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Costimulation-Dependent Expression of MicroRNA-214 Increases the Ability of T Cells To Proliferate by Targeting *Pten*

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T cell activation requires signaling through the TCR and costimulatory molecules, such as CD28. MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression posttranscriptionally and are also known to be involved in lymphocyte development and function. In this paper, we set out to examine potential roles of miRNAs in T cell activation, using genome-wide expression profiling to identify miRNAs differentially regulated following T cell activation. One of the miRNAs upregulated after T cell activation, miR-214, was predicted to be capable of targeting *Pten* based on bioinformatics and reports suggesting that it targets *Pten* in ovarian tumor cells. Upregulation of miR-214 in T cells inversely correlated with levels of phosphatase and tensin homolog deleted on chromosome 10. In vivo, transcripts containing the 3′ untranslated region of *Pten,* including the miR-214 target sequence, were negatively regulated after T cell activation, and forced expression of miR-214 in T cells led to increased proliferation after stimulation. Blocking CD28 signaling in vivo prevented miR-214 upregulation in alloreactive T cells. Stimulation of T cells through the TCR alone was not sufficient to result in upregulation of miR-214. Thus, costimulation-dependent upregulation of miR-214 promotes T cell activation by targeting the negative regulator *Pten*. Thus, the requirement for T cell costimulation is, in part, related to its ability to regulate expression of miRNAs that control T cell activation. ***The Journal of Immunology*****, 2010, 185: 000–000.**

T cell activation is a highly regulated process that requires the coordination of several sequential events that function to drive T cells to a differentiated state. Activation requires signaling through the TCR upon recognition of peptide-MHC complexes on the surface of APCs, and the delivery of costimulatory signals. The CD28 costimulatory pathway plays a central role in activating signaling pathways, such as the PI3K pathway, that promote T cell survival, cytokine production, and differentiation (1). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) controls T cell activation and maintains self-tolerance by negatively regulating signaling pathways that lead to activation (2). PTEN negatively regulates the AKT signaling pathway by dephosphorylating phosphatidylinositol 3,4,5-triphosphate, a second messenger generated by PI3K that promotes the recruitment of 3-phosphoinositide-dependent protein kinase 1 to the TCR signaling complex. Costimulation through CD28 overcomes negative regulation of TCR signaling by PTEN, supporting the notion that costimulation is needed to overcome the function of PTEN and allow for T cell activation (3). However, the mechanisms leading to costimulation-dependent regulation of PTEN remain unclear.

MicroRNAs (miRNAs) are small endogenous noncoding RNAs that are ~21–22 nt in length (4, 5). Most genes encoding miRNAs are transcribed by RNA polymerase II, although some can be transcribed by polymerase III (6–8). Primary transcripts encoding miRNAs are processed through a series of steps into pre-miRNA stem loops of ~60 nt by an miRNA processing complex that includes the RNase II enzyme Drosha and its partner DGCR8 (Pasha) (9, 10). Pre-miRNAs are transported to the nucleus by Exportin-5 in a RAS-related nuclear protein–GTP–dependent manner and then further processed into a 21- to 22-nt duplex by the RNase II enzyme Dicer. The functional miRNA strand is next loaded into the RNA-induced silencing complex. Mature miRNAs then guide the RNA-induced silencing complex to complementary target genes and repress gene expression by destabilizing the target mRNA or by repressing translation. miRNAs play a key role in several disease processes and development through their ability to control gene expression posttranscriptionally.

Evolutionarily conserved miRNAs exist in all species analyzed to date, many of which have been shown to regulate vertebrate development and to be implicated in the development of cancer (10–16). miRNAs also play a key part in lymphocyte development and function (17–19). Given the emerging role of miRNAs in the control of lymphocyte development and function, we set out to examine potential roles of miRNAs in T cell activation. PTEN is targeted by multiple miRNAs, including miR-21 in human hepatocellular cancers (20), miR-26a in human glioma tumors (21), miR-216a and miR-217 in glomerular mesangial cells (22), and miRNA cluster 17–92 contributing to lymphomagenesis (23). It therefore appears that PTEN can be controlled by multiple miRNAs; however, the ability of a given miRNA to regulate PTEN seems to be cell type specific. miRNAs that control PTEN in T cells have not been defined.

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Abbreviations used in this paper: 3′ UTR, 3′ untranslated region; DDAO-SE, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester; miRNA, microRNA; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

To examine the function of miRNAs in T cell activation, particularly control of PTEN, we performed genome-wide miRNA expression profiling. We identified a relatively limited number of miRNAs that were either up- or downregulated after T cell activation. One of the miRNAs shown to be upregulated after T cell activation, miR-214, was predicted to be capable of targeting *Pten* based on bioinformatics and reports suggesting that it is capable of targeting *Pten* in tumor cells (24). Our data show that miR-214 targets *Pten* in activated T cells and that its expression leads to increased T cell proliferation. Furthermore, upregulation of miR-214 was dependent on CD28 costimulation. Our results therefore suggest that costimulation through CD28 overcomes negative regulation of TCR signaling by allowing for increased expression of miR-214, which in turn regulates *Pten* expression. Thus, the requirement for T cell costimulation is in part related to its ability to regulate expression of miRNAs that control T cell activation.

Materials and Methods

Mice

All mice were obtained from The Jackson Laboratory (Bar Harbor, ME), housed using microisolator conditions in autoclaved cages, and maintained on irradiated feed and autoclaved acidified drinking water. Female 4- to 6-wk-old mice were used in all experiments. All experiments were performed in compliance with Institutional Guidelines.

T cell isolation and activation

Total T cells were purified by magnetic bead-based cell sorting. $CD4^+$ and $CD8^+$ T cells were then purified by fluorescence-based cell sorting, as described (25). T cells were activated by being stimulated with 2 μg/ml anti-CD3 (2C11) and 1 μg/ml CD28 (37.51) (American Type Culture Collection, Manassas, VA) in DMEM supplemented with L-glutamine, penicillin-streptomycin, sodium pyruvate (Mediatech, Manassas, VA), nonessential amino acids (Invitrogen, Carlsbad, CA), and 10% FCS (Hyclone, Brookfield, WI).

miRNA array

Total RNA was extracted from unstimulated and stimulated (activated) T cells, with the miRNeasy Kit (Qiagen, Valencia, CA), and used for microarray analysis, as previously described (26). Total RNA from three separate preparations of unstimulated and activated T cells was pooled and used for microarray analysis. Microarray assays and statistical analysis were performed by LC Sciences (Houston, TX; www.lcsciences.com), using miRNA probe sequences from mmu-miRBase 11.0 (Sanger Institute, Cambridge, U.K.; http://microrna.sanger.ac.uk/sequences).

Viruses

To generate MMP-LUC-3′-PTEN and MMP-LUC-MUT-3′-PTEN, oligonucleotides encoding the 3′ untranslated region (3′ UTR) of *Pten* containing either the miR-214 target sequence or a mutated sequence, respectively, were cloned into pMIR-REPORT (Applied Biosystems/Ambion, Austin, TX) downstream of luciferase. The fragment from each construct containing luciferase and either the miR-214 target sequence or mutated target was then cloned into the pMMP retroviral vector (kindly provided by Richard Mulligan, Harvard Medical School, Boston, MA) to generate MMP-LUC-3′-PTEN or MMP-LUC-MUT-3′-PTEN. To generate pLL3.7–pre-miR-214, oligonucleotides encoding the pre-miR-214 sequence were cloned into the XhoI site of pLL3.7 (27) downstream of the U6 promoter. Vesicular stomatitis virus G envelope protein pseudotyped viruses were prepared by packaging the retroviral or lentiviral vectors in 293T cells by transient transfection using the calcium phosphate method, as previously described (28, 29). Functional titers of viral supernatants were determined in 293T cells by analyzing expression of GFP by flow cytometry.

Validation of viral vectors

HIO80 cells were obtained from Fox-Chase Cancer Center (Philadelphia, PA) and grown in 199/MDCB 105 (1:1) medium supplemented with 5% FCS and 2 μg/ml porcine insulin (Sigma-Aldrich, St. Louis, MO). HIO80 cells were cotransfected with a 1:2 ratio of pMIR-Report to pre-miR-214–GFP or pLL3.7-GFP vector control constructs using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's specifications. Transfection efficiency was calculated by measuring GFP expression on a FACSCalibur flow cytometer (BD Biosciences, San Jose. CA). Luciferase assays were performed 48 h after transfection, using SteadyLite reagent (PerkinElmer, Wellesley, MA), and measured on a Wallac Microbeta Trilux Luminometer (PerkinElmer).

Bone marrow transduction and transplantation

Bone marrow cells were harvested from mice treated 7 d prior with 5-fluorouracil and transduced, as described previously (30). Bone marrow infections were performed at a multiplicity of infection of at least 1 for retroviruses or 5 for lentiviruses. Then 4×10^6 bone marrow cells were used to reconstitute lethally irradiated (11 Gy) recipients.

Real-time PCR

Real-time PCR using miRNA-specific stem-loop primers for reverse transcription and TaqMan probes for mature murine miRNA was performed in accordance with the manufacturer's protocols (Applied Biosystems, Foster City, CA) and analyzed using an Applied Biosystems 7900HT Real-Time PCR system. Data analysis was performed using the Applied Biosystems SDS Software package, version 2.2. Primers specific for snoRNA202 in mouse or RU48 in human cells were obtained from Applied Biosystems and used as controls in all assays, following the manufacturer's instructions. The relative quantification was calculated by dividing miR-214 expression by the endogenous control. All assays were performed in triplicate.

Western blot

Western blots were performed as previously described (31), using a PTEN-specific rabbit mAb 138G6 (Cell Signaling Technology, Cambridge MA). Mouse mAb β-actin (C4) was used to detect β-actin as a loading control (Sigma-Aldrich). Western blots were quantified using ImageJ software version 10.2 (National Institutes of Health, Bethesda MD).

Luciferase reporter assay

Luciferase assays were performed using SteadyLite reagent (PerkinElmer) according to the manufacturer's instructions and analyzed on a Wallac Microbeta Trilux Luminometer.

Proliferation assay

To measure proliferation, purified T cells were labeled with 0.6 μM CellTrace Far Red DDAO (Invitrogen) for 6 min at room temperature. Labeled T cells (3×10^6) were activated for 72 h. Cell proliferation was then assessed by flow cytometry and analyzed using FlowJo software version 6.3 (Tree Star, Ashland, OR).

Popliteal lymph node assay

C57BL/6 mice were injected in the footpad with 2×10^7 BALB/c splenocytes. In some cases, mice were also injected i.p. with 250 μg CTLA4-Ig. T cells were purified from draining popliteal lymph nodes and analyzed for miR-214 expression by RT-PCR. PTEN levels were assessed by Western blot.

Statistical analysis

All statistical calculations were performed using GraphPad Prism 4.0a software (GraphPad Software, San Diego, CA). Values of p were determined using the Student two-tailed t test for independent samples. Values of $p < 0.05$ were considered statistically significant.

Results

Differential expression of miRNAs following T cell activation

To begin to examine the role of miRNAs in T cell activation, we performed expression profiling on miRNA isolated from activated and resting T cells. T cells were purified from the spleens of C57BL/6 mice and stimulated with anti-CD3 and -CD28 mAbs for 72 h (activated) or used to prepare miRNA without further stimulation (resting). A portion of each population was labeled with CFSE to monitor T cell activation based on proliferation. Approximately 89% of T cells stimulated with anti-CD3 and -CD28 underwent proliferation, indicating that they were activated (data not shown). Genome-wide miRNA expression profiling of 599 murine miRNAs present in miRBase release 11, using μParaflo microfluidic biochip array technology, revealed significant differences

in the expression of 145 miRNAs in unstimulated and activated T cells (Supplemental Fig. 1, $p < 0.01$). Of the miRNAs observed to be differentially expressed, 61 were upregulated in activated T cells and 84 were downregulated (see Supplemental Material). Narrowing our analysis to transcripts with mean signal intensities >500 revealed 32 miRNAs that were upregulated (Supplemental Table I) and 43 that were downregulated (Supplemental Table II) in activated T cells.

miR-214 is upregulated in T cells upon stimulation

Our array data revealed that miR-214 is significantly upregulated upon T cell activation ($p = 5.63 \times 10^{-6}$ between activated and resting, Supplemental Table I). Bioinformatics analysis revealed that *Pten* is a potential target of miR-214 (32). Moreover, in human ovarian cancer cells, miR-214 has been shown to induce cell survival and resistance to chemotherapeutic agents by targeting PTEN (24). These observations led us to hypothesize that upregulation of miR-214 following T cell activation may function to regulate PTEN expression, thereby affecting T cell activation. To test this hypothesis, we first set out to confirm that miR-214 is upregulated in T cells upon activation by performing stem-loop TaqMan real-time PCR assays. Consistent with our microarray data, we observed that miR-214 is significantly upregulated in T cells after stimulation with anti-CD3 and -CD28 (Fig. 1*A*). To further characterize the role of miR-214 in T cell proliferation, we isolated $CD4^+$ and $CD8^+$ T cells and examined miR-214 expression at various time points following stimulation with anti-CD3 and -CD28. miR-214 is rapidly upregulated in both $CD4^+$ and $CD8^+$ T cells upon stimulation (Fig. 1*B*). Further analysis revealed that miR-214 is upregulated in naive and memory $CD4^+$ and $CD8^+$ T cells after stimulation with anti-CD3 and -CD28 (Fig. 2). Upregulation of miR-214, however, was observed to be significantly higher in $CD4^+CD62^{hi}CD44^{hi}$ memory T cells than in naive $CD4^+$ T cells, peaking at 48 h after stimulation (Fig. 2*A*). In contrast, miR-214 appeared to be upregulated more rapidly in naive ($Ly6c^{lo}$ $CD44^{lo}$) $CD8^+$ T cells when compared with memory ($Ly6c^{hi}$ $CD44^{hi}$) $CD8^+$ T cells (Fig. 2*B*); however, at 72 h, expression of miR-214 was the same in each group. Upregulation of miR-214 inversely correlated with levels of *Pten* mRNA. *Pten* mRNA levels were substantially reduced in both $CD4^+$ and $CD8^+$ T cells at all time points analyzed (Fig 3A). Upregulation of miR-214 inversely correlated with levels of PTEN protein in naive and memory $CD4^+$ T cells (Fig. 3*B*). This effect was observed to a lesser extent in naive $CD8^+$ T cells (Fig. 3*B*). Downregulation of PTEN was not observed in memory $CD8^+$ T cells until 72 h (Fig. 3*B*), a point at which we observed miR-214 to be significantly upregulated (Fig. 2*B*). At 72 h, levels of *Pten* mRNA were decreased in both CD4 and CD8 cells (Fig. 3*B*), correlating with an increase in miR-214 (Fig. 1*B*) and a decrease in PTEN protein levels (Fig. 3*B*).

The 3′ UTR of Pten *is targeted during T cell activation*

miRNAs function by targeting mRNA. Bioinformatics analysis suggested that the 3′ UTR of *Pten* contains a miR-214 targeting site. To examine whether the 3′ UTR of *Pten* is regulated during T cell activation, we generated retroviruses in which the 3′ UTR of *Pten* containing the miR-214 target sequence was cloned downstream of luciferase (MMP-LUC-3′-PTEN). We also generated retroviruses

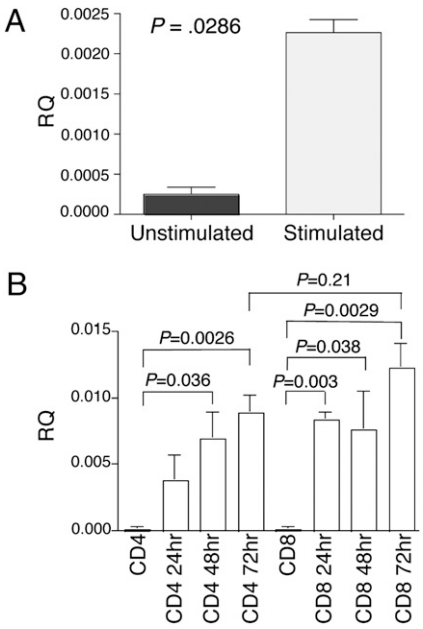

FIGURE 1. *A*, miR-214 expression is increased in activated T cells. T cells were isolated from the spleen of C57BL/6 mice, and the level of miR-214 transcript was assessed by PCR immediately after isolation (unstimulated) or after activating with anti-CD3 and -CD28 mAbs for 72 h (stimulated). *B*, Expression of miR-214 in $CD4^+$ and $CD8^+$ T cells over time after activation. The level of miR-214 expression in $CD4^+$ or $CD8^+$ T cells was assessed by PCR in either unstimulated or activated T cells at the indicated time points. Data shown are representative of at least three experiments.

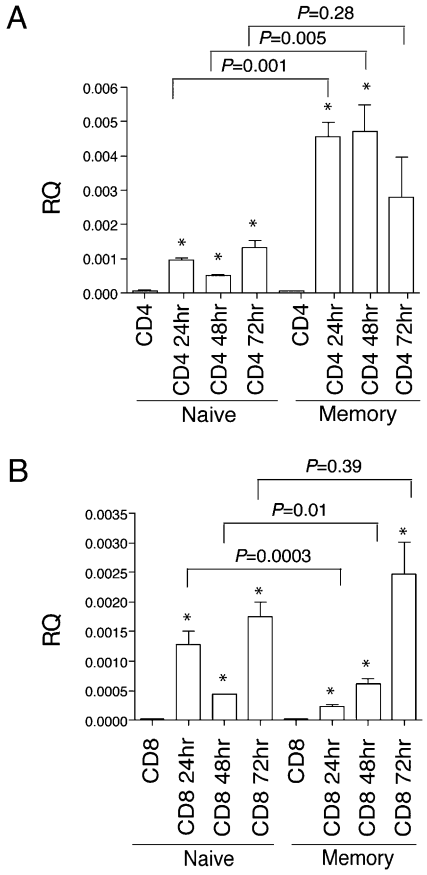

FIGURE 2. miR-214 expression in T cell subsets following activation. Expression of miR-214 in naive and memory $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells. T cells were isolated from the spleens of C57BL/6 mice. $CD4^+$ ($CD44^{hi}CD62L^{hi}$) and $CD8^+$ ($CD44^{hi}Ly6c^{hi}$) memory T cell populations, as well as naive $CD4^+$ ($CD44^{lo}$ $CD62L^{hi}$) and $CD8^+$ ($CD44^{lo}Ly6c^{lo}$) populations, were purified by FACS. Purified T cells were then used to prepare RNA or were stimulated with anti-CD3 and -CD28 Abs prior to assessment of miR-214 expression by PCR. Data shown are representative of at least three experiments.

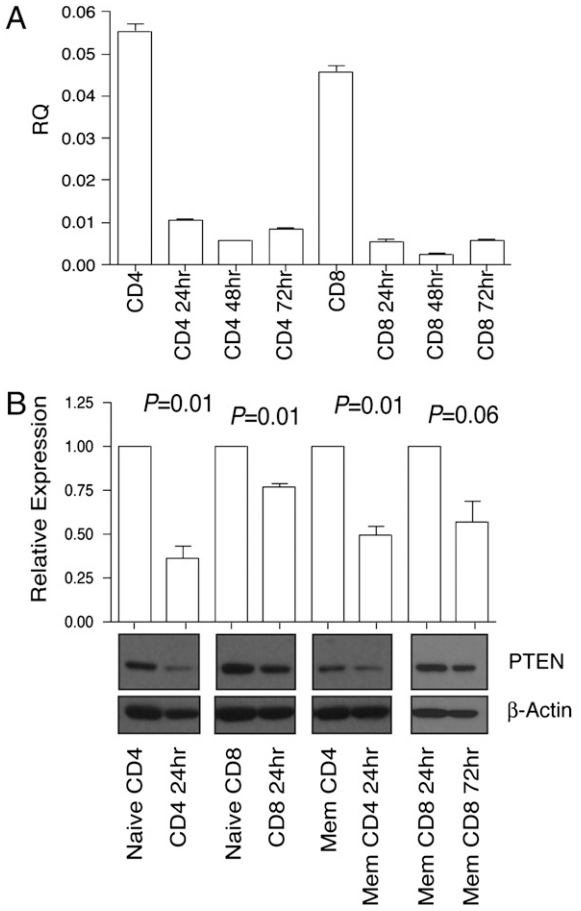

FIGURE 3. *Pten* mRNA and protein levels decrease in activated T cells and are inversely correlated with miR-214 expression. *A*, CD4 and CD8 T cells were purified from the spleen of C57BL/6 mice, and the level of *Pten* mRNA was assessed by PCR immediately after isolation, or after activating with anti-CD3 and -CD28 mAbs for the indicated time points. *B*, T cells were isolated from the spleens of C57BL/6 mice. $CD4^+$ ($CD44^{hi}$ $CD62L^{hi}$) and $CD8^+$ ($CD44^{hi}Ly6c^{hi}$) memory T cell populations, as well as naive $CD4^+$ ($CD44^{lo}$ $CD62L^{hi}$) and $CD8^+$ ($CD44^{lo}Ly6c^{lo}$) populations, were purified by FACS. The level of PTEN protein was assessed by Western blot immediately after isolation, or after activating with anti-CD3 and -CD28 mAbs for the indicated time points. Data were normalized to β-actin controls run on the same gel, using ImageJ software. Normalized data are shown in the bar graph and based on Western blotting shown at the bottom. Representative data from at least two independent experiments are shown.

in which the 3′ UTR of *Pten* containing a mutated miR-214 target sequence was cloned downstream of luciferase (MMP-LUC-MUT-3′-PTEN). To validate lentiviral constructs, HIO80 cells, which do not express endogenous miR-214, were transfected with MMP-LUC-3′-PTEN or MMP-LUC-MUT-3′-PTEN. The resulting lines were then transfected with either pLL3.7–pre-miR-214 or pLL3.7 lentiviral vectors. After 48 h, expression of miR-214 was assessed by PCR (Fig. 4*A*). Cells transfected with pre-miR-214 expressed significantly more miR-214 than did cells transfected with control plasmid. Pre-miR-214 activity was then examined on the basis of luciferase activity. Expression of pre-miR-214 significantly decreased the level of luciferase activity in cells transfected with MMP-LUC-3′-PTEN (Fig. 4*B*). A reduction in luciferase activity was not observed in cells transfected with MMP-LUC-MUT-3′-PTEN (Fig. 4*B*). Expression of miR-214 also led to a decrease in PTEN protein expression in HIO80 cells, compared with cells transfected with control plasmid alone (Fig. 4*C*), confirming that our lentivirally encoded miR-214 was capable of downregulating PTEN expression.

To test the function of miR-214 in vivo, bone marrow was harvested from 3- to 4-wk-old female C57BL/6 mice treated 7 d prior with 150 mg/kg 5-fluorouracil and transduced with either MMP-LUC-3′-PTEN or MMP-LUC-MUT-3′-PTEN and then used to reconstitute lethally irradiated C57BL/6 mice, as previously described (30). When hematopoietic reconstitution was complete, mice reconstituted with either MMP-LUC-3′-PTEN or MMP-LUC-MUT-3′-PTEN transduced bone marrow were sacrificed and T cells were isolated. Stimulation of T cells from mice receiving MMP-LUC-3′-PTEN transduced bone marrow with anti-CD3 and -CD28 led to a significant decrease in luciferase activity when compared with the level of activity observed in unstimulated T cells (Fig. 5*A*). We did not observe a decrease in luciferase activity in T cells isolated from mice receiving MMP-LUC-MUT-3′-PTEN transduced bone

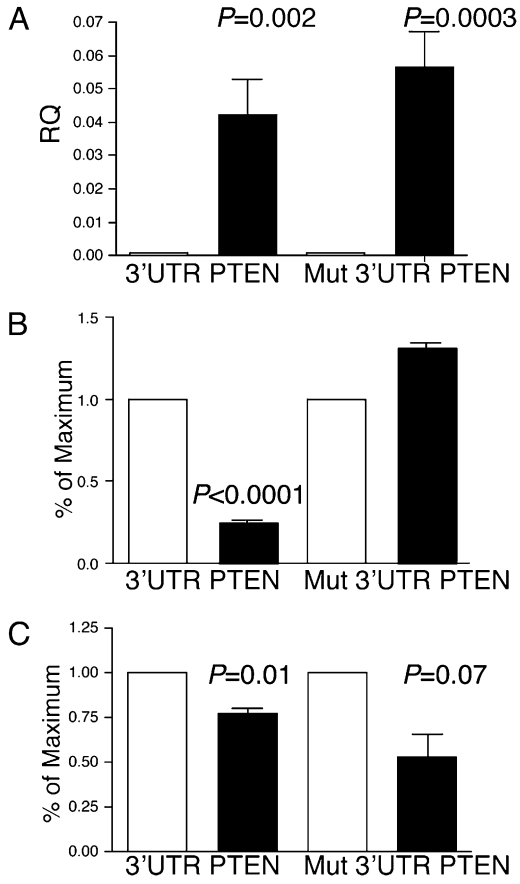

FIGURE 4. miR-214 targets the 3′ UTR of *Pten* in cell lines. HIO80 cells, which do not express endogenous miR-214, were transfected with either MMP-LUC-3′-PTEN or MMP-LUC-MUT-3′-PTEN. Resulting cell lines were then transfected with pLL3.7–pre-miR-214 (black bars) or pLL3.7 control vectors (white bars). *A*, After 48 h, RNA was isolated and miR-214 expression was assessed by PCR. Shown are representative data from one of two experiments. *B*, Expression of miR-214 targets transcripts containing the 3′ UTR of *Pten.* At 48 h after transfection of cells expressing either MMP-LUC-3′-PTEN or MMP-LUC-MUT-3′-PTEN with pLL3.7–pre-miR214 or pLL3.7, luciferase activity was measured. Transfection efficiency was measured by detecting the percentage of cells expressing GFP by flow cytometry. Data were normalized for transfection efficiency and presented as a percentage of the luciferase activity of the pLL3.7 vector alone. Shown are representative results from one of three experiments. *C*, miR-214 downregulates expression of endogenous PTEN. Shown are relative PTEN levels in cells transfected with pLL3.7–pre-miR214 or pLL3.7. Data are shown as percentage of the expression detected in cells transfected with pLL3.7 vector alone. Shown are representative data from one of two experiments.

marrow following stimulation with anti-CD3 and -CD28 (Fig. 5*B*); however, we did observe a trend toward increased luciferase activity, which is most likely the result of T cell proliferation. These data suggest that upon T cell activation, transcripts containing the 3′ UTR of *Pten* matching the miR-214 seed sequence are targeted and downregulated.

T cells constitutively expressing miR-214 show an increase in proliferation after stimulation

To further examine the role of miR-214 in T cell activation, the sequence encoding pre-miR-214 was cloned into the lentiviral vector pLL3.7 (27) to generate pLL3.7–pre-miR-214. In this vector, pre-miR-214 is expressed under the control of the U6 promoter and GFP is expressed under the control of the CMV promoter. Vesicular stomatitis virus G pseudotyped viral particles were then produced using either the pLL3.7–pre-miR-214 lentiviral construct or pLL3.7 alone. Bone marrow was then harvested from C57BL/6 mice and transduced with either pLL3.7–pre-miR-214 or pLL3.7 control virus and used to reconstitute lethally irradiated C57BL/6 mice. When hematopoietic reconstitution was complete, splenocytes were harvested from reconstituted mice, stained with 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE), and stimulated with anti-CD3 and -CD28 for 72 h. We then gated on GFP^+ $CD4^+$ or $CD8^+$ T cells and examined proliferation based on DDAO-SE dye dilution. We found that GFP^+ T cells derived from pLL3.7–pre-miR-214 virally transduced progenitors showed enhanced proliferation when compared with GFP^+ T cells from mice receiving pLL3.7 control transduced bone marrow (Fig. 6*A*, 6*B*). These data indicate that expression of miR-214 in T cells increases their capacity to proliferate upon stimulation.

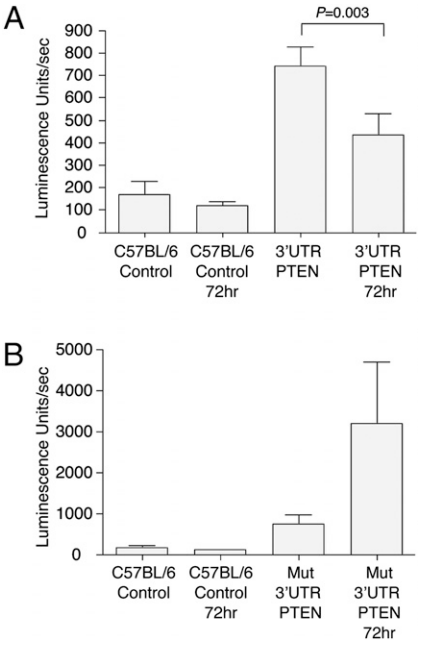

FIGURE 5. Transcripts containing the miR-214 target sequence in the 3′ UTR of PTEN are downregulated in activated T cells. Mice reconstituted with either MMP-LUC-3′-PTEN (*A*) or MMP-LUC-MUT-3′-PTEN (*B*) transduced bone marrow were sacrificed, and T cells were isolated. Luciferase activity was then measured in freshly isolated T cells or in T cells following stimulation with anti-CD3 and -CD28 for 72 h. Luciferase activity in unstimulated and activated T cells from control naive C57BL/6 mice was used to establish background in all experiments. Representative data from two independent experiments containing six mice per group are shown.

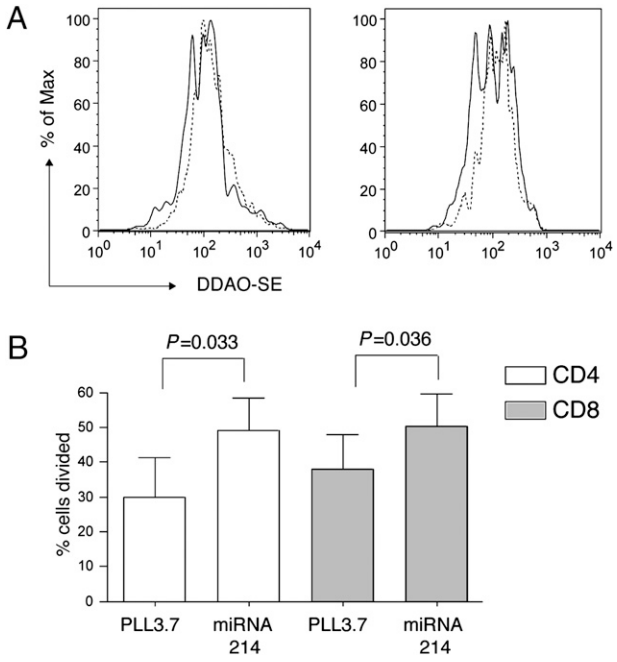

FIGURE 6. *A*, Expression of miR-214 increases proliferation of $CD4^+$ and $CD8^+$ T cells. Splenocytes were harvested from mice reconstituted with pLL3.7–pre-miR-214 (solid line) or pLL3.7 transduced bone marrow (dotted line), stained with DDAO-SE, and stimulated with anti-CD3 and -CD28 for 72 h. Shown is proliferation of GFP^+ CD4 (*left panel*) and CD8 (*right panel*) T cells based on DDAO-SE dye dilution. Data shown are from a representative experiment. *B*, Percentage of cells expressing miR-214 that divided after stimulation. Shown is proliferation of GFP^+CD4^+ (white bars) and GFP^+CD8^+ (gray bars) T cells from mice reconstituted with either pLL3.7–pre-miR-214 (miRNA-214) or pLL3.7 control transduced bone marrow. Proliferation was analyzed by flow cytometry based on DDAO-SE dilution. Cells from seven mice per group were analyzed. Shown are representative data.

Costimulatory blockade resulted in a decrease in miR-214 expression in alloreactive T cells

To understand if costimulation is required for miR-214 upregulation in T cells, we injected fully allogeneic BALB/c splenocytes into the footpads of untreated C57BL/6 mice or C57BL/6 mice that had been treated with CTLA4-Ig. Then, 5 d later, T cells from popliteal lymph nodes draining the injected footpads were isolated and miR-214 expression was analyzed by PCR. Expression of miR-214 was significantly increased in T cells isolated from mice injected with allogeneic cells, compared with C57BL/6 controls (Fig. 7*A*). Treatment with CTLA4-Ig resulted in a significant reduction in miR-214 expression in T cells from mice injected with allogeneic cells. Analysis of PTEN levels by Western blotting revealed an inverse correlation between miR-214 expression and PTEN. T cells from mice injected with allogeneic cells exhibited relatively low levels of PTEN when compared with control T cells from C57BL/6 mice (Fig. 7*B*). T cells from mice treated with CTLA4-Ig that were injected with allogeneic cells exhibited levels of PTEN similar to those observed in control T cells from C57BL/6 mice (Fig. 6*B*). To confirm that miR-214 is upregulated in a costimulation-dependent fashion, we purified T cells from the spleens of C57BL/6 mice and stimulated them with either anti-CD3 mAb alone or anti-CD3 in conjunction with anti-CD28. After 48 h, levels of miR-214 had substantially increased in T cells stimulated with anti-CD3 and -CD28 (Fig. 7*C*). In contrast, levels of miR-214 in T cells stimulated with anti-CD3 alone remained similar to those in control unstimulated T cells (Fig. 7*C*). As previously demonstrated (Fig. 3*B*), levels of PTEN substantially

decreased in T cells stimulated with anti-CD3 and -CD28. In contrast, levels of PTEN did not decrease in T cells stimulated with anti-CD3 alone, consistent with the observation that miR-214 is not upregulated in these cells (Fig. 7*D*). Together, these results suggest that miR-214 is upregulated in T cells upon Ag encounter in a CD28-dependent fashion.

Discussion

Several recent reports have shown that miRNAs play an important role in T cell development and function (33–36). We set out to identify miRNAs that regulate T cell activation. Using genome-wide miRNA expression profiling, we identified miRNAs that are differentially expressed in unstimulated and activated T cells. The data obtained from our microarray analysis led us to examine potential targets of miRNAs upregulated after T cell activation, focusing on potential targets known to play a role in T cell activation. Based on bioinformatics, miR-214 has been suggested to target PTEN (32). Moreover, in human ovarian cancer cells, miR-214 has been shown to induce cell survival and resistance to chemotherapeutic agents by targeting PTEN (24). Because of the importance of PTEN in controlling T cell activation, we therefore set out to examine whether miR-214 expression following T cell activation may function in regulating *Pten*. We reasoned that upregulation of miR-214 following T cell activation may serve to downregulate PTEN, thereby promoting T cell activation. Based on our array data, miR-214 was upregulated ~16-fold following activation with anti-CD3 and -CD28. PCR analysis revealed that miR-214 is rapidly upregulated after stimulation of either CD4 or CD8 T cells with anti-CD3 and -CD28. Upregulation of miR-214 inversely correlated with both levels of PTEN mRNA and protein in both naive T cells and memory $CD4^+$ T cells. Luciferase reporter assays formally demonstrated that the 3′ UTR of *Pten* matching the miR-214 seed sequence is targeted in activated T cells. Functionally, expression of miR-214 in T cells increased their capacity to proliferate upon stimulation. Moreover, costimulatory blockade using CTLA4-Ig resulted in a decrease in miR-214 expression in alloreactive T cells, suggesting that miR-214 is upregulated in T cells upon Ag encounter in a CD28-dependent fashion. Together these results support the idea that miR-214 plays a part in controlling T cell activation through its ability to target *Pten.* Although miR-214 has been shown to be important in development, muscle cell differentiation, cardiac hypertrophy, and protection from apoptosis (37–41), to our knowledge, a role for miR-214 in T cells has not been demonstrated.

Mammalian microRNAs can act either through the degradation of their target mRNA or through transcriptional blockade, which affects the levels of protein produced but not expression of mRNA. Our data indicate that levels of *Pten* mRNA, which is targeted by miR-214, are substantially reduced in activated T cells. This finding suggests that miR-214 acts through degradation of *Pten* mRNA in T cells.

Consistent with other reports, we observed that several miRNAs are either up- or downregulated in T cells following stimulation (42). However, in that report, analysis of expression levels by microarray led to the identification of relatively few miRNAs (miR-16, miR-142-3p, miR-142-5p, miR-150, miR-15b, and let-7f) that are significantly downregulated in virus-specific effector $CD8^+$ cells, compared with naive T cells, and relatively few miRNAs that are upregulated. Our results greatly expand the number of miRNAs observed to be up- or downregulated as a consequence of T cell activation and confirm other reports related to the role of miRNAs in lymphoid cells. Consistent with our data are the

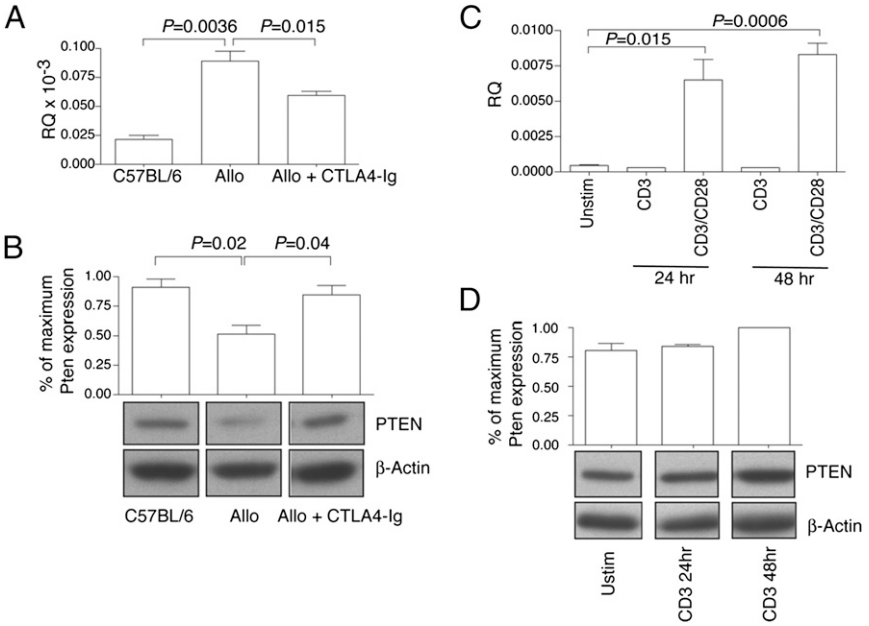

FIGURE 7. Expression of miR-214 in alloreactive T cells is upregulated in a CD28-dependent fashion. *A*, T cells were isolated from popliteal lymph nodes of C57BL/6 mice injected in the footpad with 2×10^7 allogeneic BALB/c splenocytes in the presence or absence of CTLA4-Ig. miR-214 expression was analyzed by PCR. T cells from naive C57BL/6 mice were used as controls. Shown is one representative experiment of three. *B*, PTEN levels in alloreactive T cells inversely correlate with miR-214. T cells were isolated from popliteal lymph nodes of C57BL/6 mice injected in the footpad with 2×10^7 allogeneic BALB/c splenocytes in the presence or absence of CTLA4-Ig. PTEN levels were determined by Western blot, as in Fig. 3. T cells from naive C57BL/6 mice were used as controls. Shown is one representative experiment of two. *C,* miR-214 expression in activated T cells is costimulation dependent. T cells were isolated from the spleens of C57BL/6 mice, and the level of miR-214 transcript was assessed by PCR immediately after isolation, or after activating with anti-CD3 or anti-CD3 and anti-CD28 mAbs for 24 or 48 h. *D*, PTEN expression is inversely correlated with miR-214 levels. T cells were isolated from the spleens of C57BL/6 mice, and the level of PTEN was assessed by Western blot immediately after isolation, or after activating with anti-CD3 mAb for 24 or 48 h, as in Fig. 3. Data shown are representative.

observation that during differentiation of naive T cells into Th1 or Th2 effectors, miR-150, miR-26a, and let-7d levels decrease and the observation that miR-150 expression in naive T cells is rapidly downregulated upon TCR engagement (43). Interestingly, miR-155 is upregulated by the transcription factor FOXP3 and critical for T regulatory cell function (35). Our array data show that miR-155 is upregulated in T cells following TCR stimulation, suggesting that activation of T cells with anti-CD3 and -CD28 enhances miRNAs required for T regulatory cell development (35).

We focused on miRNAs that might control *Pten* expression, because PTEN plays a critical role in regulating T cell activation. Although PTEN has been shown to be regulated by miRNAs in a cell type-specific fashion, miRNAs that regulate *Pten* in T cells have not been defined. PTEN is targeted by miR-21 in human hepatocellular cancers (20), by miR-26a in human glioma tumors (21), and by miR-216a and miR-217 in glomerular mesangial cells (22). Stimulation of T cells with anti-CD3 and -CD28 resulted in the downregulation of miR-21 and miR-26a, suggesting that these miRNAs may not be involved in regulation of *Pten* expression in T cells. miR-216a and miR-217 were not expressed in T cells. Expression of the miRNA 17–92 cluster has been shown to contribute to lymphoproliferative disease through its effects on *Pten* and *Bim* and lead to enhanced survival and proliferation of T cells overexpressing this miRNA cluster (23). miR-20a was the only member of the miR-17–92 cluster analyzed that was differentially expressed at significant levels in activated T cells; however, this miRNA was downregulated in activated T cells. This observation suggests that the miR-17–92 gene cluster may not be responsible for downmodulation of *Pten* in activated T cells. We also observed that expression of miR-181a, which has previously been shown to modulate T cell sensitivity by regulating multiple phosphatases (34), was downregulated in activated T cells. This observation is consistent with the observation that primed T cells contain fewer copies of miR-181a when compared with naive T cells.

It has previously been suggested that costimulation through CD28 is required for T cell activation to overcome negative regulation by PTEN (3). Our data suggest that stimulation through CD28 is required to upregulate miR-214, which in turn targets the 3′ UTR of *Pten,* thereby reducing PTEN levels and promoting T cell activation. Our results thus provide a link between the need for costimulation through CD28 and expression of an miRNA that decreases expression of a negative regulator of T cell activation. To our knowledge, a link between costimulation through CD28, regulation of PTEN, and miRNAs has not been previously described. This observation raises the possibility of manipulating miRNA expression for the purpose of altering T cell responsiveness.

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Disclosures

The authors have no financial conflicts of interest.

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