified are stage 0 as TCRβ$^+$CD1d tetramer$^+$CD24$^+$, stage 1 as TCRβ$^+$CD1d tetramer$^+$CD24$^-$CD44^{lo}NK1.1$^-$, stage 2 as TCRβ$^+$CD1d tetramer$^+$CD24$^-$CD44^{hi} NK1.1$^-$ and stage 3 as TCRβ$^+$CD1d tetramer$^+$CD24$^-$ CD44$^+$NK1.1$^+$. (**B**) The summary of thymic iNKT cell frequencies (left) and absolute numbers (right) in the stage 0 (ST0), stage 1 (ST1), stage 2 (ST2) and stage 3 (ST3) of miR-183C KO mice. (**C**) The maturation status of thymus iNKT cells was assessed by examining the surface expression of NK1.1, CD69, and CD122 on gated iNKT cells. CD69 and CD122 were further analyzed in the subsets of iNKT cells based on NK1.1 expression. Numbers adjacent to outlined areas indicate the percentage of indicated populations. (**D**) The summary of thymic iNKT cell frequency of NK1.1, CD69, and CD122 expression from miR-183C KO mice. (**E**) The maturation status of spleen iNKT cells was assessed by examining the surface expression of NK1.1, CD69, and CD122 on gated iNKT cells. Numbers adjacent to outlined areas indicate percentage of indicated populations. The summary of splenic iNKT cell frequencies of NK1.1, CD69, and CD122 expression from miR-183C KO mice are shown in right panel. Data are from four independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with WT controls.

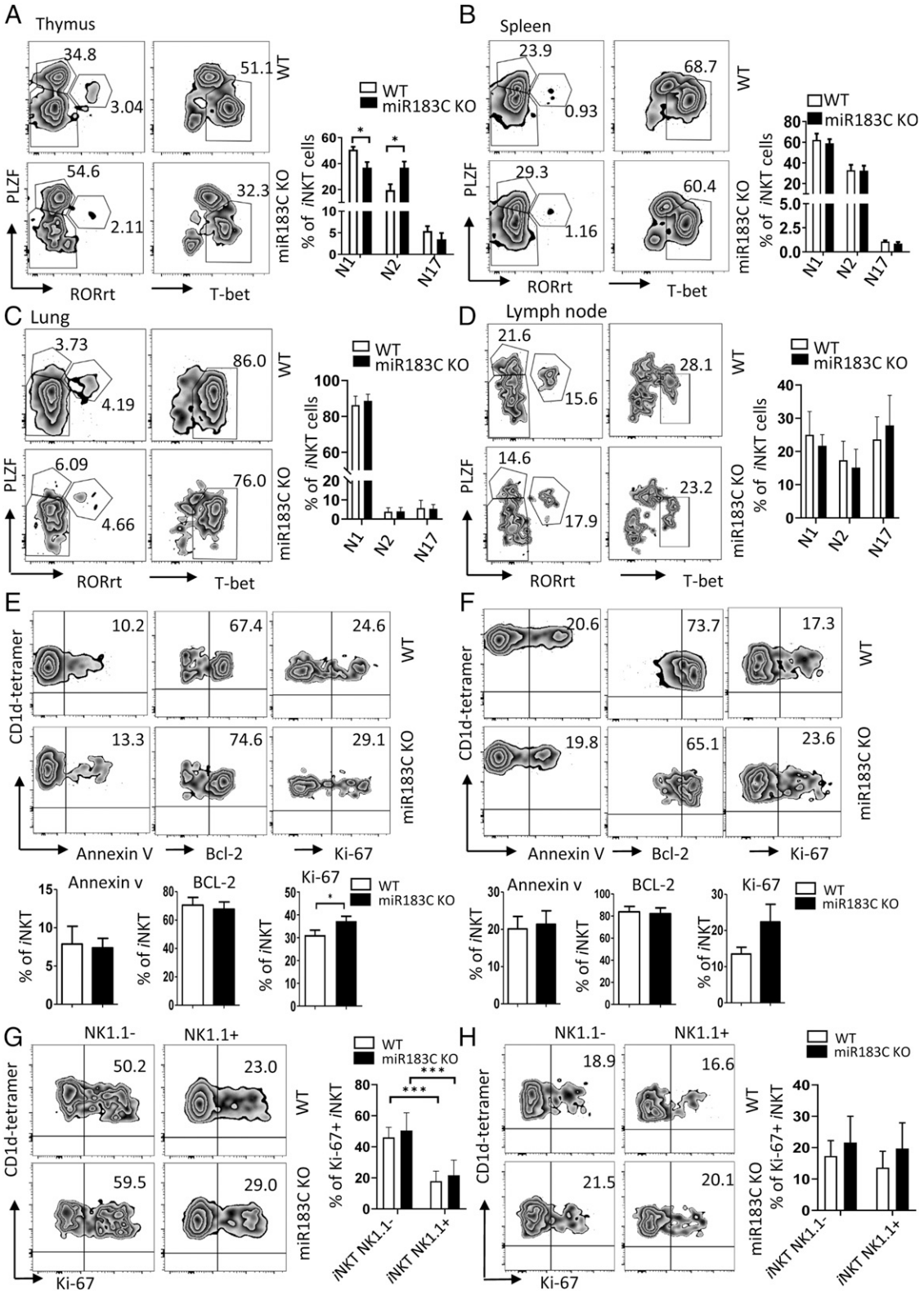

FIGURE 3. The role of miR-183C in iNKT cell lineage differentiation and homeostasis. Expression of transcription factors in thymus (**A**), spleen (**B**), lung (**C**), and LN (**D**) iNKT cells from WT and miR-183C KO mice assessed by intracellular staining for PLZF versus RORγt (left) or PLZF versus T-bet (right). Identified are NKT1 (N1) as PLZF^{lo}T-bet^{+}, NKT2 (N2) as PLZF^{hi}T-bet^{-}RORγt^{-} and NKT17 (N17) as T-bet^{-} PLZF^{int} RORγt^{+}. Numbers adjacent to outlined areas indicate percentage of indicated populations. Left panels show the summary of frequencies of thymus (A), spleen (B), lung (C), and LN (D) iNKT sublineages. (**E** and **F**) Representative flow cytometric plots showing the frequencies of iNKT cells stained for Annexin V, Bcl-2, and Ki-67 in thymus (E) and spleen (F) from WT and miR-183C KO mice. Frequency of Annexin V, Bcl-2, and Ki-67 expression in indicated populations in thymus and spleen iNKT cells was summarized in the lower panel. (**G** and **H**) Representative flow cytometric plots showing the percentages of iNKT cell Ki-67 expression in the subpopulations of iNKT cells (NK1.1^{-} and NK1.1^{+}) in thymus (G) and spleen (H) from WT and miR-183C KO mice. Frequency of Ki-67 expression in indicated subpopulations in thymus and spleen iNKT cells were summarized in the left panel. Data are from four independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with WT controls.

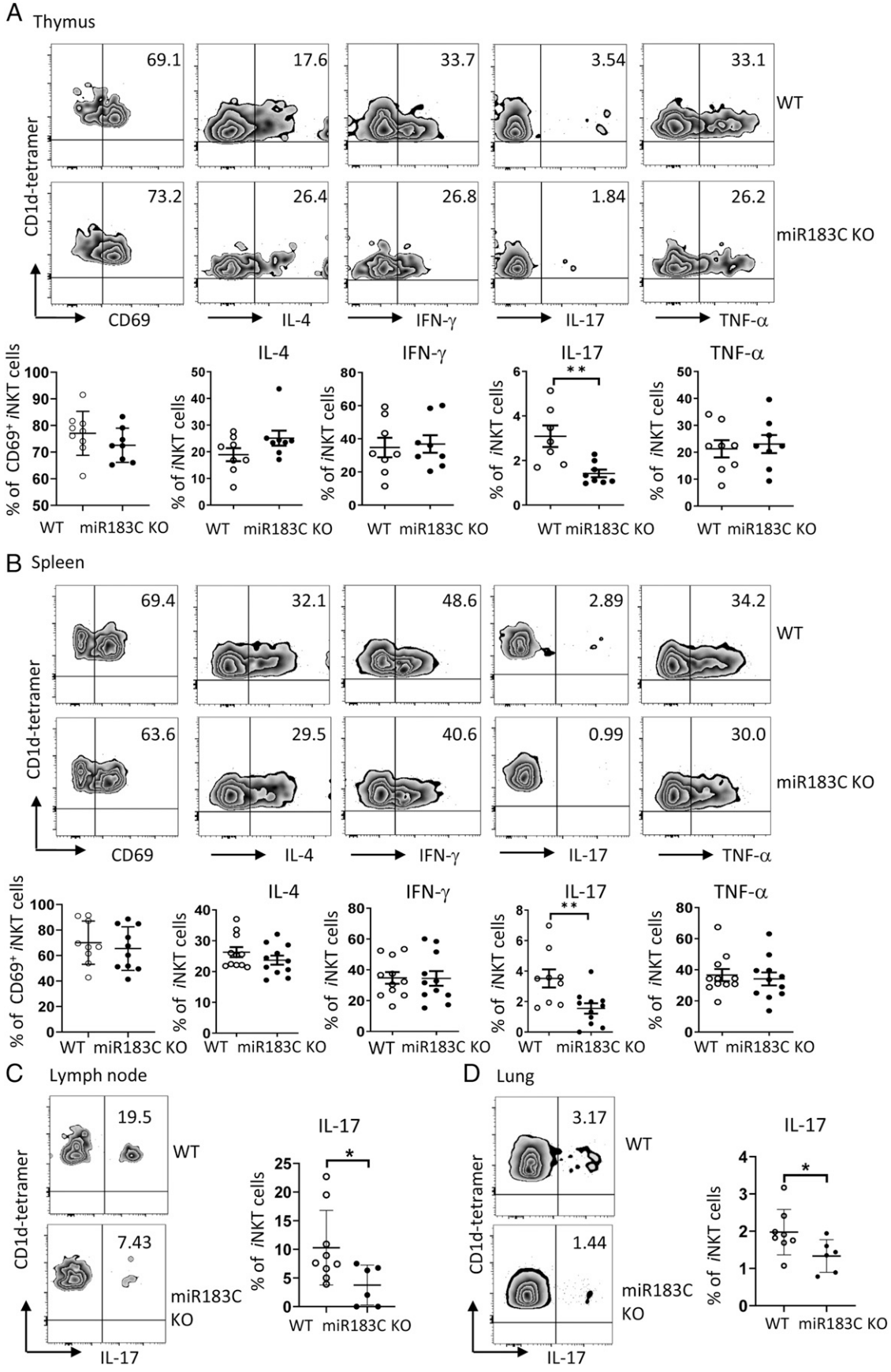

FIGURE 4. The role of miR-183C in iNKT cell cytokine production. (**A** and **B**) Flow cytometric plots showing the expression of CD69 and IL-4, IFN-γ, IL-17, and TNF-α production of iNKT cells from thymus (A) or spleen (B) of miR-183C KO and WT controls after in vitro P/I stimulation. Lower panels show the summary of frequencies of iNKT cells expressing CD69 or producing IL-4, IFN-γ, IL-17, and TNF-α from miR-183C KO versus WT controls. (**C** and **D**) IL-17 production of iNKT cells from LN (C) and lung (D) of miR-183C KO and WT controls after in vitro P/I stimulation. Data are from three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with WT controls.

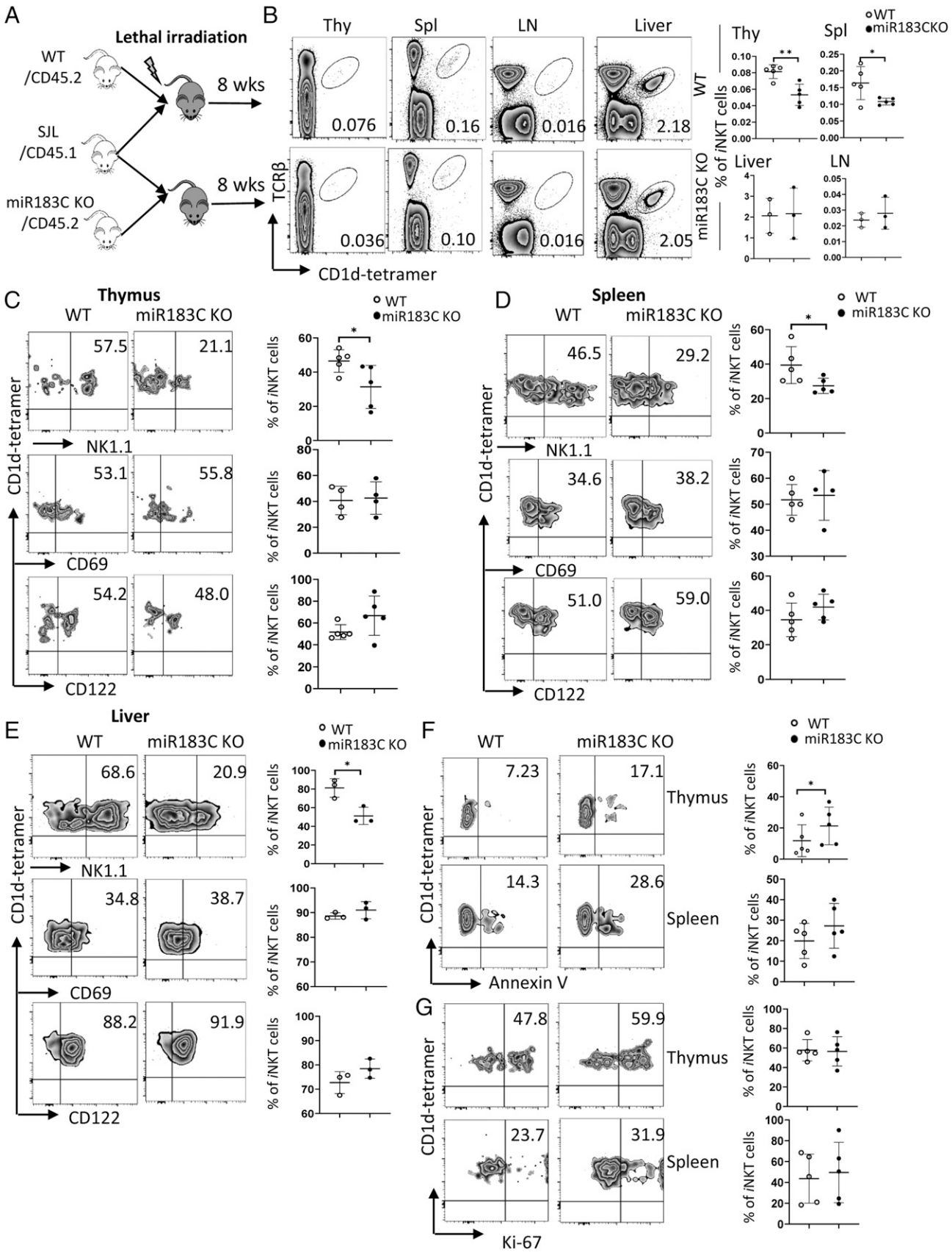

FIGURE 5. miR-183C regulation of iNKT cell development and differentiation is cell autonomous. (**A**) Donor BM harvested from age- and gender-matched SJL (CD45.1$^+$) mice and miR-183C KO (CD45.2$^+$) or WT control (CD45.2$^+$) mice with CD3 deletion were cotransferred at 1:1 ratio to 8-wk-old B6.SJL recipient mice that were lethally irradiated. (**B**) Representative flow cytometric plots showing the percentages of iNKT cells in thymus (Thy), spleen (Spl), LN and liver from CD45.2$^+$ WT and CD45.2$^+$ miR-183C KO BM-derived cells. The frequencies of iNKT cells in CD45.2$^+$ population from indicated organs were shown in right panels. (**C**–**E**) Flow cytometric plots showing the NK1.1, CD69, and CD122 expression in thymic iNKT cells (C), spleen iNKT cells (D), and liver iNKT cells (E) from CD45.2$^+$ WT and CD45.2$^+$ miR-183C KO mice. Bar graph showing *(Figure legend continues)*

miR-183C regulation on iNKT cell development and maturation is cell autonomous

Because the miR-183C deletion model used in our study is conventional instead of tissue specific, it is difficult to decipher whether the defect of iNKT cells identified in the mice is a direct effect of miR-183C on iNKT cells and their precursors or the consequence of changed BM or thymus environments involved in iNKT cell development and differentiation. To answer this question, we performed mixed BM chimera experiments in which BM cells from miR-183C KO or WT littermates ($CD45.2^+$) were mixed with BM cells from SJL mice ($CD45.1^+$) at 1:1 ratio and transferred into lethally irradiated B6.SJL recipient mice (Fig. 5A). Analysis of chimera mice 8-wk after BM transfer revealed that lower frequencies of thymic and splenic iNKT cells were derived from miR-183C KO BM compared with those derived from WT controls, albeit comparable LN and liver iNKT cell frequencies were observed (Fig. 5B). In addition, miR-183C KO BM-derived thymus iNKT cells showed defective maturation, based on NK1.1 expression, but comparable CD69 and CD122 expression (Fig. 5C). Consistent with the thymus iNKT phenotype, spleen and liver iNKT cells derived from miR-183C KO BM showed defective NK1.1 but comparable maturation markers CD69 and CD122 compared with that from WT BM (Fig. 5D, 5E). To further identify the cause of reduced iNKT cell numbers derived from KO BM, we evaluated the Annexin V and Ki-67 expression in thymus and spleen iNKT cells. As shown in Fig. 5F and 5G, both thymus and spleen iNKT cells from KO BM showed comparable proliferation capacity compared with iNKT cells from WT BM, which is consistent with the iNKT phenotype from original miR-183C KO mice. Nevertheless, thymus iNKT cells from KO BM showed clearly elevated Annexin V binding, whereas spleen iNKT cells from the KO BM showed the similar trend of change, albeit not statistically significant. This result does not recapitulate the phenotype observed in the original miR-183C KO mice, indicating that the elevated apoptosis may be one of the major factors causing the defective thymus iNKT cell development. Overall, data from BM chimeras indicated that vast majority of the defective iNKT cell development and maturation observed in miR-183C KO mice are cell intrinsic, whereas cell-extrinsic factors may mask the cell-autonomous defect in homeostasis in the iNKT cells with miR-183C deletion.

miR-183C regulates iNKT cell development, lineage differentiation, and function through targeting multiple signaling molecules

To further investigate the potential molecular mechanisms of miR-183C–mediated iNKT regulation, we sorted thymus iNKT cells from miR-183C KO and WT littermate controls. Because iNKT cells from miR-183C KO showed defective NK1.1, a related lineage development, and a distinct gene expression program was displayed by different developmental stages (16), we sorted thymus iNKT cells of both miR-183C KO and WT controls based on NK1.1 expression to get a more reliable target analysis (Fig. 6A). Total RNAs from sorted $NK1.1^-$ and $NK1.1^+$ iNKT cells from both miR-183C KO and WT littermate controls were purified and quantitative RT-PCR (qRT-PCR) was used to evaluate related potential miR-183C target molecule expression levels. Recent studies indicated that miR-183C could target Foxo1, Foxo3, Egr1, and Egr2, which are potentially related to iNKT development, differentiation, and homeostasis (5, 12, 17). All four genes expressed at a relatively higher level in $NK1.1^+$ iNKT cells compared with their $NK1.1^-$ counterpart from WT mice (Fig. 6A), which is in a reciprocal pattern of the miR-183C expression (Fig. 1A). This result suggests that these molecules may be the targets and under the tonic active regulation of miR-183C during the iNKT cell differentiation. More interestingly, both $NK1.1^+$ and $NK1.1^-$ iNKT cells with miR-183C deletion showed upregulated expression of all four genes, except for Egr1 in $NK1.1^+$, compared with their counterparts from WT controls. This further supports that Foxo1, Foxo3, Egr1, and Egr2 are the potential targets of miR-183C in iNKT cells during their development and differentiation. These results suggest that miR-183C are active regulators in iNKT development, differentiation, and function through targeting multiple molecular pathways.

iNKT effector function requires Foxo1

IL-17–producing NKT17 cells are key players in autoimmune diseases. In this study, we found that deletion of miR-183C resulted in an impairment in NKT17 effector function (Fig. 4A). A previous study demonstrated that the miR-183C regulate Th17 cells by negatively regulating transcriptional factor Foxo1 expression (12). Given the upregulated Foxo1 and defective IL-17 production in iNKT cells with miR-183C deletion, we speculated that Foxo1 deletion would result in elevated NKT17 effector function. Consistent with our expectation, both thymic and splenic iNKT cells from Foxo1-deletion mice produced excessive IL-17 upon P/I stimulation compared with WT controls, although no gross alteration in iNKT cell frequencies in thymus and spleen were identified in these mice (Fig. 6B, 6C). These results further indicate that miR-183C control NKT17 effector function, potentially through targeting Foxo1.

Discussion

miRNAs have been identified as key regulators of immune cell development and function as well as disease pathogenesis. Several individual miRNAs, such as miR-150, miR-155, miR-181 ab, Let-7, and miR-17-92 family clusters have been shown to play a role in iNKT cell development (14, 18–21). Nevertheless, the overall and specific roles of miRNAs in the molecular networks that regulate iNKT cell positive selection, lineage specification, acquisition of functional activity, and homeostasis remain poorly understood. In the current study, our data demonstrated that miR-183C regulates iNKT cell development, homeostasis and effector function through targeting multiple transcription factors, which reflects previous observations that miRNAs target different molecules in different cell contexts.

iNKT cells with miR-183C deletion showed perturbed IL-17 production, although the differentiation of NKT17, based on RORγt expression, remains unchanged compared with WT controls. A recent study by Ichiyama et al. (12) demonstrated that miR-183C enhanced the IL-17 production and pathogenic function in Th17 cells through targeting Foxo1, which inhibited RORγ*t*-induced IL-1R1 expression and subsequent IL-17 production without affecting RORγt expression. In concordance

the summary of frequencies of NK1.1, CD69, and CD122 expression in thymus (C) spleen (D), and liver (E) iNKT cells from $CD45.2^+$ WT and $CD45.2^+$ miR-183C KO BM-derived cells. (**F** and **G**) Flow cytometric plots showing the Annexin V binding (F) and Ki-67 (G) staining in thymus and spleen iNKT cells from $CD45.2^+$ WT and $CD45.2^+$ miR-183C KO BM-derived cells. The summary frequencies of Annexin V and Ki-67 expression in indicated iNKT cells is shown in the right panel. Data are from three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with WT controls.

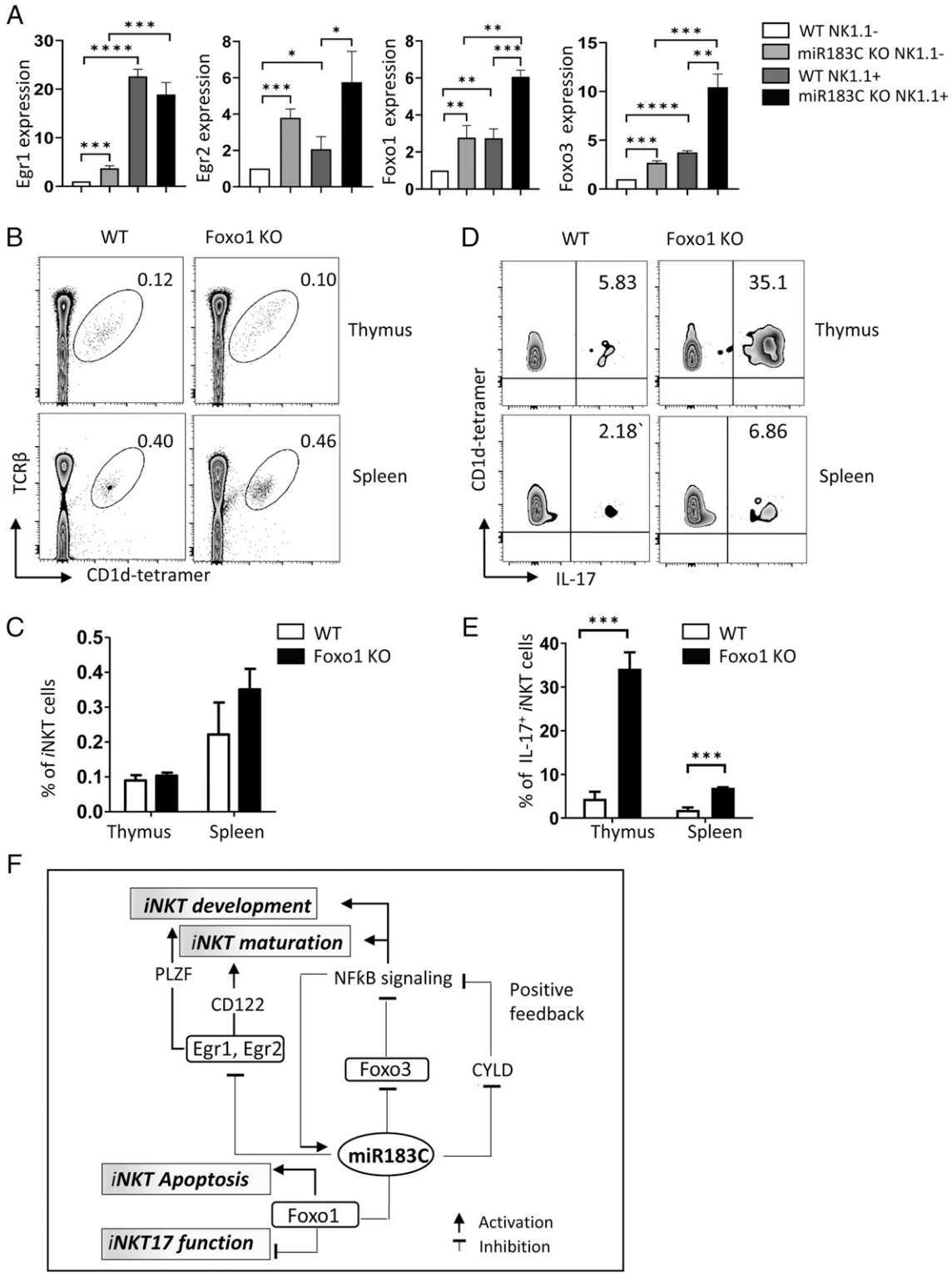

FIGURE 6. miR-183C regulates iNKT cell development, lineage differentiation, and function through targeting multiple signaling molecules. (**A**) qRT-PCR analysis of Egr1, Egr2, Foxo1, and Foxo3 expression in purified thymic NK1.1$^-$ and NK1.1$^+$ iNKT cell populations from miR-183C KO mice and WT controls. Related gene expression are presented as relative to expression level of GAPDH. Data presented are the representative of two independent experiments. (**B**) Representative flow cytometric plots showing the percentages of iNKT cells in the thymus and spleen of Foxo1 KO mice and WT littermate controls. Numbers adjacent to outlined areas indicate percentage of indicated populations. (**C**) Frequencies of iNKT cells in the indicated organs of Foxo1 KO versus WT control mice. (**D**) Flow cytometric plots showing the percentages of thymus and spleen iNKT cells producing IL-17. (**E**) Summary of frequencies of IL-17–producing iNKT cells from thymus or spleen of Foxo1 KO and WT control mice upon in vitro P/I stimulation for 4 h. (**F**) The speculated schematic model of miR-183C regulation of iNKT cell development, maturation, homeostasis, and function through targeting multiple signaling molecules and pathways. Data are from two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with WT controls.

with this study, we observed an upregulation of Foxo1 in iNKT cells with miR-183C deletion. In addition, we found that iNKT cells with Foxo1 deletion had dramatically increased IL-17 production capacity, which further indicates that miR-183C is involved in maintaining NKT17 effector function through targeting Foxo1.

As Foxo3 can bind NF-κB RelA in the cytosol and prevent RelA nuclear translocation (22), upregulation of Foxo3 could result in an interrupted NK-κB signaling pathway. The NF-κB signaling pathway is known to be critical for iNKT cell differentiation. NF-κB–deficient mice, including NF-κB1, c-Rel, and RelA KO mice, showed perturbed iNKT maturation, especially in RelA KO mice (23), which closely resembled the iNKT phenotypes in miR-183C KO mice, particularly with regard to the interrupted stage 3 maturation. As Foxo3 is one of the potential targets of miR-183C in iNKT cells (Fig. 6A), these data strongly suggest that the defect of iNKT cell maturation in miR-183C KO mice are largely if not uniquely associated with the upregulation of Foxo3. In addition, miR-182 directly suppressed cylindromatosis, an NF-κB negative regulator, to promote NF-κB activation in glioma (24). Furthermore, miR-182 is upregulated after RET-induced NF-κB translocation into the nucleus via binding of NF-κB to the miR-182 promoter (25). Therefore, this evidence supports the notion that miR-183C regulates iNKT cell differentiation and maturation through targeting multiple NF-κB signaling pathway–related molecules, which cooperate additively in controlling NK-κB activation, and the reciprocal regulation between miR-182 and NF-κB may form a positive feedback loop in promoting iNKT cell development and differentiation (Fig. 6F).

Egr1 and Egr2 are among the earliest transcription factors induced by TCR signaling and play a critical role in activating the survival program associated with the positive selection of T cells (26). Furthermore, they are also involved in iNKT cell development and differentiation through positive regulation of the iNKT lineage-specific transcription factor PLZF as well as IL-2rβ (CD122) (27). As potential targets of miR-183C, Egr1 and Egr2 were both upregulated in iNKT cells of miR-183C KO mice (Fig. 6A) Given the positive regulatory role of Egr2 on PLZF, the upregulation of PLZF and augmented NKT2 differentiation may be a result of the dysregulated Egr1 and Egr2, which may also explain the dissociation of normal CD122 expression and defective maturation in iNKT cells with miR-183C deletion (Fig. 6F).

Although iNKT cells going through apoptosis are comparable in miR-183C KO mice and WT controls, KO BM-derived iNKT cells in the BM chimeras manifested dramatically elevated apoptosis, indicating the cell-intrinsic effect of miR-183C in the homeostasis of iNKT cells. As a potential target of miR-183C, Foxo1 could be either proapoptotic or antiapoptotic through its antioxidative stress capability (28, 29). The ultimate outcome may be determined by the basal levels of Foxo1 as well as the overall context of transcriptional landscapes of related cells (30, 31). Therefore, miR-183C regulates the homeostasis of iNKT cells, at least partially, through targeting Foxo1.

Both miR-183 and miR-182 were found to be transcriptionally upregulated by TGF-β in separate studies (24, 32), whereas TGF-β orchestrates lineage expansion and maturation of iNKT cells through the concerted action of different pathways of TGF-β signaling (33, 34). Thus, considering the role of miR-183C in iNKT developmental and functional regulation, miR-183C may be involved in TGF-β–mediated iNKT cell regulation.

Overall, our data demonstrate the subtle but indispensable role of miR-183C in iNKT cell development, differentiation, homeostasis, and effector function regulation through potentially targeting multiple molecules important for related signaling pathways (Fig. 6F).

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Disclosures

The authors have no financial conflicts of interest.

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