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Lipid phosphatases identified by screening a mouse phosphatase shRNA library regulate T-cell differentiation and Protein kinase B AKT signaling

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Screening a complete mouse phosphatase lentiviral shRNA library using high-throughput sequencing revealed several phosphatases that regulate CD4 T-cell differentiation. We concentrated on two lipid phosphatases, the myotubularin-related protein (MTMR)9 and -7. Silencing MTMR9 by shRNA or siRNA resulted in enhanced T-helper (Th)1 differentiation and increased Th1 protein kinase B (PKB)/AKT phosphorylation while silencing MTMR7 caused increased Th2 and Th17 differentiation and increased AKT phosphorylation in these cells. Irradiated mice reconstituted with MTMR9 shRNA-transduced bone marrow cells had an elevated proportion of T-box transcription factor T-bet expressors among their CD4 T cells. After adoptive transfer of naïve cells from such reconstituted mice, immunization resulted in a greater proportion of T-box transcription factor T-bet–expressing cells. Thus, myotubularin-related proteins have a role in controlling in vitro and in vivo Th-cell differentiation, possibly through regulation of phosphatidylinositol [3,4,5]-trisphosphate activity.

CD4 T-cell activation and differentiation requires an intricate signaling network in which activity of both phosphatases and kinases ensures a delicate balance. As examples of tyrosine, lipid, and serine/threonine phosphatases critical for these processes, we cite CD45, protein tyrosine phosphatase receptor type C, which functions as an essential regulator of T- and B-cell antigen receptor signaling (1); phosphatase and tensin homolog (PTEN), which opposes the action of PI3-kinase (PI3K), regulates the cell cycle, and acts as a tumor suppressor (2); and calcineurin, a calcium-dependent serine/threonine protein phosphatase, which regulates nuclear factor of activated T cells (NFAT) translocation and cytokine production (3).

By contrast to the intensive focus on kinases in lymphocyte responses, phosphatases are less well understood. Here, we report screening of a mouse phosphatase lentiviral shRNA library in mouse T-helper (Th)1 CD4 T lymphocytes, aiming to identify phosphatases involved in the regulation of Th-cell differentiation and/or plasticity. We identified and studied in detail two lipid phosphatases, myotubularin-related protein (MTMR) 9 and -7.

Lipid phosphatases regulate the metabolism of phosphoinositides (PIs). PIs are ubiquitous in cellular membranes, interact with the lipid-binding domains of a wide variety of signaling proteins, and function as important second messengers in the spatial and temporal organization of signaling pathways and vesicle trafficking (4, 5). Although the roles and regulation of many cellular lipids are not well understood, the picture for PI[3,4,5]-trisphosphate (PI[3,4,5]P_3) has been relatively well established (5, 6). The amount of PI[3,4,5]P_3 in resting T lymphocytes is low. Upon stimulation, PI3K adds a phosphate at position 3 of the inositol ring of PI[4,5]P_2, converting it into PI[3,4,5]P_3, which, in turn, activates protein kinase B (PKB) (also known as AKT), a key downstream effector kinase (7–9). CD4 T cells lacking p110δ, the main PI3K catalytic subunit isoform used in T-cell receptor (TCR) and cytokine signaling, show attenuated TCR-stimulated AKT phosphorylation, decreased proliferation, and impaired Th1 and Th2 cytokine production (10, 11). Conversely, constitutive expression of active AKT leads to increased proliferation and enhanced Th1/Th2 cytokine production (12).

The amount of PI[3,4,5]P_3 and the level of AKT activation are tightly controlled by several mechanisms, including breakdown of PI[3,4,5]P_3, down-regulation of the amount and activity of PI3K, and the dephosphorylation of AKT (13). PTEN is a major negative regulator of PI[3,4,5]P_3. It removes the 3-phosphate from the inositol ring of PI[3,4,5]P_3 to generate PI[4,5]P_2 and serves as a sentinel phosphatase controlling the basal level of PI[3,4,5]P_3 under physiological conditions, attenuating PI3K-dependent signaling including AKT activation (14, 15).

MTMRs are 3-phosphatases that hydrolyze PI[3]P and PI[3,5]P_2, generating PI and PI[5]P, respectively. They are a highly conserved family consisting of 15 members, namely myotubularin 1 and MTMR1 through -14, each possessing nonoverlapping functions (16–18). PI[3]P is the only 3-phosphoinositide that exists at a significant amount in resting mammalian cells; it has been reported to mediate endocytosis, autophagy, and many other cellular processes (5, 19).

As noted above, we screened a mouse phosphatase shRNA library on differentiated Th1 cells, identifying several phosphatases involved in regulating T differentiation/plasticity. Among these are two MTMR family members, MTMR9 and MTMR7. Silencing *Mtmr9* led to stronger Th1 differentiation, whereas knocking down *Mtmr7* expression increased Th2 and Th17 differentiation; both were associated with enhanced AKT phosphorylation in particular cell types. Irradiated mice reconstituted from *Mmtr9* shRNA-transduced bone marrow progenitors showed a higher percentage

Significance

We identified a series of lipid phosphatases that regulate differentiation of naïve CD4 T cells to their major phenotypic states, T-helper (Th)1/Th2/Th17. Previously, phosphatase and tensin homolog (PTEN) was the only lipid phosphatase shown to function in this process. To identify these phosphatases, we used a unique "phosphatase-wide" screening procedure. Our results demonstrate that myotubularin-related protein (MTMR) 9 and MTMR7 play critical roles in Th differentiation. Knocking down MTMR9 enhances Th1 priming, and such Th1 cells show increased protein kinase B (PKB)/AKT phosphorylation. Knocking down MTMR7 increases Th2 and Th17 priming; these cells show enhanced AKT phosphorylation.

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of memory phenotype cells expressing T-box transcription factor T-bet and naïve cells from these mice had an increased propensity to express T-bet upon immunization. Thus, these lipid phosphatases participate in regulating T-cell differentiation, possibly by functioning as fine tuners of PI3K/AKT activity.

Results

Screening Design. To study the role of phosphatases in CD4 T-cell differentiation/plasticity, we used a pooled 1,043-member shRNA library of mouse phosphatases that we introduced in naïve CD4 T cells from T-bet green reporter (TBGR) mouse in which Zoanthus green fluorescent protein ZS-green (ZSG) reports T-bet expression (20). These cells were then primed to become Th1 cells; those that had integrated the shRNA were drug-selected and then "switched" to Th2 conditions. Cells that showed greater or lesser degrees of switching, as measured by loss or retention of ZSG, were purified, and the representation of the shRNAs in both sets of cells determined by high-throughput (HT) sequencing.

The generation of the pooled library is described in *SI Materials and Methods*. To achieve relatively unbiased identification of individual shRNAs in the switched and unswitched cells, we carried out HT sequencing of the 21-bp unique sense strand of each shRNA. For this purpose, we used a strategy that involved disruption of the stem–loop structure of the shRNA hairpin by XhoI digestion. This strategy is described in *SI Materials and Methods* and Fig. S1.

Phosphatase shRNA Library Screening. $CD62L^{bright}CD44^{dull}ZSG^{negative}$ CD4 T cells were sorted from TBGR mice, transduced with the phosphatase shRNA lentivirus pool, and primed under conventional Th1 conditions (IL-12, anti–IL-4) (Fig. 1*A*). Four days later, cells were transferred to puromycin-containing medium for 2 d. Surviving $ZSG^{positive}$ cells were purified by cell sorting, followed by a 4-d culture under conventional Th2 differentiation conditions (IL-4, anti–IL-12, anti–IFN-γ). Afterward, cells were cultured in IL-7–containing medium for 7 d. ZSG-positive and -negative cells were purified by cell sorting, and HT sequencing of the integrated shRNAs was carried out. We opted for a Th1/Th2 "switch" system, so that we could examine two populations of relatively similar size, to avoid the possible distortions from the comparison of two disproportionately represented sets.

A total of ∼40% of the shRNAs were represented in the library after excluding those with fewer than 10 "reads." Results from samples into which two separate barcoded adaptors were incorporated were highly consistent. Setting the criteria for enrichment at ≥fivefold more representation in the ZSG-positive or -negative populations, we obtained an initial group of 47 shRNA constructs encoding 42 unique genes, as listed in Fig. 1*B*.

To validate these 47 shRNA candidates, we digested genomic DNA from the two populations with XhoI and amplified the fragments with a PCR primer complementary to each of the individual shRNA 21-bp specific sequences at the 3′ end and with a U6 promoter-specific primer at the 5′ end. Forty-four of the 47 candidates were successfully amplified. The relative representations of these 44 shRNAs determined by HT sequencing and PCR amplification are plotted in Fig. 1*C*. There was a good correlation, with only three discordances between the two methods.

We then individually transfected each of the 47 shRNAs into naïve CD4 T cells and repeated the differentiation/switching analysis. We also verified that the switch procedure we used did not result in substantial apoptosis on the part of the switched cells (Fig. S2). Results from 34 shRNAs correlated with the screening data, with a similar number favoring either promotion or inhibition of switching. Ten shRNAs were indistinguishable from the nonsilencing control (NC) shRNAs, and three revealed activities opposite from that observed in the screening assay. Additionally, 10 shRNAs that had not enhanced or retarded switching in the HT screen (neutral shRNAs) were tested in the switch assay. Eight functioned similarly to the NC, and two favored either more or less ZSG expression. Thus, one could estimate that an shRNA that was "positive" in the screening assay was ∼10-fold [(34/13)/(2/8)] more likely to have such an activity in the test assay than was a neutral shRNA.

The 10 most impressive shRNAs, in terms of magnitude of effect, were MTMR9, MTMR7, inositol polyphosphate-5-phosphatase B (INPP5b), receptor-type tyrosine-protein phosphatase S (PTPrs), dual specificity phosphatase 6 (DUSP6), protein tyrosine phosphatase, non-receptor type 22 (PTPn22), V-type proton ATPase subunit e 1 (ATP6v0e), serine/threonine-protein phosphatase with EF-hands 1 (PPEF1), alkaline phosphatase intestinal (ALPI), and dual specificity phosphatase 13 (DUSP13). We excluded PPEF1,

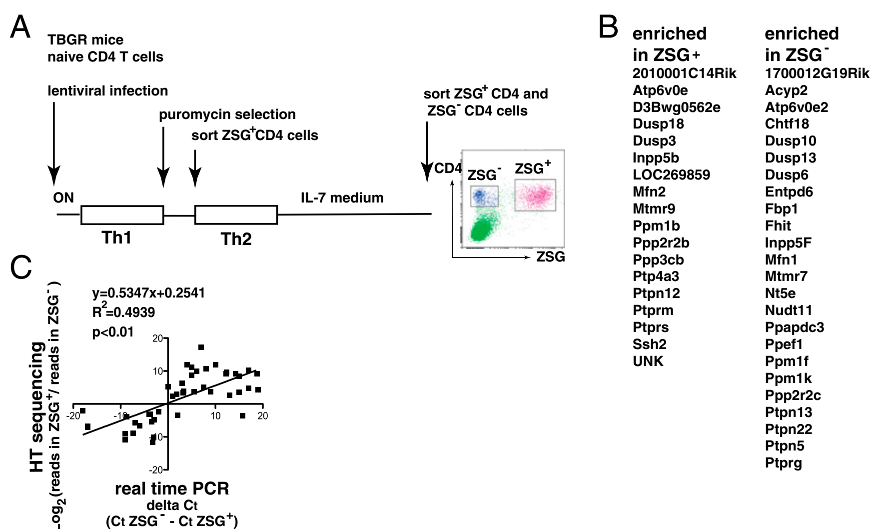

Fig. 1. Phosphatases screening system. (*A*) $CD62L^{bright}CD44^{dull}ZSG^{-}$CD4 T cells sorted from TBGR reporter mice were transduced with pooled shRNA lentiviruses. On the next day, cells were washed and primed under conventional Th1 conditions. Four days later, cells were cultured in puromycin-containing medium for 2 d. T-bet–ZSG^{+} cells were sorted and cultured under Th2 conditions for 4 d. T-bet–ZSG^{+} and T-bet–$ZSG^{−}$ cells were separated by cell sorting. (*B*) List of phosphatase shRNAs enriched in T-bet–ZSG^{+} and T-bet–$ZSG^{−}$ sets determined by shRNA screening. (*C*) Relative abundance of T-bet–ZSG^{+} vs. T-bet–$ZSG^{−}$ enriched phosphatase shRNAs determined by HT sequencing and real-time PCR analysis. Ct, cycle threshold.

ALPI and DUSP13 from further study because of their relatively low abundance in CD4 T cells. INPP5b, PTPrs, DUSP6, PTPn22, and ATP6v0e showed interesting impact on Th cell differentiation and deserve further analysis. Three of the shRNAs, MTMR9, MTMR7, and INPP5b, stood out by virtue of their common function in the regulation of PIs. In particular, both MTMR9 and MTMR7 belong to myotubularin family, and, furthermore, two separate shRNAs targeting MTMR9 scored in the screening assay. We chose MTMR9 and MTMR7 for more focused study.

MTMR9 Silencing Leads to Increased IFN-γ Production. MTMR9 shRNA was enriched in the ZSG-positive cell population. When introduced individually into naïve CD4 T cells, cells expressing MTMR9 shRNA showed stabilized ZSG expression in the Th1/Th2 switch system (Fig. 2*A*). Consistent with an enhanced proportion of ZSG-expressing cells, MTMR9 shRNA-transfected cells exhibited decreased IL-4 production and enhanced IFN-γ production after they had been subjected to the switch protocol (Fig. 2*B*). To further verify the effect of silencing MTMR9, four different shRNA clones were tested in Th1/Th2 switch system (Fig. 2 *C* and *D*). Three out of four constructs caused decreased *Mtmr9* mRNA levels, and each stabilized ZSG expression. Furthermore, cells expressing the least *Mtmr9* displayed the most abundant ZSG expression (Fig. 2 *C* and *D*).

Multiple factors participate in regulating the switch of Th1 to Th2 cells; among them could be a greater or lesser degree of commitment during the initial Th1 differentiation culture. We first examined the role of MTMR9 in regulating T-bet expression in naïve cells that were primed with antigen/antigen-presenting cell (APCs) without addition of polarizing cytokines or antibodies in the medium (Fig. 3*A*). Silencing MTMR9 in ovalbumin (OVA)-specific TBGR cells cultured with 100 nM OVA leads to stronger ZSG induction and elevated IFN-γ production when cells were challenged with phorbol 12-myristate 13- acetate (PMA)/ionomycin at the conclusion of the priming culture. Next, we introduced MTMR9 shRNA and primed the cells under Th1 conditions but with a limited amount of exogenous IL-12 (10 pg/mL) (Fig. 3*B*). Substantially, more IFN-γ production was observed in MTMR9 shRNA-transduced cells than in those transduced with a NC shRNA. The role of MTMR9 in Th1 differentiation was carefully examined using a series of dilutions of IL-12 during priming (Fig. 3*C*). Silencing MTMR9 resulted in greater IFN-γ–producing capacity under all priming conditions but was most prominent in the cells primed with limited amounts of IL-12. By contrast, silencing MTMR9 caused a marginal increase in Th17 differentiation, as determined by IL-17 production, and no change in Th2 differentiation, as measured by IL-4 production (Fig. 3*D*). Limiting the amount of IL-4 in Th2 priming culture and the amount of TGF-β/IL-6 in Th17 culture did not lead to greater difference (Fig. S3).

To further establish the specificity of "knocking down" MTMR9 in enhancing Th1 priming, four separate MTMR9-specific siRNAs were introduced into naïve CD4 T cells, followed by Th1 priming (Fig. 3 *E* and *F*). Only MTMR9-3 siRNA led to a substantial (~50%) *Mtmr9* mRNA diminution, and only it resulted in enhanced IFN-γ production, consistent with the observation in the shRNA-transduced cells. This is of particular importance because the siRNA-sequence composition is distinct from the shRNA sequence, strongly arguing against an off-target effect.

Effect of MTMR9 Silencing in Bone Marrow Chimeras. To test the effect of knocking down MTMR9 in vivo, the puromycin-resistance gene in the MTMR9 shRNA and NC shRNA constructs were replaced by a cDNA encoding Thy1.1; therefore, the expression of the shRNA could be reported by the expression of Thy1.1. Lineage-negative (Lin^-) bone marrow cells from TBGR OVA transgenic mice were isolated and transduced with MTMR9-Thy1.1 or NC-Thy1.1 lentivirus (Fig. 4*A*). The next day, such transduced, Lin^- bone marrow cells were transferred into sublethally irradiated recombination activating gene 1 $(Rag1)^{-/-}$ C57BL/6 mice. CD4 T cells in the blood of recipient mice were tested 8 wk later for T-bet–ZSG expression (Fig. 4*B*). The percentage of $Thy1.1^+$ CD4 T cells expressing ZSG in mice that had received MTMR9 shRNA-transduced Lin^- bone marrow cells from TBGR OVA transgenic donors was more than sevenfold greater than in recipients of NC shRNA-transduced cells from the same donors. Splenocytes from above recipient mice were also analyzed (Fig. 4 *C* and *D*). There were twice as many ZSG^{high} $Thy1.1^+CD44^{bright}$ CD4 T cells in the spleens of mice that had received MTMR9 shRNA-transduced Lin^- bone marrow cells than in those that had received NC shRNA-transduced cells.

Fifty thousand naïve ZSG-negative CD4 T cells from mice that had received either MTMR9 shRNA-transduced TBGR OVA transgenic Lin^- bone marrow cells or similar cells that had been infected with a NC lentivirus were transferred i.v. to C57BL/6 recipient mice. The recipients were immunized on the next day with OVA and LPS. A substantially higher percentage of ZSG^{high} CD4 T cells were induced in the immunized animals that had received cells from donors that had themselves received

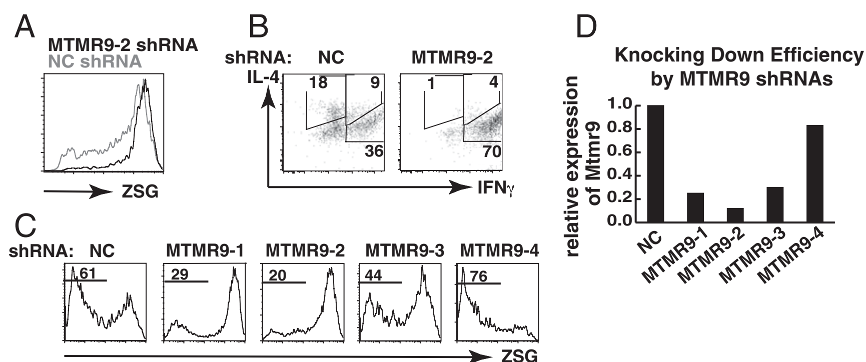

Fig. 2. MTMR9 shRNA-transduced cells showed stabilized ZSG expression and enhanced IFN-γ production. (*A* and *B*) Cells were transduced with MTMR9 shRNA or NC shRNA and cultured as described in Fig. 1*A*. Three days after cells were cultured in IL-7–containing medium, they were tested for their T-bet–ZSG expression (*A*) and were stimulated with PMA and ionomycin for 4 h, and cytokine production was examined by intracellular staining (*B*). (*C* and *D*) Four different MTMR9 shRNA lentiviral clones were individually examined in Th1/Th2 switch system as described in Fig. 1*A*. Seven days after being cultured in IL-7 medium, T-bet–ZSG expression was measured (*C*). Live CD4 cells were sorted and *Mtmr9* mRNA levels were measured by real-time PCR (*D*).

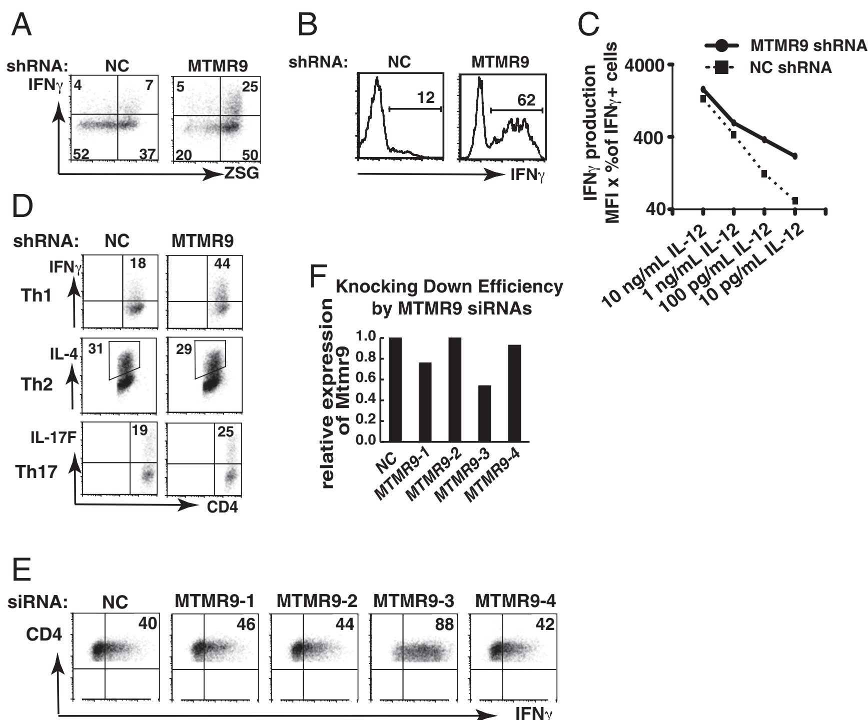

Fig. 3. Knocking down MTMR9 enhances Th1 differentiation. (*A*) CD62L^{bright}CD44^{dull}ZSG^{-} CD4 T cells were sorted from OVA transgenic TBGR mice, transduced with MTMR9 or NC shRNA lentivirus, and then cultured with splenic dendritic cells and 100 nM OVA peptide alone. At the end of 4-d priming, cells were cultured in puromycin-containing medium for 48 h and then stimulated with PMA plus ionomycin for 4 h to examine ZSG expression and cytokine production. (*B* and *C*) CD62L^{bright}CD44^{dull} CD4 T cells were sorted from OVA transgenic CD45.2 C57BL/6 mice, transduced, and cultured with T-depleted splenocytes as APCs under Th1 conditions. (*B*) The concentration of IL-12 was 10 pg/mL. (*C*) IL-12 concentration was varied from 10 pg/mL to 10 ng/mL. (*D*) Cells were sorted and transduced as above and then cultured under Th1, Th2, or Th17 conditions for 4 d; 10 pg/mL IL-12 was used in Th1 culture, 5,000 units/mL IL-4 in Th2 culture, and 5 ng/mL TGF-β and 5 ng/mL IL-6 in Th17 culture. After 48 h in puromycin-containing medium, cells were restimulated with 1 μM OVA in the presence of CD45.1 T-depleted splenocytes for 6 h to test their cytokine production. (*E*) One million CD4 T cells were isolated from OVA transgenic Rag1$^{-/-}$ C57BL6 mice by negative selection and transfected with 1 μM Mtmr9 siRNAs or NC siRNA by Amaxa Nucleofector. The cells were then differentiated under Th1 conditions using 10 pg/mL IL-12 for 4 d and placed in IL-2-containing medium for 2 d. Some of the cells were stimulated by PMA plus ionomycin for 4 h to check cytokine production. (*F*) *Mtmr9* mRNA levels were determined in the remainder of the cells by real-time PCR.

MTMR9 shRNA-transduced Lin^{-} bone marrow compared with mice that had received naïve cells from control donors (Fig. 4*E*).

Sorted naïve CD4 T cells from mice that had received either MTMR9 shRNA-transduced TBGR OVA Lin^{-} bone marrow cells or similar cells that had been infected with a NC lentivirus were also cultured under Th1 conditions with 10 pg/mL IL-12 (Fig. 4 *F* and *G*). The MTMR9 shRNA-expressing cells responded to Th1 differentiation with greater expression of ZSG than did cells expressing the NC shRNA (Fig. 4*F*). ZSG^{high} cells derived from donors with silenced *Mtmr9* expression produced more IFN-γ than ZSG^{high} cells from control donors (Fig. 4*G*). Efficient knock down of *Mtmr9* mRNA levels in these Th1 cells was confirmed by analysis of *Mtmr9* mRNA expression in sorted Thy1.1^{+} CD4 T cells (Fig. 4*H*).

Elevated AKT Activation in Response to MTMR9 Silencing. MTMR9 belongs to a large and highly conserved family of lipid phosphatases. As phosphoinositide phosphatases, MTMRs take part in controlling the biosynthesis and degradation of PIs. There is growing evidence that MTMRs can regulate AKT signaling; it has been established that AKT activation amplifies Th cell differentiation and cytokine production. Phosphorylation of AKT S473 is a commonly used surrogate for determining PI[3,4,5]P_3 levels. We measured AKT S473 phosphorylation in MTMR9 shRNA-transduced Th1, Th2, and Th17 cells (Fig. 5*A*). We used a PTEN shRNA as a positive control because PTEN directly converts PI[3,4,5]P_3 to PI[4,5]P_2. Indeed, PTEN shRNA-transduced cells have a substantially higher level of AKT activation in Th1, Th2, and Th17 cells (Fig. 5*A*). Compared with a NC shRNA construct, knocking down MTMR9 led to an elevated level of AKT phosphorylation in Th1 cells (Fig. 5*A*) ($P = 0.004$; Fig. 5*B*), although not as robustly as was achieved by silencing PTEN. MTMR9 silencing showed no impact on AKT phosphorylation in Th2 and Th17 cells. The effect of silencing MTMR9 on AKT phosphorylation is likely to be the mechanism through which it enhances Th1 differentiation because knocking out PTEN increases AKT phosphorylation and results in greater differentiation of Th1 cells (Fig. 5 *C* and *D*) (21, 22).

Knocking Down MTMR7 Enhances Th2 and Th17 Differentiation. In contrast to MTMR9 shRNA, MTMR7 shRNA was enriched in the ZSG-negative cell population in our switch assay. This effect of knocking down MTMR7 was confirmed first by individually testing four MTMR7 shRNA constructs in the Th1/Th2 switch system (Fig. 6*A*). All four led to accelerated down-regulation of ZSG expression, decreased IFN-γ, and enhanced IL-4 production. All four shRNA constructs also significantly decreased *Mtmr7* mRNA expression (Fig. 6*B*).

Reducing expression of *Mtmr7* resulted in increased Th2 and Th17 differentiation but no change in Th1 differentiation (Fig. 6*C*). PTEN knockdown enhanced differentiation in all three culture conditions and knock down of MTMR9, as before, enhanced Th1 but not Th2 or Th17 differentiation.

Silencing MTMR7 led to enhanced AKT phosphorylation in Th17 cells but not in Th1 or Th2 cells. When each of these cell types were activated by stimulation with peptide in the presence

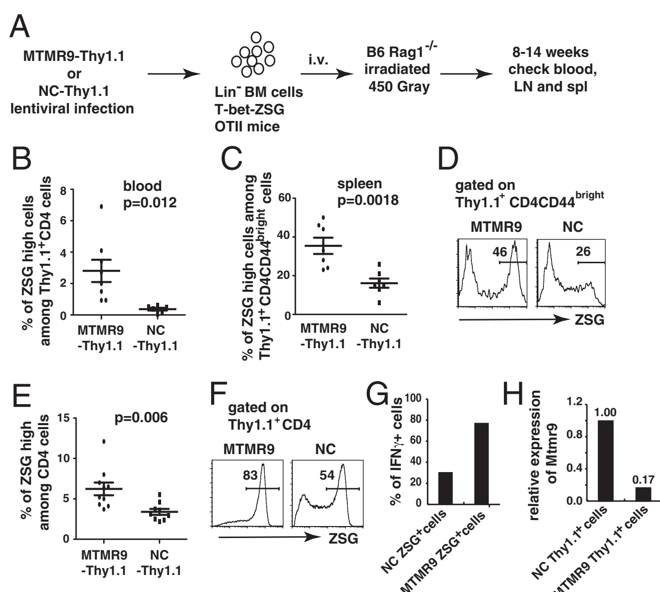

Fig. 4. In vivo effect of knocking down MTMR9 expression. (*A*) Schematic outline of the bone marrow chimera experiment. Bone marrow cells were isolated from TBGR OVA transgenic mice and lineage-positive cells were depleted. Lin^- bone marrow cells were transduced with lentivirus of MTMR-Thy1.1 or NC shRNA-Thy1.1. On the next day, such transduced cells were i.v. injected into irradiated recipient $Rag1^{-/-}$ C57BL/6 mice. (*B*–*D*) ZSG expression was examined in CD4 T cells in blood (*B*) and spleens (*C* and *D*) of reconstituted mice. Error bars represent SEM. (*E*) A total of 50,000 $Lin^-CD25^-CD45RB^+ZSG^-$ naïve CD4 T cells were sorted from above mice and i.v. transferred into C57BL/6 mice. On the next day, mice were immunized with 0.5 mg of OVA and 25 μg of LPS at footpads. T-bet–ZSG expression in CD4 T cells in draining lymph nodes was examined 6 d after immunization. (*F* and *G*) Part of the $CD25^-CD45RB^+ZSG^-$ naïve CD4 T cells sorted in *E* were cultured with 10 pg/mL exogenous IL-12, 10 μg/mL anti–IL-4, and 1 μM OVA in the presence of T-depleted splenocytes. Four days later, cells were washed and cultured in 10 ng/mL IL-2–containing medium for 2 d and then restimulated with 1 μM OVA in the presence of CD45.1 T-depleted splenocytes for 6 h to test their cytokine production and T-bet–ZSG expression. (*H*) $Thy1.1^+CD4$ cells from above cultures were sorted and *Mtmr9* mRNA level was measured by real-time PCR.

of APCs overnight, both Th2 and Th17 cells but not Th1 cells showed elevated AKT phosphorylation (Fig. 6*D*).

Expression Patterns of *Mtmr9* and *Mtmr7* mRNA. In an effort to understand the differential effects of knocking down MTMR9 and -7, we measured the expression of their mRNAs in naïve CD4 T cells and in Th1, Th2, and Th17 cells (Fig. 7). *Mtmr9* is abundantly expressed in naïve CD4 T cells and in cells of each of the Th lineages (Fig. 7*A*). Its expression level is not substantially altered by TCR stimulation. By contrast, *Mtmr7* is expressed at low levels in naïve CD4 T cells and is up-regulated by ∼30-fold in Th17 cells but only modestly increased in similarly cultured Th1 and Th2 cells (Fig. 7*B*). Its expression level declines when cells are rested in culture for a 10-d period. Two-hour stimulation of rested cells with PMA and ionomycin caused striking up-regulation of *Mtmr7* expression in Th2 cells but not in Th1 or Th17 cells. Thus, there is a correlation between expression level of MTMR7 and the consequence of its knockdown, although the peak times of expression vary in Th2 and Th17 cells. *Pten* mRNA levels were generally similar in each of the three cell populations (Fig. 7*C*).

Discussion

With the greater availability of HT sequencing technology, pooled RNAi screens coupled with HT sequencing provide a practical way to perform large-scale screens in individual laboratories. Here, we report screening a mouse phosphatase shRNA library to identify those phosphatases that play a selective role in the Th differentiation process.

For simplicity, we chose a screen that made optimal use of our T-bet reporter strain; the screen involved the switch of differentiated Th1 cells to the Th2 phenotype, that is, of ZSG-positive to -negative cells. Forty-seven phosphatase shRNAs were enriched in either the ZSG-positive or ZSG-negative cell populations. Individually introducing these shRNAs into naïve cells validated 34 of them. Ten with particularly striking effects were considered further, and, of those, three were lipid phosphatases. In this report, we describe our detailed analysis of two lipid phosphatases, both members of the MTMR family, MTMR9 and -7.

Cells in which MTMR9 expression was diminished by either shRNA or siRNA demonstrated a preferential retention of T-bet–ZSG expression in our screening assay and a greater degree of Th1 differentiation, as judged by T-bet–ZSG expression and IFN-γ production. The increased Th1 responses of naïve CD4 T cells in which MTMR9 expression was diminished correlated with stronger AKT phosphorylation in nonstimulated MTMR9 "knocked-down" Th1 cells.

By contrast, silencing MTMR7 led to less retention of T-bet–ZSG in our screening assay and, to a greater degree, of Th2 and Th17 differentiation in priming assays. CD4 T cells in which MTMR7 had been knocked down showed more AKT phosphor-

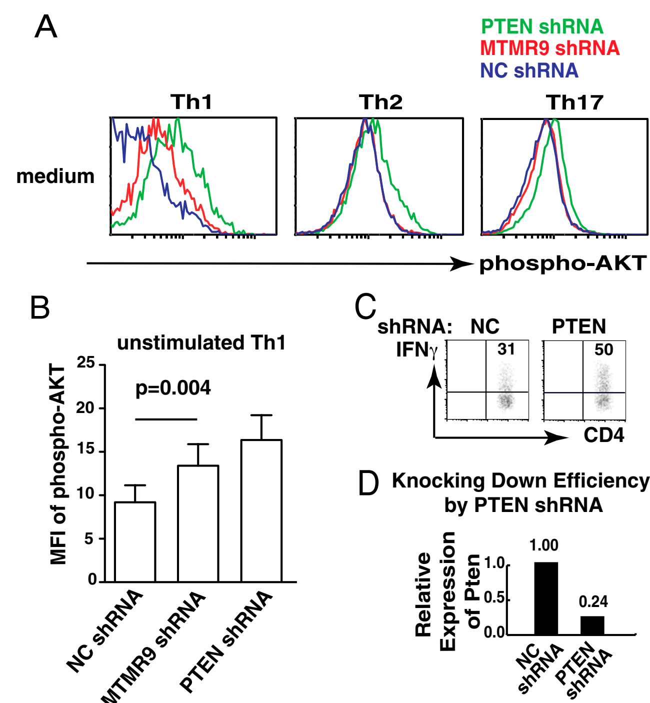

Fig. 5. AKT phosphorylation in differentiated *Mtmr9* "silenced" cells. (*A*) $CD62L^{bright}CD44^{dull}$ CD4 T cells were sorted from OVA transgenic CD45.2 C57BL/6 mice. Cells were transduced and cultured as described in Fig. 3*B*. After incubation in puromycin-containing medium for 3 d, cells were fixed and AKT phosphorylation was examined by flow cytometry. (*B*) Mean fluorescence intensities of phospho-AKT in unstimulated Th1 cells that had been transduced with NC, MTMR9, or PTEN shRNAs. The data represent the means and SEs of five independent experiments. (*C*) Cells were sorted as in Fig. 3*D*, transduced with a NC or PTEN shRNA, and then cultured under Th1 conditions for 4 d; 10 pg/mL IL-12 was used in the Th1 culture. After 48 h in puromycin-containing medium, cells were restimulated with 1 μM OVA in the presence of CD45.1 T-depleted splenocytes for 6 h to test their cytokine production. (*D*) The cultured cells, after incubation in puromycin-containing medium for 48 h, were purified and *Pten* mRNA levels measured by real-time PCR.

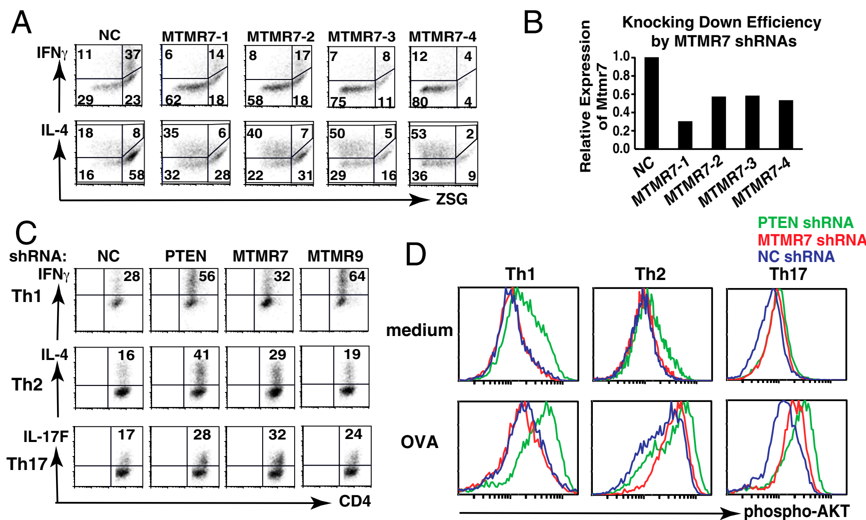

Fig. 6. Knocking down MTMR7 enhances Th2 and Th17 differentiation. (*A*) Four different MTMR7 shRNA lentiviral clones were individually examined in the Th1/Th2 switch system as described in Fig. 1*A*. Seven days after being cultured in IL-7 medium, cells were stimulated with PMA plus ionomycin to examine cytokine production and ZSG expression. (*B*) Live CD4 cells were sorted and *Mtmr7* mRNA expression measured by real-time PCR. (*C*) CD62L^{bright}CD44^{dull} CD4 T cells were sorted from OVA transgenic CD45.2 C57BL/6 mice. Cells were transduced as before and cultured under Th1, Th2, and Th17 conditions; 10 pg/mL IL-12 was used in Th1 culture, 500 units/mL IL-4 in Th2 culture, and 5 ng/mL TGF-β and 5 ng/mL IL-6 in Th17 culture. At the end of the 4-d differentiation, cells were cultured in puromycin-containing medium for 3 d, and a portion of the cells was restimulated with 1 μM OVA in the presence of CD45.1 T-depleted splenocytes for 6 h to test their cytokine production. (*D*) The remainder of the cells were mixed CD45.1 T-depleted splenocytes and either medium or 1 μM OVA. On the next day, cells were fixed and AKT phosphorylation was examined by flow cytometry.

ylation in nonstimulated Th17 cells and in both Th2 and Th17 cells that had been challenged with peptide and APCs.

We validated the effect of diminishing expression of *Mtmr9* in vivo in antigen-responding CD4 T cells using Rag1$^{-/-}$ recipients of OVA TCR transgenic bone marrow progenitors that had been transduced with an MTMR9 shRNA or a NC shRNA. Eight weeks after transfer, recipients of MTMR9-silenced progenitors had a greater representation of ZSG-positive cells among their CD4CD44^{bright} T cells than their counterparts that had received NC shRNA-transduced progenitors. When naïve CD4 T cells from mice that had received MTMR9 knocked-down progenitors were themselves transferred into wild-type mice, immunization elicited a response in which a greater percentage of cells expressed ZSG.

Thus, MTMR9 and MTMR7 regulate Th cell differentiation, effects that correlated, in part, with their ability to modulate AKT activity in differentiated T cells.

It has previously been shown that constitutive expression of active AKT led to increased proliferation and Th1/Th2 cytokine production, and we showed here that knocking down PTEN enhanced Th1, Th2, and Th17 differentiation. AKT regulates a wide range of downstream effectors, including the mammalian targets of rapamycin (mTOR1 and -2) (23) and several forkhead box protein O subclass (FoxO) transcription factors (24). Loss of mTOR complex 1 and 2 (mTORC1 and mTORC2) activity has been reported to lead to impaired differentiation into Th1 and Th2 cells both in vitro and in vivo (25, 26). Deficiency in both mTORC1 and mTORC2 activity also leads to a failure of Th17 differentiation (25).

FoxO family transcription factors have emerged as particularly important for lymphocyte function. Phosphorylation of FoxO family members by AKT triggers their translocation from the nucleus to the cytoplasm, where FoxOs form a complex with 14-3-3 proteins.

It has been proposed that unless PI3K-AKT lifts a restraint imposed by FoxO transcription factors, the *Il4* and *Ifng* loci fail to become fully accessible and CD4 T cells fail to complete Th differentiation (27). Indeed, FoxO3a-deficient T cells and Foxj1-deficient T cells show increased proliferation and exaggerated Th1 and Th2 cytokine production (28, 29).

MTMRs are lipid phosphatases that specifically remove the 3-phosphate from PI[3]P and PI[3,5]P_2, generating PI and PI[5]P, respectively. PI[3]P is the only 3-phosphoinositide that exists in significant amounts in resting mammalian cells. It has been linked to endocytosis and autophagy (30, 31). PI[3,5]P_2 makes up <0.1% of phosphoinositols and can only be generated by the action of 5-kinase on PI[3]P. In T cells, PI[3,5]P_2 can be induced by hyperosmotic conditions, UV treatment, and IL-2 (32). Silencing MTMRs raises the level of PI[3]P and PI[3,5]P_2 in T cells.

MTMRs have been reported to negatively regulate the PI3K-AKT pathway (33, 34). Expression in *Schizosaccharomyces pombe* of MTMR1, but not a catalytically inactive mutant, diminished cell growth (33). An MTMR8 antisense injected into zebrafish embryos caused increased AKT phosphorylation, which could be reduced by coinjection of an MTMR8 mRNA (34).

How do MTMRs regulate PI3K/AKT activity? One mechanism through which diminishing MTMRs could enhance PI3K/AKT activity is by increased endocytosis during immune synapse function as a result of elevated PI[3]P (35). Furthermore, knocking down both MTMR1 and MTMR2 caused accumulation of PI[3]P that, in turn, delayed EGF receptor turnover rate, leading to a sustained elevated AKT activation (36).

Alternatively, given that the functions and regulation of PIs are generally not well understood, PI[3]P may contribute to AKT activation in an unexpected manner. For example, PI[3]P has been reported to be required for the activity of the Ca^{2+}-activated K^+ channel KCa3.1 in human CD4 T cells (37). Such activation is inhibited by MTMR6-mediated dephosphorylation of PI[3]P and its resultant depletion from the plasma membrane adjacent to KCa3.1 (38). Indeed, although PI[3]P is highly enriched in endosomes, it has also been identified on the plasma membrane, where its function remains to be elucidated (39–43).

As we have shown, silencing MTMR9 resulted in elevated AKT phosphorylation in Th1 cells and enhanced differentiation of naïve cells to the Th1 phenotype. Knocking down MTMR7 caused increased AKT activation and cytokine production in Th2 and Th17 cells. These activities could be explained by the regulatory features of MTMRs and the expression pattern of these two phosphatases.

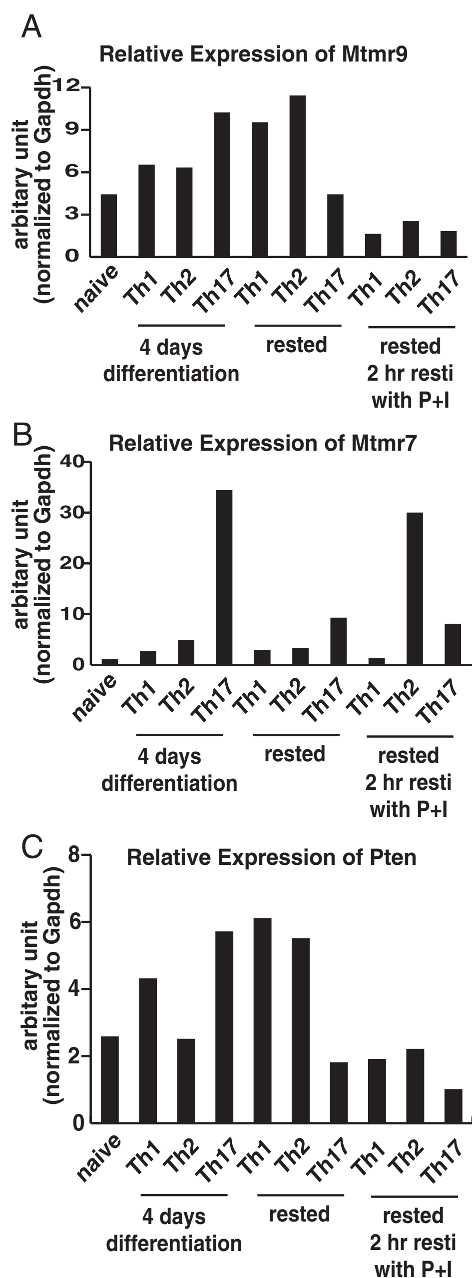

Fig. 7. Expression pattern of *Mtmr9*, *Mtmr7*, *and Pten*. CD62L^{bright}CD44^{dull}CD4 T cells were sorted and stimulated with anti-CD3/CD28 microbeads under Th1, Th2, or Th17 conditions for 4 d. Microbeads were removed, and cells were cultured in IL-2–containing medium (Th1 and Th2 cells) or IL-23–containing medium (Th17 cells). Cells were then restimulated with PMA plus ionomycin for 2 h. Cells were collected at indicated time points, and *Mtmr9* (*A*), *Mtmr7* (*B*), or *Pten* (*C*) mRNA expression was measured by real-time PCR.

Nine MTMRs possess catalytic activity; the remaining members of the family are not catalytically active because they lack the critical cysteine residue in their active site. However, a catalytically inactive MTMR may heterodimerize with a catalytically active family member and, by doing so, enhance its partner's function through stabilizing the protein, increasing its catalytic activity, and/or targeting it to a special subcellular location (44–49). MTMR9 is an inactive phosphatase and has been reported to bind to MTMR6 (48), MTMR7 (47), and MTMR8 (49).

Whether MTMR9 and MTMR7 function through binding to each other in Th cells remains unclear. However, *Mtmr9* is expressed in all lineages of Th cells. By contrast, *Mtmr7* is expressed at a very low level in Th1 cells, suggesting MTMR9 may have a partner other than MTMR7 in these cells accounting for their insensitivity to MTMR7 shRNA. The failure of MTMR9 shRNA to enhance Th2 and Th17 differentiation may be accounted for by larger amounts of the catalytically active heterodimer or the presence of yet another MTMR in these cells. An extensive search for the expression of the MTMRs could shed light on understanding this family of phosphatases.

Many of the PIs serve as important second messengers; PI3K-AKT signaling stands in the center of a multitude of potent and diverse transduction cascades. Regulation of PI3K-AKT signaling to ensure that it is properly balanced occurs at multiple levels, including diverse phosphatases targeting kinases, lipids, and various adaptor proteins (13). Our observation that MTMR9 and MTMR7 regulate AKT activity in T cells adds to the important controllers of this signaling system. These lipid phosphatases, in company with PTEN and potentially other phosphatases, contribute to the precise control of T-cell activation and effector cytokine production, providing an opportunity for more accurate therapeutic manipulation.

Materials and Methods

Mice and Cell Culture. CD62L^{bright}CD44^{dull}CD4 T cells were purified from OVA TCR transgenic C57BL/6 OT-II mice, TBGR reporter mice, or TBGR OVA TCR transgenic C57BL/6 mice by cell sorting. All of the mice were bred and maintained in the National Institute of Allergy and Infectious Diseases (NIAID)-specific pathogen free animal facility, and the experiments were performed under protocols approved by the NIAID Animal Care and Use Committee. T-depleted splenocytes were used as APCs. CD4 T cells were primed with APCs, OVA (1 μM), or anti-CD3 (3 μg/mL) together with anti-CD28 (3 μg/mL) under Th1, Th2, and Th17 conditions as described previously (50). CD4 T cells cultured with peptide/APCs only was performed as described before (51).

shRNA Lentiviral Infection. Generation of the pooled shRNA library is described in *SI Materials and Methods*; 293FT "packaging" cells were transfected with shRNA plasmid DNA or NC shRNA plasmid DNA, together with the packaging plasmid pCMVΔ8.9 and the envelope plasmid pHCMV-G using the Fugene 6 transfection reagent (Roche Diagnostics) according to the manufacture's protocol. Packaging plasmid pCMVΔ8.9 and envelope plasmid pHCMV-G were kindly provided by N. Hacohen (Harvard Medical School, Cambridge, MA). Lentivirus-containing supernatants were collected at 48 and 72 h after transfection, pooled, and stored at −80 °C.

Naïve CD4 T cells (5×10^6) were seeded into one well of a six-well plate in 8 mL of lentiviral supernatant, supplemented with IL-7 (1 ng/mL), 2-mercaptoethanol (50 μM), and Polybrene (8 μg/mL) (Sigma). The cells were centrifuged at $900 \times g$ for 90 min at room temperature and then incubated in above medium overnight. Cells were then centrifuged to remove the viral supernatant and cultured in fresh T-cell differentiation medium. After 4 d of T-cell differentiation, cells were placed in fresh medium containing puromycin (5 μg/mL) (Sigma) and cytokines, IL-2 for Th1 cells, IL-7 for Th2 cells, and IL-23 for Th17 cells. Forty-eight hours later, puromycin-resistant cells were purified by cell sorting.

siRNA Transfection. CD4 T cells were isolated from OT-II Rag1$^{-/-}$ mice and further enriched by negative selection using the pan-T isolation kit (Miltenyi Biotec). CD4 T cells (1×10^6) were transfected with 1 μM siRNA (Dharmacon) using the Amaxa Nucleofector kit (Lonza). After 24 h, the cells were changed to fresh T-differentiation medium and primed as described above. Fours days later, cells were collected for analysis of mRNA expression and cytokine producing capacity.

RNA Purification and Real-Time PCR. Total RNA was isolated using RNeasy mini kit (Qiagen); first-strand cDNA was prepared using SuperScript III (Invitrogen). All PCRs were performed on a 7900HT sequence detection system (Applied Biosystems). The TaqMan universal PCR SuperMix and all of the primer and probe sets were purchased from Applied Biosystems. The relative expression level of each given gene has been normalized to the expression level of *Gapdh*.

Intracellular Staining. In vitro-differentiated Th1 and Th2 cells were stimulated by the addition of OVA (1 μM) in the presence of CD45.1 T-depleted spleen cells for 6 h or with PMA (10 ng/mL) and ionomycin (1 μM) for 4 h, in the presence of monensin. Harvested cells were fixed with fixation buffer (1 vol of 16% paraformaldehyde diluted with 3 vol of PBS), washed with 0.1% BSA-con-

taining PBS, and stored at 4 °C. To determine cytokine production, cells were incubated with permeabilization buffer (PBS supplemented with 0.1% BSA/0.1% Triton X-100) and various antibodies for 20 min.

To test AKT phosphorylation, cells were fixed with 4% paraformaldehyde, followed by 2 h of incubation in 90% methanol at −20 °C. Alexa Fluor 647 mouse anti-AKT (pS473) was from BD PharMingen.

Plasmid Reconstruction. A Thy1.1 cDNA fragment was obtained by PCR amplification from a Thy1.1 cDNA plasmid. The primers 5′-GTG-GAT-CCA-TGA-ACC-CAG-CCA-TCA-GCG-T-3′ and 5′-GAG-GTA-CCT-CAC-AGA-GAA-ATG-AAG-TCC-AG-3′ were designed complementary to ∼20-bp sequences of Thy1.1 tailed by BamHI or Acc65I restriction enzyme recognition sequences. The plasmid DNAs pLKO.1-MTMR9-3 and pLKO.1-NC were treated with BamHI and Acc65I. The desired DNA fragments were gel-purified (Qiagen), mixed together with the Th1.1 cDNA fragment and T4 ligase (New England Biolabs).

Bone Marrow Chimera. Bone marrow cells were isolated from T-bet–ZSG OVA transgenic C57BL/6 mice. Cells with lineage-specific markers were depleted with the Miltenyi Biotec depletion kit. The remaining cells were infected with MTMR9-Thy1.1 or NC-Thy1.1 shRNA lentivirus as described above. The medium was supplemented with 5 ng/mL SCF and 5 ng/mL IL-3. On the next day, the cells were washed and injected into $Rag1^{-/-}$ C57BL/6 recipient mice that have been irradiated with 450 Gy. The mice were treated with 0.67 mg/mL trimethoprim and 0.13 mg/mL sulfamethoxazole via drinking water immediately after irradiation, and the treatment lasted for 5 wk. Blood, spleen, and lymph nodes were examined 8–14 wk after transfer.

Immunization. $CD45RB^{hi}CD25^{-}ZSG^{-}$ naïve CD4 T cells were sorted from bone marrow-reconstituted mice described above and transferred into C57BL/6 mice through retroorbital injection, 50,000 cells per recipient mouse. The next day, the recipient mice were immunized with 500 μg of OVA and 25 μg of LPS in footpads. Six days later, popliteal lymph nodes and inguinal lymph nodes were collected and analyzed.

Statistical Analysis. Student *t* test was used to determine statistical significance of the difference between two groups.

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