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mir-181a-1/b-1 Modulates Tolerance through Opposing Activities in Selection and Peripheral T Cell Function

Steven A. Schaffer,§ Christina Loh,§ Song Wang,§ Christopher P. Arnold,§ Robert C. Axtell,§ Evan W. Newell,* Garry Nolan,§† K. Mark Ansel,§‖ Mark M. Davis,§†,§ Lawrence Steinman,§ and Chang-Zheng Chen*§†,**

Understanding the consequences of tuning TCR signaling on selection, peripheral T cell function, and tolerance in the context of native TCR repertoires may provide insight into the physiological control of tolerance. In this study, we show that genetic ablation of a natural tuner of TCR signaling, mir-181a-1/b-1, in double-positive thymocytes dampened TCR and Erk signaling and increased the threshold of positive selection. Whereas mir-181a-1/b-1 deletion in mice resulted in an increase in the intrinsic reactivity of naïve T cells to self-antigens, it did not cause spontaneous autoimmunity. Loss of mir-181a-1/b-1 dampened the induction of experimental autoimmune encephalomyelitis and reduced basal TCR signaling in peripheral T cells and their migration from lymph nodes to pathogenic sites. Taken together, these results demonstrate that tolerance can be modulated by microRNA gene products through the control of opposing activities in T cell selection and peripheral T cell function. The Journal of Immunology, 2015, 195: 000–000.

The vertebrate immune system has the potential to generate complex TCR repertoires capable of recognizing vast numbers of Ags (1). The diverse TCR repertoire inevitably contains those that have high affinity for self-antigens and are capable of attacking self when not eliminated or suppressed. This conundrum is resolved through two well-established tolerance mechanisms: central tolerance, which prevents self-reactive T cells in the periphery. It is now accepted that the mechanism behind central tolerance is positive and negative selection in the thymus (1, 2). This notion is supported by manipulation of TCR signal strength as a key component of the discrimination between positive and negative selection (3). Some T cells bearing TCRs with little to no affinity for self will also be eliminated due to “neglect.” The remaining T cells expressing TCRs with low and intermediate affinity are positively selected to mature and contribute to the peripheral T cell repertoire (1, 4, 5). A low level of self-recognition is necessary for proper T cell activation and homeostasis (1, 2, 6). The strength of the TCR signal at the CD4 and CD8 double-positive (DP) developmental stage of thymocyte development, which is dictated by the affinity between TCR and peptide/MHC complexes, is central to all three T cell fates during selection and peripheral tolerance (3, 7, 8).

Previous studies commonly used mice with a single transgenic TCR that recognizes a defined Ag. For example, male but not female mice expressing a TCR recognizing a Y chromosome-encoded Ag exhibit a dramatic reduction in the number of DP cells in the thymus, demonstrating that developing T cells exposed to their cognate Ags are deleted. Several other studies have reached similar conclusions using other transgenic TCRs (9, 10). These studies with single transgenic TCRs have been instrumental to understanding the selection process; however, they suffer some serious drawbacks. The precocious expression of TCR transgenes before the DP stage and their high expression levels complicate these findings. Moreover, in the monoclonal environment of a single transgenic TCR mouse, thymocytes face competition over limited positively selecting ligands, which may promote additional TCRs locus rearrangement (11). Nevertheless, analyses using transgenic TCRs and their cognate Ags, superantigen, and anti-CD3 administration all implicate TCR signal strength as a key component of the discrimination between positive and negative selection (12). This notion is supported by manipulation of TCR signaling complex components Zap70 (13) and by altering the number of ITAMs on chains of CD3 (14).

However, it remains a challenge to study the selection of diverse TCRs against defined Ags or a broad-spectrum of endogenous Ags. Intriguingly, thymic T cells are known to be much more sensitive than their counterparts in the periphery (15). This heightened sensitivity to Ag in the thymus compared with in the periphery is thought to serve two purposes: first, high sensitivity provides the
necessary positively selecting signals to the developing T cell while ensuring that the same self-ligands do not provide a sufficient signal to activate the postselection T cells in the periphery, and, second, the increase in sensitivity widens the “safety net” of negative selection, preventing the escape of autoreactive T cells (4). Thus, tuning TCR sensitivity to Ags and TCR signal strength during selection may permit the analysis of selection and tolerance in the context of the full spectrum of TCRs and endogenous Ags.

Interestingly, mir-181a-1/b-1 has been identified as a tuner of T cell sensitivity to Ags (16). This gene produces two mature microRNAs (miRNAs), miR-181a and miR-181b. miR-181a is highly expressed in developing T cells and downregulated in peripheral T cells (16). High levels of miR-181a potentiate TCR signaling, whereas low levels make T cells less sensitive to stimulation through their TCR (16). miR-181a targets several negative regulators of TCR signaling, including PTPN22, SHP2, DUSP5, and DUSP6 (16). These genes encode phosphatases that suppress TCR signaling at several points. PTPN22 dephosphorylates Lck (17, 18), SHP2 mediates dephosphorylation of CD3ζ (19), and DUSP5 and DUSP6 dephosphorylate Erk (20). Importantly, miR-181a expression is dynamically regulated and correlates with the change of intrinsic T cell sensitivity in various T cell populations. Its function in modulating TCR signaling and T cell selection in vitro (16, 21) indicates that miR-181a is an intrinsic T cell sensitivity regulator during T cell development and maturation. Thus, the discovery of miR-181a as an intrinsic TCR signaling regulator suggests a method to manipulate TCR signal strength during selection, permitting analysis of selection and central tolerance in the context of the full spectrum of TCRs and endogenous Ags.

In this study, we characterized the effects of mir-181a-1/b-1 deletion on selection, peripheral T cell function, and tolerance in the context of native TCR repertoires and demonstrate that mir-181a-1/b-1 enhances TCR signal strength in vivo. Our study provides insight into how careful tuning of TCR signal strength during development impacts selection, peripheral T cell function, and tolerance. In contrast to detrimental effects of genetic ablation of structural components of TCR signaling (such as Zap70), mice lacking mir-181a-1/b-1 appear normal under unperturbed conditions (22–24). The fact that mir-181a-1/b-1 knockout mice have no major structural defects allowed us to study tolerance in an intact immune system and helped to reveal how quantitative and spatiotemporal aspects of peripheral T cell function may be regulated to counter the detrimental effects of mir-181a-1/b-1 deletion on selection and tolerance.

Materials and Methods

Mouse strains

Wild-type control C57BL/6d mice (stock no. 000664) were obtained from The Jackson Laboratory. mir-181a-1/b-1 knockout mice were generated as previously described (24). All mice were housed at the Stanford Research Animal Facility in accordance with National Institutes of Health and the Stanford University Administrative Panel on Laboratory Animal Care guidelines. For Western blot, quantitative PCR (qPCR), calcium flux, phosphoflow cytometry, and tetramer analyses, we generally used pooled cells from two or three mice for each individual analysis and then repeated the experiments at least two more times with cells from additional cohorts of mice.

Quantitative real-time PCR

Relative and absolute quantitative real-time PCR (qRT-PCR) analyses were carried out to determine the relative changes or absolute copy/cell changes in miRNA expression in various T cell populations as previously described (16). Briefly, for absolute quantification of miRNA expression by qRT-PCR, a known amount of a synthetic standard miRNA (mir-122, which is not present in T cells) was spiked into sorted T cells (preserved in TRIzol reagent, Life Technologies) at a fixed ratio of cells to spiked synthetic mir-122. Total RNA was extracted and used in qRT-PCR analyses following the manufacturer’s protocols. mir-122, mir-181a, and mir-181b probe/primer sets (Life Technologies) were used to measure the relative abundances of mir-181a or mir-181b and then compared with the mir-122 levels in corresponding samples. All measurements were carried out in triplicate and repeated at least three times with independent RNA samples. To determine the absolute miRNA copy numbers, we also determined standard curves by carrying out qRT-PCR analyses on RNA samples with known concentrations of synthetic mir-181a, mir-181b, and mir-122. The relative concentration of miR-181a or miR-181b to 100 ng total RNA was converted to copies/cell using the spiked mir-122 copies/cell as a reference, providing an estimate of the number of mir-181a or mir-181b molecules present in each cell of the measured sample. Certain relative qRT-PCR data are from a larger qRT-PCR array study performed on several thymocyte subsets. To this end, sorted T cells were resuspended in Cells-to-CT buffer (Thermo Fisher Scientific) and analyzed using the TaqMan OpenArray miRNA panel (Life Technologies). Raw cycle threshold (Ct) values were median centered, and relative abundance was calculated using 2^[-ΔCt] where ΔCt is the difference in median-centered Cts between total DP cells and the cell type of interest.

Calcium flux

Cells were collected from various lymphoid organs of wild-type and mir-181a-1/b-1 knockout mice. Wild-type cells were stained with 8 μg/ml Alexa Fluor 488 NHS ester (Life Technologies) for 20 min at room temperature and mixed with 3 μg/ml mir-181a-1/b-1 knockout cells at a 1:1 ratio. We found that tracing dye Alexa Fluor 488 had no effect on calcium flux (data not shown). Mixed cells were stained for surface markers in FACS buffer (PBS plus 2% FBS) for 10 min at room temperature followed by washing with HBSS (with Ca2+ and Mg2+ and without phenol red) with 1% FBS and were then stained with Indo-1 AM (Life Technologies) at a final concentration of 2 μM at 37°C for 30 min. Following washing and resuspending at room temperature for 30 min, cells were analyzed on an LSR II (BD Biosciences) equipped with a 555-nm UV laser. After 30 s of collection, anti-CD3 (clone 145-2C11) was added to a final concentration of 5 μg/ml and acquisition was resumed. After 2 additional minutes, anti-hamster Ig (Jackson ImmunoResearch Laboratories) was added to a final concentration of 15 μg/ml to crosslink TCRs. Acquisition was continued for 5 min total. Calcium flux was analyzed using FlowJo (Tree Star) by comparing the ratio of emission of Indo-1 on the Indo-1 blue (450/50 nm) channel to the Indo-1 green (525/50 nm) channel.

Tetramer enumeration

For naive tetramer enrichment, single-cell suspensions with RBCs lysed (PharmLyse, BD Biosciences) were prepared from the spleens harvested from wild-type and mir-181a-1/b-1 knockout mice and suspended in IVMIM plus 10% FBS. PE-conjugated myelin oligodendrocyte glycoprotein (MOG)35–55 tetramer (National Institutes of Health Tetracer Facility) or PE-conjugated HY-SMCY(KCSRNRQYL):Db tetramer was added to the cell suspension at a 1:100 dilution. Cells were incubated for 3 h at 37°C in 5% CO2 (for MOG tetramer) or 1 h at 4°C (for HY tetramer). HY-tetramer–stained samples were also costained with lymphocytic choriomeningitis virus gp33:D6 tetramer conjugated to PE-Cy7 as a negative control. After washing, cells were resuspended in PBS and 0.5% FBS, and anti-PE microbeads (Miltenyi Biotec) were added to a concentration of 100 million particles/ml. Samples were incubated at 4°C for 30 min and then washed. Cells were applied to prewashed MACS LS columns. Following three washes, the bound fraction was eluted with MACS buffer and resuspended in FACS buffer (PBS plus 2% FBS).

Tetramer-enriched cells (bound fractions) were stained with fluorophore-conjugated Abs to the following markers: CD3ε, B220, CD11b, CD11c, Ter119, NK1.1, F4/80, γ/δ TCR, Gr-1, CD44, CD4, and CD8. Following staining, dead cells were excluded by Live/Dead Aqua staining (Life Technologies). Tetracer+ cells from each bound fraction were counted using FITC CaliBRITE beads (BD Biosciences). The samples were collected on a BD LSR II and analyzed using FlowJo (Tree Star). For postimmunization tetramer enrichment, wild-type and mir-181a-1/b-1 knockout mice were injected with 100 μg MOG35–55 peptide in 100 μl CFA. On day 10, spleens were harvested and treated as above.

Experimental autoimmune encephalomyelitis

Wild-type C57BL/6d and mir-181a-1/b-1 knockout female mice (8–12 wk of age) were injected s.c. with a total of 100 μg MOG35–55 peptide in 100 μl CFA followed by i.p. injection of 300 ng Bordetella pertussis toxin on days 0 and 2 after MOG immunization. Mice were monitored daily, and beginning on day 7 after immunization, mice were evaluated for EAE clinical symptoms and assigned a score from 0 to 5 (0, no symptoms; 1, any...
CD8. HY+CD8SP cells are thymocytes that are positive for the HY TCR positive for CD3 and CD4. CD8 splenocytes are positive for CD3 and are thymocytes that are positive for CD4 and not CD8. CD8SP cells are CCR7+. Effector memory T cells are CD3+ and CD44+ but CCR7

DUSP6 Ab (EPR129Y; ab76310) was obtained from Abcam, and anti–phospho-Thr202/Tyr204 Erk1/2 was obtained from Cell Signaling Technology, anti-DUSP6 Ab (EPR129Y; ab76310) was obtained from Abcam, and anti–phospho-Thr202/Tyr204 Erk1/2.

**Aldrich), layered onto 70% Percoll, and centrifuged at 400 g**

**deletion in thymocytes upregulates DUSP6 and PTEN expression levels (copies/cell) in thymocytes with or without mir-181a knockout mice (CD54.2*)**

**knockout study (23), no evidence of direct regulation of putative binding sites through loss of function analyses. In contrast, we found that putative miR-181a binding sites from the PTEN gene did not mediate repression in a reporter assay and that**

**mixed bone marrow chimeras**

**Lethally irradiated (9.5 Gy) C57BL/6 hosts were transplanted with 10 million bone marrow cells containing equal parts of wild-type (CD54.1*) and mir-181a-1/b-1 knockout (CD54.2*) donor cells. Blood was collected 10–12 wk after bone marrow transplantation to validate reconstitution.**

**Intracellular cytokine staining**

Single-cell suspensions were obtained from the draining lymph nodes, spleens, and spinal cords of mice immunized with MOG peptide and cultured in the presence of 20 nM PMA, 1 μM ionomycin, and 5 μg/ml brefeldin A for 4 h. After stimulation, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% saponin, and stained with anti-CD4, anti–IFN-γ, and anti–IL-17A Abs. In the mixed bone marrow chimera experiments, anti-CD45.2 Ab was also included.

**Mixed bone marrow chimeras**

Lethally irradiated (9.5 Gy) C57BL/6 hosts were transplanted with 10 million bone marrow cells containing equal parts of wild-type (CD54.1*) and mir-181a-1/b-1 knockout (CD54.2*) donor cells. Blood was collected 10–12 wk after bone marrow transplantation to validate reconstitution.

**CNS mononuclear cell extraction**

Spinal cords of mice immunized with MOG35-55 peptide were dissected, cut into pieces (<2 mm), and digested with collagenase D in the presence of DNase I (Sigma-Aldrich) at 37°C. Following collagenase digestion, the tissue pieces were dissociated mechanically to liberate infiltrating cells. Cells were resuspended in 80% Percoll (Sigma-Aldrich), layered onto 70% Percoll, and centrifuged at 400 × g for 25 min. Cells in the interface between the two densities of Percoll were collected for analysis.

**Results**

**mir-181a-1/b-1 deletion in thymocytes upregulates DUSP6 and dampens Erk signaling**

**mir-181a-1/b-1 germline knockout mice (24) were used to characterize the function of mir-181a in TCR signaling and selection.**

**DP thymocytes can be separated based on their sizes and CD69 expression. We found that miR-181a was dynamically regulated during the process of positive and negative selection (Fig. 1A).**

**Preselection thymocytes are small, early activated DP cells are small and express CD69 (DP small), and postselection thymocytes are large and blast-like (DP blast) (25, 26).**

**miR-181a expression correlates with the intrinsic regulation of T cell sensitivity during selection and development as previously noted (16, 21). Moreover, we found that germline deletion of mir-181a-1/b-1 essentially abolished mir-181a and mir-181b expression in DP thymocytes (Fig. 1B), whereas deletion of mir-181a-2/b-2 did not cause a significant reduction in mature mir-181a and mir-181b levels in DP thymocytes (data not shown). Thus, mir-181a-1/b-1, but not mir-181a-2/b-2, contributes nearly all mature mir-181a and mir-181b in DP thymocytes and may be critical for T cell selection.**

**Loss of mir-181a-1/b-1 resulted in an ∼52% increase in DUSP6 protein expression in thymocytes (Fig. 1C): DUSP6 is a validated miR-181a target in mouse (16) and human T cells (27). These results further confirmed that DUSP6 is a physiological target of miR-181a in thymocytes through loss of function analyses. In contrast, we found that putative miR-181a binding sites from the PTEN gene did not mediate repression in a reporter assay and that loss of mir-181a-1/b-1 had no significant effect on PTEN protein expression (Fig. 1C). Although PTEN was identified as an miR-181a target in another mir-181a-1/b-1 knockout study (23), no evidence of direct regulation of putative binding sites through a reporter assay and no quantitative analysis of PTEN Western blot data in the knockout mice were provided in that study.**

**Supporting the function of DUSP6 in controlling Erk signaling, we observed a >50% decrease in basal Erk phosphorylation in mir-181a-1/b-1 knockout DP and CD4SP thymocytes (Fig. 1D), and significant decreases of p-Akt levels in mir-181a-1/b-1**

**FIGURE 1.** miR-181a expression and function in DP thymocytes. (A) Differential regulation of miR-181a expression during positive and negative selection, miR-181a expression in DP subsets, fractionated based on their sizes (small versus blast) and activation status (CD69 expression), was determined by qPCR (n ≥ 4, error bars are SD). (B) miR-181a expression levels (copies/cell) in thymocytes with or without mir-181a-1/b-1 were determined by qPCR (n ≥ 4, error bars are SD). (C) Effects of mir-181a-1/b-1 deletion on the levels of DUSP6 and PTEN in thymocytes as determined by Western blot. (D and E) Effects of mir-181a-1/b-1 deletion on the levels of (D) p-Erk and (E) p-Akt in thymocytes as determined by intracellular flow cytometry analyses (n = 4, *p < 0.05, unpaired t test).
knockout DP and CD4SP thymocytes. Taken together, these findings provide strong genetic evidence that mir-181a-1/b-1 controls DUSP6 expression and Erk and Akt signaling, extending previous studies of miR-181a function in mouse and human T cells using overexpression or anti-sense inhibition (21, 27).

mir-181a-1/b-1 knockout mice exhibit defects in thymocyte TCR signaling

As mir-181a-1/b-1 was shown to regulate Erk and Akt signaling in thymocytes, we carried out calcium flux analysis to investigate its role in controlling TCR signal strength in thymocytes using a FACS-based calcium flux assay. We found that loss of mir-181a-1/b-1 had varied effects on calcium flux in different T cell subsets pre- and poststimulation (Fig. 2). Deletion of mir-181a-1/b-1 resulted in a drastic decrease in the peak calcium flux and total calcium flux over time in DP T cells, but not in other T cell populations, after TCR crosslinking (Fig. 2A–D). Intriguingly, mir-181a-1/b-1-null DP thymocytes had distinct calcium flux kinetics with the rate of calcium flux significantly lower in these cells than in other T cell populations (Fig. 2D), yielding the calcium flux profiles that resembled those elicited by positive-selecting Ags (28, 29). Loss of mir-181a-1/b-1 decreased basal calcium flux in splenic CD4 and CD8 T cells but not in thymocyte populations (Fig. 2E). That loss of mir-181a-1/b-1 causes a strong decrease in TCR signaling strength in DP cells as indicated by the reduction in total calcium flux and its kinetics, which is consistent with the fact that preselection DP thymocytes have high levels of miR-181a (Fig. 1A). Together, these results indicate that mir-181a-1/b-1 deletion may impact T cell selection and T cell reactivity to Ags in peripheral T cells.

Effects of loss of mir-181a-1/b-1 on selection against a defined TCR

The above findings (Figs. 1, 2) suggest that mir-181a-1/b-1 knockout mice may serve as a model to examine the consequences of altering TCR signaling strength on positive and negative selection. These mice are largely normal. They have no major developmental defects or apparent autoimmunity (24), thus permitting us to investigate how changing TCR signaling strength in DP cells affects the selection of the naive TCR repertoire. Moreover, this mouse model can be used to examine how altering TCR signaling strength affects either the selection of T cells with a defined TCR or the selection of T cells with diverse TCRs against a defined Ag. The information obtained from these mice complements knowledge obtained using transgenic TCR models, mutant signaling machinery, and in vitro models of T cell development.

We first examined how miR-181a controls the selection of T cells with a defined TCR, the HY TCR, which recognizes a Y chromosome-encoded HY-SMCY self-antigen (30). In this model, developing T cells are deleted in males but not females at the DP stage. We generated HY TCR transgenic mir-181a-1/b-1 wild-type, heterozygous, and knockout mice and examined their thymi. Loss of mir-181a-1/b-1 did not rescue male DP thymocytes from negative selection, but it did quadruple the percentage of CD8SP cells (positively selected HY TCR transgenic cells) in females (Fig. 3A, 3B). Mature, selected T cells have high TCR expression and are not CD69+. Thymocytes in mir-181a-1/b-1 knockout females had high levels of surface TCR and their CD8SP thymocytes had low levels CD69 expression, indicating that these cells are mature postselection CD8SP thymocytes (Fig. 3C, 3D). These results demonstrate that loss of mir-181a-1/b-1 does not decrease TCR signaling strength enough to rescue male HY TCR+ DP thymocytes from negative selection, moving them into the positive selection window. However, the increase in positively selected CD8SP thymocytes indicates that some fraction of HY TCR T cells are negatively selected in females by endogenous Ags and that mir-181a-1/b-1 deletion rescues some of these cells from negative selection. Thus, loss of mir-181a-1/b-1 may change the negative selection threshold of a defined HY-specific TCR against unknown self-antigens in female mice (Supplemental Fig. 1A).

Loss of mir-181a-1/b-1 does not affect the size of the naive autoreactive T cell pool

We then examined how loss of mir-181a-1/b-1 affected selection of the naive TCR repertoire against defined self-antigens using a tetramer enumeration strategy (31). We chose two Ags for this

![FIGURE 2. Effects of mir-181a-1/b-1 deletion on calcium flux. (A and B) Total calcium flux (integrated area under the curve) for indicated T cell subsets analyzed (A) after and (B) before stimulation. (C) Maximum calcium flux in T cell subsets following stimulation. (D) The rates of calcium flux in DP thymocytes with and without mir-181a-1/b-1. A representative plot is shown. (E) Representative calcium flux profile in indicated T cell subsets. Thymocytes and splenocytes from mir-181a-1/b-1 knockout and wild-type mice were fluorescently labeled and combined, loaded with Indo-1 calcium-sensitive fluorescent dye, and stimulated by adding anti-CD3 followed by crosslinking. Calcium flux as indicated by Indo-1 400 nm/475 nm ratio was assessed by flow cytometry before and after stimulation (n = 5; **p < 0.01, ***p < 0.001, unpaired t test).](http://www.jimmunol.org/)

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analysis: one is the male-specific Ag HY-SMCY, a class I–
restricted antigenic peptide, and the other is MOG35–55, a class
II–restricted antigenic peptide. We pooled cells from the spleens
and lymph nodes of three or more wild-type or
mir-181a-1/b-1
knockout mice, used magnetic beads to enrich tetramer-bound
T cells (31), and determined the number of tetramer-specific T cells
per mouse. The enumeration analyses were independently repeated
on three different cohorts of knockout mice. We found that
mir-181a-1/b-1
deletion did not significantly alter the number or
frequency of naive HY-specific CD8 T cells in male
mir-181a-1/

b-1
knockout mice (Fig. 4A–C) or MOG tetramer–specific CD4
T cells (Fig. 4D, 4E).

Assuming that the frequency of TCR affinities for a specific Ag
follows a Gaussian distribution, shifting the threshold of positive
selection would have distinct outcomes on the size of the naive
T cell repertoire depending on the width of the positive selection
window and its position on the curve (Supplemental Fig. 1B). If the
width of the positive selection window is wide and located on the
lower affinity side of the curve, loss of
mir-181a-1/b-1
would
reduce T cell sensitivity, shifting the window to the right (closer to
the middle of the curve). This would result in a significant increase
in the frequency of naive HY-specific CD8 T cells in male
mir-181a-1/

b-1
knockout mice (Fig. 4A–C) or MOG tetramer–specific CD4
T cells (Fig. 4D, 4E).

Based on the above analysis, we decided to test whether MOG-
specific T cells in
mir-181a-1/b-1
knockout mice are more reactive
toward MOG than those from wild-type mice. We first immunized
wild-type mice with MOG and used tetramer enrichment to enu-
merate MOG-reactive T cells in the spleen over time. MOG-reactive
T cells expanded in the first 7 d after immunization and mildly
contracted afterward (Supplemental Fig. 2). We next examined
mir-181a-1/b-1
knockout mice 10 d after immunization to enu-
merate MOG-reactive CD4 T cells. Following immunization,

mir-181a-1/b-1
knockout mice had about twice as many activated (CD44+)
MOG-reactive CD4 T cells per spleen as did wild-type mice (Fig.
4F, 4G), suggesting that
mir-181a-1/b-1
knockout MOG-reactive
T cells have a higher reactivity toward MOG than those cells in
wild-type mice. Thus, deletion of
mir-181a-1/b-1
did not change the
pool size of MOG-specific naive T cells, but increased their reac-
tivity against MOG peptide, presumably through the selection of
a more reactive naive MOG-specific repertoire.

Loss of
mir-181a-1/b-1
dampens the induction of a
T cell–mediated autoimmune disease

Given the function of
mir-181a-1/b-1
in controlling selection and
reactivity of postselection naive T cells to self-antigen, we tested
how loss of
mir-181a-1/b-1
affects tolerance. Under steady-state

FIGURE 3. Effects of
mir-181a-1/b-1
deletion on selection of HY T cells. (A) Representative FACS plots of CD4 and CD8 expression on thymocytes in
wild-type, heterozygous, and knockout male and female mice. (B) The effects of
mir-181a-1/b-1
deletion on the percentage of total DP, total CD8SP, HY

TCR

+ DP, and HY

TCR

+ CD8SP in total thymocytes (t test, significant p value indicated). (C) HY TCR transgenic thymocytes with wild-type,
heterozygous, null
mir-181a-1/b-1
alleles were analyzed for (C) HY TCR expression and (D) expression of positive selection markers CD5 and CD69 (n ≥ 3). Representative plots are shown.
T cells recognizing HY and MOG self-antigens in naive or immunized mice. (A) Gating scheme used to identify HY and MOG tetramer+ cells. (B-G) The activation status (CD44) and the total number of Ag-specific T cells per spleen in naive or immunized were determined by tetramer staining and FACS analyses. (B) The activation status and (C) the total numbers of HY tetramer+ CD8 T cells in naive mice. (D) The activation status and (E) the total numbers of MOG tetramer+ CD4 T cells in naive mice. (F) The activation status and (G) the total numbers of MOG tetramer+ CD4 T cells at day 10 following MOG immunization. Each point in (C), (E), and (G) represents analysis of pooled cells from three mice (*p < 0.05, unpaired t test).

conditions, mir-181a-1/b-1 germline knockout mice do not develop apparent autoimmune symptoms, indicating that altering selection as a result of mir-181a-1/b-1 deletion is not sufficient to break tolerance. However, it is not known whether these mice might be more susceptible to induction of autoimmune disease than wild-type mice. To address this question, we asked whether mir-181a-1/b-1 knockout mice were more susceptible to experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis that is dependent on MOG-reactive T cells. We immunized groups of wild-type and mir-181a-1/b-1 knockout mice and assessed disease progression in each group over time. Wild-type mice developed disease at day 8 after induction and had an average clinical score of 3 at day 11, whereas knockout mice remained disease-free at this time point (Fig. 5A). mir-181a-1/b-1 knockout mice began to develop disease at day 11, and clinical scores increased over time but remained significantly lower than those of wild-type mice until day 16. Eventually disease scores of both wild-type and knockout mice plateaued with wild-type mice having somewhat higher disease scores. During the period from day 0 to day 16, mir-181a-1/b-1 knockout mice developed disease at a lower rate than did wild-type mice based on analysis of disease incidence (Fig. 5B). Thus, mir-181a-1/b-1 deletion significantly delays disease onset and dampens disease clinical severity. These results demonstrate that loss of mir-181a-1/b-1 inhibits the immune response against self-antigens under a perturbed condition. This is despite the fact that its effects on selection and reactivity of naive T cells for self-antigen (Fig. 4) would suggest the opposite.

Loss of mir-181a-1/b-1 inhibits brain infiltration of encephalitogenic T cells

There are several potential ways in which loss of mir-181a-1/b-1 might alter effector T cell function. Loss of mir-181a-1/b-1 could 1) render effector T cells nonfunctional, 2) dampen effector T cell sensitivity to Ag, 3) alter effector T cell differentiation, or 4) alter migration to the site of disease. The fact that mir-181a-1/b-1 knockout mice eventually developed EAE suggests that mir-181a-1/b-1-null T cells are functional in EAE pathogenesis (Fig. 5A, 5B). We measured the basal phosphorylation state of Erk in peripheral CD4 and CD8 T cells lacking mir-181a-1/b-1 in naive mice and found that loss of mir-181a-1/b-1 did not alter the basal levels of phosphorylation of Erk in naive, central memory, or effector memory T cells (Supplemental Fig. 3A–C). This result indicates that mir-181a-1/b-1-null peripheral T cells have no major Erk signaling defects. Similarly, calcium flux analysis (Fig. 2) indicated that although deletion of mir-181a-1/b-1 resulted in a significant decrease of total integrated calcium flux in the prestimulation T cells, there was no difference following stimulation (Fig. 2A, 2B). These observations suggest that loss of mir-181a-1/b-1 increases the threshold for activation in peripheral T effector cells; however, it is possible that such an effect may be compensated by the selection of TCRs with higher reactivity against self-antigens (Fig. 4), which should elicit stronger TCR signaling. Nonetheless, these effects on TCR signaling in peripheral T cells are not sufficient to prevent them from being activated or from becoming pathogenic. Thus, by method of elimination, it is likely that loss of mir-181a-1/b-1 in peripheral T cells counters detrimental effects on selection by affecting T cell differentiation or migration to the site of disease.

Both migration to the site of disease and effector T cell differentiation into encephalitogenic T cells are critical for autoimmune disease pathogenesis (32, 33). To monitor infiltration of inflammatory cells into the CNS, we collected cells from the CNS and spleens of wild-type and mir-181a-1/b-1 knockout mice after EAE induction. H&E stains of spinal cord sections revealed that overall mononuclear cell infiltration in the knockout mice was indistinguishable from that in wild-type mice (Fig. 5C), suggesting that there is no gross defect in migration of inflammatory cells into the CNS in mir-181a-1/b-1 knockout mice. Further analysis of MOG-specific CD4 T cells in the CNS revealed that there was a slight reduction of activated MOG-specific T cells in the CNS of knockout mice compared with wild-type mice after EAE induction (Fig. 5D). Because there were significantly more MOG-reactive CD4 T cells in the periphery of mir-181a-1/b-1 knockout mice (Fig. 4), loss of mir-181a-1/b-1 clearly compromises the migration of activated Ag-specific T cells into the CNS. Next, we asked whether loss of mir-181a-1/b-1 altered effector helper T cell differentiation into Th1 or Th17 subtypes, which are important for EAE pathogenesis. mir-181a-1/b-1 deletion caused an increase in Th1 differentiation in vitro under both nonpolarizing (Supplemental Fig. 3D) and Th1-polarizing conditions (Supplemental Fig. 3E) and in the spleens of EAE mice (Fig. 5E). Similarly, mir-181a-1/b-1 deletion also caused a slight increase in Th17 differentiation in the spleens of EAE mice (Fig. 5E). Whereas mir-181a-1/b-1 deletion caused a strong increase in the percentage of IFNγ+ CD4 T cells in the CNS during EAE onset, it resulted in a significant reduction in the percentage of IL-17A+ CD4 T cells (Fig. 5F). These results
were transplanted with 10 million bone marrow cells containing induced EAE (Fig. 6A). Lethally irradiated (9.5 Gy) C57BL/6 hosts contributed to the hematopoietic compartment and then bone marrow chimeras with equal wild-type and knockout contributions in the spleens at these time points. These results indicate that loss of IL-17A–producing cells in the CNS was 20–40% of total IL-17A+ and IFN-γ+ T cells in the hematopoietic compartment in the CNS. Interestingly, the 3′ untranslated region (UTR) of the mRNA encoding S1PR1 contains two predicted miR-181a binding sites (35), suggesting that these results demonstrate that there is an overall defect in migration of encephalitogenic Th17 cells to the CNS in mir-181a-1/b-1-null mice.

Because miR-181a is expressed in the CNS, we investigated whether the decrease in IL-17A–producing cells in the CNS was due to the loss of mir-181a-1/b-1 in the hematopoietic compartment or due to its loss in cells of the CNS. To this end, we created bone marrow chimeras with equal wild-type and mir-181a-1/b-1 knockout contributions to the hematopoietic compartment and then induced EAE (Fig. 6A). Lethally irradiated (9.5 Gy) C57BL/6 hosts were transplanted with 10 million bone marrow cells containing equal parts of wild-type (CD45.1+) and mir-181a-1/b-1 knockout (CD45.2+) donor cells. In the spleens of these chimeras, mir-181a-1/b-1 knockout-derived Th17 and Th1 cells accounted for 20–30 and 40–50% of total IL-17A+ and IFN-γ+ CD4 T cells, respectively, at days 10 and 14 after EAE induction (Fig. 6B, 6C). This demonstrates that mir-181a-1/b-1 deletion caused a competitive disadvantage in Th17 differentiation, but not Th1 differentiation. In contrast, in the CNS of these chimeras, mir-181a-1/b-1 knockout-derived Th17 and Th1 cells accounted for 15–20 and 20–40% of total IL-17A+ and IFN-γ+ CD4 T cells, respectively, at days 10 and 14 after EAE induction (Fig. 6B, 6C). Importantly, the percentages of mir-181a-1/b-1 knockout-derived Th17 and Th1 cells in the CNS were significantly lower than percentages in the spleens at these time points. These results indicate that loss of mir-181a-1/b-1 in a competitive environment results in a hematopoietic-intrinsic defect in Th17 and Th1 effector T cell migration into the CNS and a competitive disadvantage in Th17 differentiation. These outcomes likely contribute to the delayed and dampened EAE phenotype observed in the mir-181a-1/b-1 knockout mice.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** mir-181a-1/b-1 knockout mice exhibit a delay in EAE induction. (A) Disease progression of mir-181a-1/b-1 knockout versus wild-type mice. A representative plot of three independent experiments is shown. Error bars are SEM; *p < 0.05, unpaired t test; n = 10/group. (B) Percentage of disease-free mice (score of 0) in wild-type and mir-181a-1/b-1 knockout mice at various time points after EAE induction (p = 0.0066, Gehan–Breslow–Wilcoxon test; n = 10/group). (C) Histological analyses of CNS inflammation. Brains and spinal cords were collected from mice 11 d after EAE induction. Spinal cord sections were stained with H&E (original magnification ×10). Representative sections from two or three mice are shown. (D–F) Brain and spinal cord–infiltrated immune cells were isolated from mice immunized with MOG35–55 peptide at day 10 postimmunization. (D) Effects of loss of mir-181a-1/b-1 on MOG tetramer+ CD4 T cells and control CLIP tetramer+ CD4 T cells in the CNS of EAE mice. (E and F) Effects of mir-181a-1/b-1 deletion on Th1 and Th17 responses in spleen (E) and CNS (F) of EAE mice. Representative plots from three independent experiments are shown.

Based on these observations, we further tested the effects of loss of mir-181a-1/b-1 on T cell migration in vitro and in vivo. The chemotaxis agent sphingosine-1-phosphate (S1P) is critical in the pathologies of multiple sclerosis and EAE (33, 34). We first measured the chemotactic response to S1P in wild-type and mir-181a-1/b-1–null T cells by using a transwell migration assay. CD4 and CD8 T cells lacking mir-181a-1/b-1 migrated less efficiently than did their wild-type counterparts in response to all concentrations of S1P measured (Fig. 7A, 7B). Interestingly, there was also a defect in migration in the absence of S1P, suggesting that there is an intrinsic mobility defect in mir-181a-1/b-1–null T cells. We next examined how mir-181a-1/b-1 deletion affected cell migration in vivo. We carried out a competitive analysis by transferring labeled wild-type and mir-181a-1/b-1–null cells into recipient mice and analyzing spleens and lymph nodes after 12 h (Fig. 7C). We found significantly fewer mir-181a-1/b-1 knockout CD4 T cells than wild-type cells in recipient lymph nodes but not in spleens (Fig. 7E), indicating that mir-181a-1/b-1 knockout T cells are defective in trafficking in vivo. A similar trend was observed with mir-181a-1/b-1 knockout CD4 T cells, albeit the difference was statistically insignificant (Fig. 7D). Taken together, these results demonstrate that mir-181a-1/b-1–null T cells have defects in migration in vitro and in vivo. Interestingly, the 3′ untranslated region (UTR) of the mRNA encoding S1P1 contains two predicted miR-181a binding sites (35), suggesting that the levels of S1P1 may be directly regulated by mir-181a-1/b-1 (Fig. 7F). Indeed, we showed that miR-181a expression repressed a luciferase reporter bearing the S1P1 3′ UTR containing these two target sites by ∼40% (Fig. 7G), and mir-181a-1/b-1 deletion...
FIGURE 6. Effects of loss of mir-181a-1/b-1 on effector T cell responses during EAE onset dissected. (A) EAE was induced in bone marrow chimeras generated by transferring equal numbers of mir-181a-1/b-1 knockout (CD45.2) and wild-type (CD45.1) cells into lethally irradiated hosts. At indicated time points, cells were obtained from spleens, brains, and spinal cords, stimulated with PMA and ionomycin, and then stained for IFN-γ and IL-17A to determine effector cell phenotype in a competitive environment. (B) and (C) Bar graphs depicting percent mir-181a-1/b-1 knockout cells (CD45.2+) out of (B) total IL-17A+ or (C) total IFN-γ+ CD4 T cells in spleen and CNS at days 10 and 14 after EAE induction (**p < 0.05, t test; representative results of two independent repeats). Representative histograms of Th1 and Th17 cells in the spleen and CNS of chimeras are shown on right.

casted an increase in S1PR1 levels in naive CD4 T cells (Fig. 7H). Given the role of miR-181a in controlling basal TCR signaling strength (16) and the link between ligand affinity and TCR signaling strength (36), these findings suggest that miR-181a may also influence T cell migration by affecting TCR signaling.

Pharmacological inhibition of miR-181a dampens EAE induction

Despite the opposing effects on tolerance as the result of loss of mir-181a-1/b-1 on selection and effector T cell function, deletion of mir-181a-1/b-1 delayed and dampened EAE pathogenesis. We therefore asked whether pharmacological inhibition of miR-181a using an antagonir (21, 37) might have a similar inhibitory effect on EAE. We injected mice with miR-181a antagonir at days 1 and 3 after MOG immunization. Early injection of the miR-181a antagonir both delayed EAE disease onset and depressed the overall disease pathology when compared with mice injected with saline control (Fig. 8). This phenotype resembled the effect of germline deletion of mir-181a-1/b-1 on EAE onset (Fig. 5). Early antagonir treatment likely affects both T cell selection and peripheral T cell function. Thymocytes spend ∼7 d in the thymus from the DP stage onward (8, 38, 39). Thus, the inhibitory effects of antagonir treatment on EAE may be primarily due to its effects on effector T cell function. These results suggest that pharmacological inhibition of miR-181a activity in T cells before migration delays and dampens EAE progression, supporting the notion that miR-181a plays a role in T cell migration during EAE onset.

Discussion

In this study, we investigated how dampening of TCR signaling can affect selection and peripheral T cell function and thereby influence tolerance through characterizing mice that lack a gene encoding a natural tuner of TCR signaling strength, mir-181a-1/b-1. By comparing T cell responses in wild-type to mir-181a-1/b-1 knockout mice, we showed that loss of mir-181a-1/b-1 dampens TCR signal strength in DP thymocytes and affects the selection of T cells with transgenic TCRs and the selection of T cells with endogenous TCRs against a self-antigen. Importantly, loss of mir-181a-1/b-1 resulted in an increase in the reactivity of peripheral T cells against a self-antigen during immunization, indicating that selection in the absence of mir-181a-1/b-1 yields T cells bearing TCRs with high reactivity against self. Paradoxically, loss of mir-181a-1/b-1 did not cause spontaneous autoimmunity, and it attenuated the development of EAE in part through inhibition of T cell migration into the pathological sites. Deletion of mir-181a-1/b-1 in peripheral T cells decreased basal TCR signaling and increased the threshold of activation, which might also render peripheral T cells more difficult to activate. Although loss of mir-181a-1/b-1 has detrimental effects on tolerance by increasing the selection of more autoreactive T cells, it also exerts opposing effects on the strength of TCR signaling and migration of peripheral T cells that dampen self-reactivity.

Notably, in this study we characterized the effects of TCR signaling strength on the selection of T cells bearing natural and diverse TCRs against endogenous Ags through genetic deletion of mir-181a-1/b-1, an intrinsic tuner of T cell sensitivity, in DP thymocytes. Such analyses avoided the limitations of using transgenic TCRs and specific Ags in characterizing the selection processes and provided unique insights into the principles underlying selection and tolerance against natural repertoires of self-antigens. We found that mir-181a-1/b-1 regulates TCR signaling in DP thymocytes (Fig. 2) and that its loss can affect the selection of T cells bearing the HY TCR (Fig. 3). Intriguingly, deletion of mir-181a-1/b-1 did not rescue HY thymocytes from negative selection in male mice but potentiated the positive selection of T cells bearing HY TCR in female mice (Fig. 3). Thus, mir-181a-1/b-1 deletion does not cause a sufficient reduction in TCR signal strength to enable the positive selection of DP cells bearing the HY TCR in male mice but does cause a sufficient reduction in TCR signal strength to enhance the positive selection of DP cells bearing the HY TCR in female mice against self-antigens. Recent evidence suggests up to 6-fold as many T cells are negatively selected than positively selected (38), lending support to the hypothesis that mir-181a-1/b-1 deletion permits the positive selection of self-lingids in female HY mice.

Moreover, our analysis of the effects of mir-181a-1/b-1 deletion on TCR selection provided insights into the affinity distribution of the endogenous TCR repertoire against specific self-lingids. Loss of mir-181a-1/b-1 did not increase the frequency of autoreactive T cells (Fig. 4), but it did appear to increase the reactivity of these cells against self-lingids (Fig. 4F, 4G). This outcome may be explained if...
the positive selection window is narrow for MOG-specific DP cells—shifting this window as the result of mir-181a-1/b-1 deletion might not measurably change the number of positively selected T cells but may yield postselection T cells with enhanced reactivity. However, it is also possible that tetramer enumeration assay may not have the sensitivity to quantify modest changes in the size and affinity of naive repertoire of MOG-specific T cells (40).

Use of the mir-181a-1/b-1 knockout mice allowed us to study the development, maintenance, and regulation of tolerance in a system where the structural components of the immune system were intact (41). Importantly, note that loss of mir-181a-1/b-1 results in quantitative changes in selection, TCR signaling and reactivity, and quantitative changes in selection, TCR signaling and reactivity, and spontaneous autoimmunity in unperturbed conditions (22–24), but differences between the knockout and wild-type mice did become apparent upon perturbation. Quantitative changes were also noted in the B cell compartment and more dramatically in the NK T cell compartment (Supplemental Fig. 4). Loss of mir-181a-1/b-1, a natural tuner of T cell signaling strength during selection, affected T cell selection, but these effects were counteracted by concomitant effects on the strength of TCR signaling in peripheral T cells and their migration to the pathological sites. These findings illustrate the importance of dosage and spatiotemporal function in tolerance and effector T cell function.

Intuitively, one would assume that the products of mir-181a-1/b-1 serve as buffers to increase the safety net of negative selection. However, mir-181a-1/b-1 germline knockout mice do not develop spontaneous autoimmunity. Clearly, despite that high mir-181a expression in DP thymocytes indeed mediates the control of the threshold for negative selection, the effects of mir-181a-1/b-1 deletion seem to be insufficient to disrupt tolerance. Moreover, the generation and suppressive capacity of thymic-derived and induced regulatory T cells in knockout mice were not different from those in wild-type mice (data not shown). Thus, deletion of mir-181a-1/b-1 did not cause the upregulation of regulatory T cells to prevent activation of the more reactive mir-181a-1/b-1–null T cells. Given that peripheral T cells in mice lacking mir-181a-1/b-1 did have a lower basal level of calcium flux (Fig. 2) and migration defects (Figs. 5–7), these findings suggest that mir-181a-1/b-1 deletion compromises spatiotemporal responses of peripheral T cells and contributes to the dampened and delayed EAE (Fig. 5).

Our study also offers a resolution to apparently conflicting studies published using other strains of mice lacking mir-181a-1/b-1 (22, 23). All three knockout strains have a severe reduction in invariant NKT cells (Supplemental Fig. 4). In agreement with Ziętara et al.
(22), we showed that loss of mir-181a-1/b-1 dampens TCR signaling in DP thymocytes as measured by calcium flux and basal phospho-Erk levels. We found no evidence that PTEN is regulated in DP thymocytes as measured by calcium flux and basal signaling activities in selection and peripheral T cell function. The authors have no financial conflicts of interest.

The primary mir-181a-1/b-1 transcript or may have unintended effects on neighboring gene expression (for instance, PTPRC, the gene encoding CD45, is within 50 kb of mir-181a-1/b-1).

It will be of interest to transiently inhibit mir-181a-1/b-1 expression during the stage of preselection DP thymocytes and restore its expression in the periphery. A set of naive T cells with higher affinity for self will be selected in the absence of mir-181a-1/b-1 but, unlike in the germine knockout mice, these cells will be functionally competent in the periphery with mir-181a-1/b-1 expression and regulation unchanged. Such analyses will help to interrogate the function of mir-181a-1/b-1 in selection and central tolerance without complications of loss of mir-181a-1/b-1 function in peripheral T cells. Despite this caveat, our findings indicate that mir-181a-1/b-1 can modulate tolerance through controlling opposing activities in selection and peripheral T cell function.

Disclosures

The authors have no financial conflicts of interest.

References


