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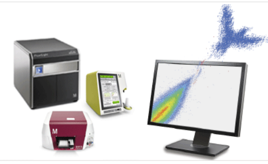

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Repression of Arginase-2 Expression in Dendritic Cells by MicroRNA-155 Is Critical for Promoting T Cell Proliferation

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Arginine, a semiessential amino acid implicated in diverse cellular processes, is a substrate for two arginases—Arg1 and Arg2—having different expression patterns and functions. Although appropriately regulated Arg1 expression is critical for immune responses, this has not been documented for Arg2. We show that Arg2 is the dominant enzyme in dendritic cells (DCs) and is repressed by microRNA-155 (miR155) during their maturation. miR155 is known to be strongly induced in various mouse and human DC subsets in response to diverse maturation signals, and miR155-deficient DCs exhibit an impaired ability to induce Ag-specific T cell responses. By means of expression profiling studies, we identified Arg2 mRNA as a novel miR155 target in mouse DCs. Abnormally elevated levels of Arg2 expression and activity were observed in activated miR155-deficient DCs. Conversely, overexpression of miR155 inhibited Arg2 expression. Bioinformatic and functional analyses confirmed that Arg2 mRNA is a direct target of miR155. Finally, in vitro and in vivo functional assays using DCs exhibiting deregulated Arg2 expression indicated that Arg2-mediated arginine depletion in the extracellular milieu impairs T cell proliferation. These results indicate that miR155-induced repression of Arg2 expression is critical for the ability of DCs to drive T cell activation by controlling arginine availability in the extracellular environment. *The Journal of Immunology*, **2014, 193: 1690–1700.**

Arginine, a semiessential amino acid playing important roles in multiple metabolic and cellular processes, is a substrate for arginases and NO synthases (NOS) (1). Arginases convert L-arginine into urea and L-ornithine, the latter serving as a substrate for the synthesis of polyamines and L-proline. NOS enzymes use L-arginine for the production of NO and L-citrulline. In addition to competing for their shared substrate, the biochemical pathways involving arginases and NOS enzymes regulate each other via feedback mechanisms (2, 3).

Mammals possess two arginases designated arginase 1 (Arg1) and arginase 2 (Arg2). Arg1 and Arg2 present 58% sequence identity at the amino acid level and are encoded by distinct genes (3). They catalyze the same biochemical reaction but differ with respect to subcellular localization and tissue distribution (4). Arg1 is a cytosolic enzyme expressed predominantly in the liver, where it functions as an enzyme in the urea cycle for the detoxification of ammonia. It also is expressed in nonhepatic tissues such as the breast, kidney, testis, salivary glands, and epidermis. Arg2 is instead localized in mitochondria and has a wide tissue distribution, being expressed most highly in the prostate, kidney, small intestine, and lactating mammary glands.

Arginases and arginine metabolism are involved in various normal biological processes as well as in diverse pathological conditions, including vascular, pulmonary, infectious, immunological, and neoplastic diseases (5, 6). Two general functions of arginases have been defined in the immune system. First, changes in arginase activity can modulate phagocytic function by affecting NO production. Second, arginase-mediated arginine depletion can inhibit T cell responses. In particular, arginine depletion recently has been implicated in tumor immunobiology (6–8). Several studies have highlighted a key role of arginine depletion resulting from increased arginase expression in the suppression of tumor-specific T cell responses (5, 6, 9–11).

Most information on the expression and function of arginases in the immune system concerns Arg1 (12). Arg1 expression is induced in macrophages by cAMP, LPS, and Th2 cytokines such as IL-4, IL-10, and IL-13 (13–18). Arg1 is also expressed in dendritic cells (DCs) and granulocytes (15, 19, 20). It has been suggested that increased Arg1 expression in macrophages leads to enhanced uptake of arginine from the extracellular microenvironment and hence to an inhibition of T cell responses (9, 21, 22). Arginine depletion induces downregulation of CD3ζ chain expression in activated T cells, leading to a deficiency in the TCR complex and the suppression of T cell proliferation (6, 22–25). In contrast to Arg1, little is known about the expression and function of Arg2 in the immune system. Arg2 expression has been documented in

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The microarray data presented in this article have been submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-1527.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Arg1, arginase 1; Arg2, arginase 2; BIC, B cell integration cluster; BM-cDC, BM conventional DC; BM-pDC, bone marrow plasmacytoid DC; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; miR155, microRNA-155; miRNA, microRNA; Mut, mutated; NOS, NO synthase; PGN, peptidoglycan; poly I/C, polyinosinic-polycytidylic acid; qRT-PCR, quantitative RT-PCR; UTR, untranslated region; WT, wild-type.

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mouse macrophages but was not found to be significantly modulated by Th1 or Th2 cytokines (15, 22, 26, 27). A recent report suggested that Arg2 expression can be induced moderately in mouse macrophages by stimulation with CpG plus IFN-γ (28).

It has become increasingly clear during the course of the past few years that microRNAs (miRNAs) exert critical functions in the development and function of the immune system. Specific miRNAs have been shown to modulate gene expression in various immune cell types. microRNA-155 (miR155) in particular was found to be a prominent player in the regulation of innate and adaptive immune responses (29–40). Regulation of the maturation, function, and maintenance of DCs involves several specific miRNAs (37). We and others recently demonstrated that miR155 is strongly induced in mouse and human DCs of various subtypes in response to a range of maturation signals and that it is required for efficient DC maturation and the activation of Ag-specific T cell responses (35, 36). miR155 is encoded by the B cell integration cluster (BIC) gene. Various genes involved in DC function have been shown to be downregulated by miR155 (29–38).

In this study, we identify Arg2 as a novel miR155 target in DCs. Experiments with mouse DCs exhibiting ectopic miR155 expression and DCs derived from miR155$^{-/-}$ mice demonstrated that miR155 represses the expression of Arg2. Bioinformatic and functional analyses confirmed that Arg2 mRNA is a direct target of miR155. Finally, in vitro and in vivo T cell activation assays demonstrated that deregulation of Arg2 expression in DCs leads to depletion of arginine in the extracellular milieu and thus induces a functional defect in miR155-deficient DCs. To our knowledge, this study provides the first evidence for posttranscriptional regulation of Arg2 and indicates that fine-tuning of Arg2 expression by miR155 in activated DCs is critical for their ability to drive T cell responses by controlling the availability of arginine in the extracellular environment.

Materials and Methods

Mice

miR155$^{-/-}$, OTII, OTII-CD45.1, and OTII-Rag2$^{-/-}$ mice have been described previously (29, 41). Mice were bred under specific pathogen-free conditions and used for experiments at 8–10 wk of age. Experiments were performed with permission of the cantonal and national veterinary authorities.

Cells and medium

The mouse DC^{2114} cell line and 293T cells were cultured as described previously (35, 42). Mouse lung myofibroblasts were cultured under 5% CO_2 in a humidified incubator in DMEM supplemented with 10% FCS and 1000 U/ml penicillin and 1000 μg/ml streptomycin. Bone marrow plasmacytoid DCs (BM-pDCs) and BM-conventional DCs (cDCs) were derived from tibia and femur bone marrow suspensions from 8- to 10-wk-old mice as described previously (35). CD11c$^+$ BM-cDCs, CD11c$^+$B220$^+$ BM-pDCs, total splenic CD11c$^+$ DCs, CD11c$^+$CD8$^+$ splenic DCs, and CD11c$^+$ CD8$^-$ splenic DCs were purified by sorting with a FACSVantageSE (BD Biosciences). DC maturation was induced with 100 ng/ml LPS (Alexis), 0.05 mg/ml polyinosinic-polycytidylic acid (poly I/C) (Amersham Biosciences), 0.2 nM CpG ODN 1826 (Trilink Biotech), 10 μg/ml peptidoglycan (PGN) (Sigma-Aldrich), 500 ng/ml PAM_3CSK_4 (InvivoGen), 200 ng/ml flagellin (InvivoGen), or 3 μg/ml imiquimod (InvivoGen). CD4$^+$ T cells from spleen and lymph nodes of WT or OTII mice were purified using a CD4$^+$ T cell isolation kit (Miltenyi Biotec). DMEM without arginine was purchased from Genaxxon Bioscience and supplemented with 4 μM $MnCl_2$, 10% FCS, 1000 U/ml penicillin, and 1000 μg/ml streptomycin. L-Arginine–free DMEM or supernatants from vector or Arg2 transduced DC^{2114} cells were supplemented with 400 μM L-arginine (Sigma-Aldrich).

Illumina expression profiling

Five hundred nanograms of total RNA was purified using TRIzol from sorted immature and mature CD11c$^+$ BM-cDCs and CD11c$^+$ B220$^+$ BM-pDCs from wild-type (WT) and miR155$^{-/-}$ mice. Labeling was performed using the Illumina TotalPrep RNA Amplification kit. Biotin-labeled cRNA was hybridized to Illumina mouse genome WG-6_V2 microarrays. Arrays were washed, stained, and scanned using Illumina's BeadStation 500 system and an iScan instrument equipped with Autoloader2. Data were normalized and analyzed using Illumina Beadstudio 3.1.3 (background correction and quantile normalization without scaling). Microarray data reported in our study have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession numbers E-MTAB-1527 (Supplemental Fig. 1A).

Lentiviral transductions

Arg2 cDNA was amplified by PCR (primers 5′-ATGACGTTTAAACGCCACCATGTTCCTGAGGAGCAGC-3′ and 5′-ATGACGTTTAAACCTAAATTCTCACACATTCTTC-3′) and cloned into the lentiviral pWPI vector, which contains a GFP expression cassette for assessing transduction efficiency (35). BIC and mutated BIC lentiviral vector constructs have been described previously. Transduction of DC^{2114} cells and BM-cDCs was performed as previously described (35) and assessed on the basis of GFP expression.

Quantitative RT-PCR

Real-time PCR of mouse miR155 was performed using the miRCURY LNA Universal RT microRNA PCR system according to the manufacturer's instructions (Exiqon). miR155 expression of was normalized using U6 snRNA and 5S rRNA (Exiqon). Mouse mRNAs were quantified by quantitative RT-PCR (qRT-PCR) as described previously (35). Expression was normalized using β-actin mRNA, TBP mRNA, or 18S rRNA. Primer sequences were as follows: mouse Arg2 forward (5′-CTGTGTCACCATGGGAGGAG-3′) and reverse (5′-GCATGAGCATCAACCCAGAT-3′); mouse Arg1 forward (5′-ATGAAGAGCTGGCTGGTGTG-3′) and reverse (5′-GCCAGAGATGCTTCCAACTG-3′). Primers sequences for mouse BIC, IL-6, and IL12-p40 have been reported previously (35).

Luciferase reporter assays

The 3′-untranslated regions (UTRs) of mouse Arg2 (270 bp) and Arg1 (402 bp) mRNAs were amplified by PCR and inserted downstream of the Renilla luciferase gene in the dual luciferase reporter plasmid psiCHECK-2 (Promega). The QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) was used to mutate the miR155 binding site. Luciferase reporter assays were performed as described previously (32).

Flow cytometry

Flow cytometry was performed with a FACSCalibur (BD Biosciences) and analyzed with WinMDI software. Staining was performed in the presence of saturating concentrations of 2.4G2 anti-FcγRII/III mAbs. Abs were as follows: anti-CD4 (RM4-5), anti-CD45R/B220 (RA3-6B2), anti-CD11c (N418), anti-CD69 (H1.2F3), anti-CD44 (IM7), anti-CD247 (CD3 ζ) (6B10.2), anti-Vα2 TCR (B20.1), anti-CD45.1 (A20), and anti-AnnexinV (BioLegend). Intracellular staining was done with the Fixation/Permeabilization kit (eBioscience). T cell proliferation was assessed by flow cytometry using an anti-human Ki67 staining kit (BD Biosciences).

Western blotting

Protein extracts were fractionated by SDS-PAGE, and Western blotting was performed using anti-mouse Arg2 (sc-20151; Santa Cruz Biotechnology), anti-mouse Arg1 (sc-20150; Santa Cruz Biotechnology), and anti-tubulin (B-5-1-2; Sigma-Aldrich).

T cell stimulation and proliferation

Purified CD4$^+$ T cells were activated in vitro with anti-CD3ε (2 μg/ml; 145-2C11) and anti-CD28 (2 μg/ml; 37.51). Purified CD4$^+$ T cells were labeled with 0.5 μM CFSE (BioLegend). Stimulated CD4$^+$ T cells were cultured in flat-bottom 96-well culture plates at 20×10^5 cells/well. Proliferation was assessed by Ki67 or CFSE staining. For in vivo experiments, 10^6–10^7 CD4$^+$ T cells purified from LNs of OTII-CD45.1 or OTII-Rag2$^{-/-}$ mice were injected i.v. into WT recipient mice. A total of 2×10^6 transduced DC^{2114} cells were loaded with 1 μg/ml OVA peptide and transferred into the recipients. Spleens were harvested after 2 or 3 d, and Vα2$^+$ OTII or OTII-CD45.1 T cell proliferation was assessed by Ki67 or CFSE staining.

ELISAs

Mouse IL6, TNF, IL12-p40, and IL-10 were quantified in culture supernatants using an ELISA kit, according to the manufacturer's instructions (eBioscience).

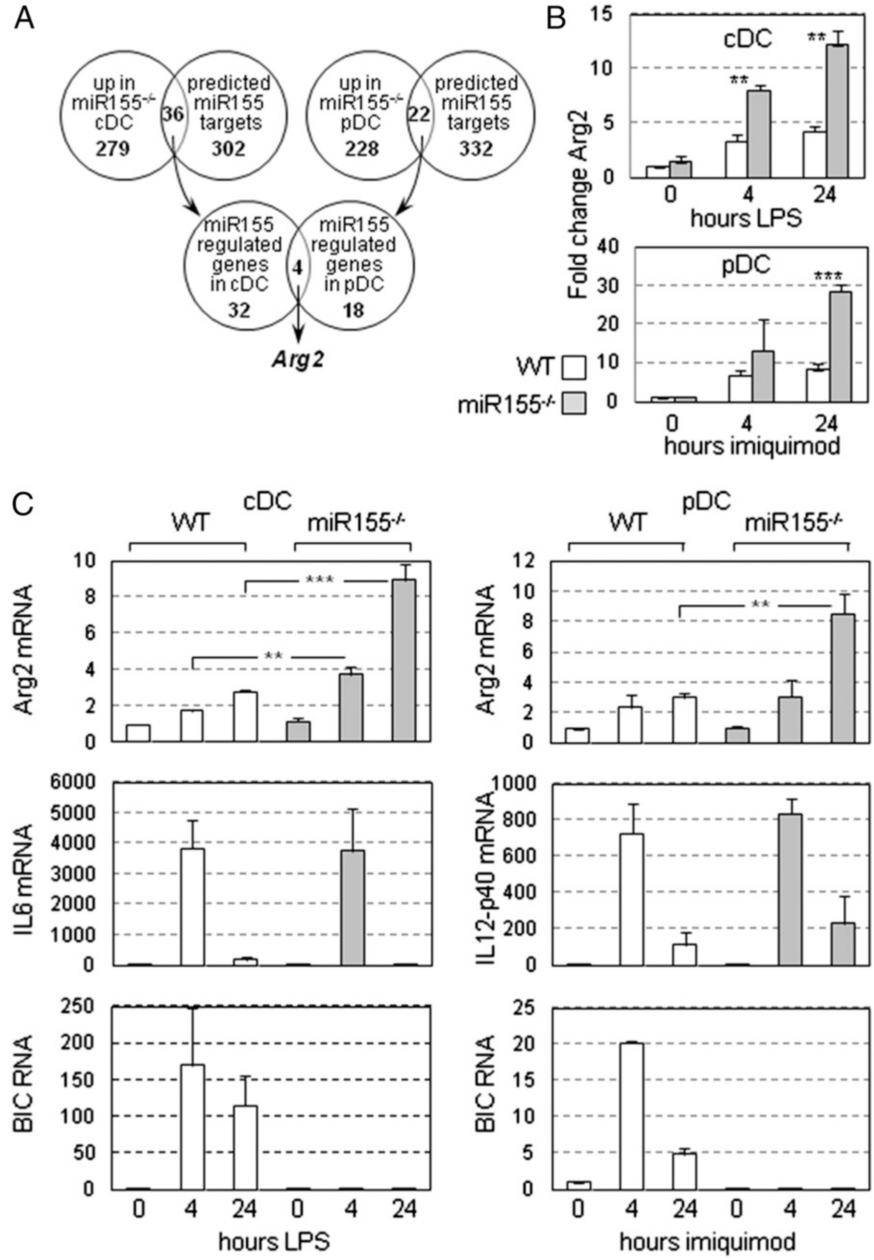

FIGURE 1. Identification of Arg2 mRNA as a novel miR155-regulated mRNA in DCs. (**A**) Schematic representation of the identification of potential miR155 targets in BM-cDCs and BM-pDCs. (**B**) Signal intensities observed for Arg2 mRNA in microarray experiments are represented for BM-cDCs and BM-pDCs from WT and miR155$^{-/-}$ mice. DCs were unstimulated (0) or activated for 4 or 24 h with LPS (BM-cDCs) or imiquimod (BM-pDCs). Results are represented as fold change relative to unstimulated WT DCs. Means and SDs were derived from three microarray experiments. (**C**) qRT-PCR was used to analyze Arg2 and BIC (miR155 precursor) expression in WT and miR155$^{-/-}$ BM-cDCs and BM-pDCs stimulated for 0, 4, and 24 h with LPS (BM-cDCs) or imiquimod (BM-pDCs). IL-6 and IL12-p40 mRNAs were used as controls for maturation. Results are represented as fold change relative to unstimulated WT DCs. Means and SDs were derived from three experiments. $**p < 0.01$, $***p < 0.001$.

Immunofluorescence microscopy

Cells were seeded on glass coverslips, cultured for 24 h in the absence or presence of CpG, fixed for 10 min at room temperature with 1% paraformaldehyde in PBS, and stained with anti–Tom-20 (a gift from L. Scorrano, University of Padua, Padua, Italy), anti-mouse Arg2, and DAPI. Cells were observed with a Zeiss Axiocam microscope using Axiovision software.

Enzymatic assays

Arginase activity was measured using the QuantiChrom Arginase Assay kit (BioAssay Systems). Arginine concentrations were determined using the PD-direct Media Analytical Services (Invitrogen). $NO3^-$ (nitrate) and $NO2^-$ (nitrite) concentrations were measured using Griess reagent and a total nitrate/nitrite colorimetric assay (Cayman Chemical) following the reduction

Table I. Analysis of miR155 targets in BM-cDCs and BM-pDCs

Gene		BM-cDCs		BM-pDCs		
Symbol	Name	Fold Change[a]	*p* Value[b]	Fold Change[a]	*p* Value[b]	miRmap[c] Score
Arg2	Arginase type II	2.88	0.00227	3.59	0.00072	80.85
Csnk1g2	Casein kinase 1 γ 2	1.65	0.00001	1.69	0.00116	76.72
Hn1l	Hematological and neurological expressed 1-like	3.34	0.03459	1.26	0.01506	84.02
Tspan14	Tetraspanin 14	2.27	0.00009	1.71	0.02060	96.25

[a]Expression in miR155$^{-/-}$ BM-DCs relative to WT BM-DCs.
[b]*t* test of expression in miR155$^{-/-}$ BM-DCs relative to WT BM-DCs.
[c]Prediction model (43).

of nitrate to nitrite. Absorbance was measured at 550 nm using a plate reader, and nitrite concentrations were determined using $NaNO_2$ as standard.

Lung histology and collagen quantification

Paraffin-embedded lung sections (5 μm) were stained with Masson Trichrome and observed in a Zeiss Axiocam microscope using Axiovision software. For collagen quantification, lungs were homogenized in 1 ml 0.5 M acetic acid containing 0.1 mg/ml pepsin overnight, and soluble collagen was measured using the Sircol collagen assay (Biocolor), according to the manufacturer's instructions. Data are expressed as micrograms of collagen per milligram of total protein.

Statistical analysis

The *p* values were calculated using the Student *t* test with two-tailed distribution and two-sample unequal variance parameters.

Results

Identification of mRNAs regulated by miR155 in DCs

Microarray experiments were performed to compare the gene expression profiles exhibited by immature and activated BM-cDCs and BM-pDCs from WT and miR155$^{-/-}$ mice (Supplemental Fig. 1A). DCs were activated for 4 or 24 h with LPS (BM-cDCs) or imiquimod (BM-pDCs). Well-established miRNA binding site prediction criteria were used to generate a list of potential targets of miR155 (Fig. 1A) (43). The list of putative targets was compared with the set of mRNAs that exhibited elevated expression in activated BM-cDCs and BM-pDCs from miR155$^{-/-}$ mice. This identified target mRNAs likely to be regulated by miR155 in BM-cDCs and/or BM-pDCs (Fig. 1A). Only four of these mRNAs (*Arg2*, *Csnk1g2*, *Hn1l*, and *Tspan14*) were found to be potential miR155 targets in both BM-cDCs and BM-pDCs (Fig. 1A, Table I). Given the growing interest in the role of arginine metabolism in the immune system, we focused our attention on the *Arg2* gene.

Regulation of Arg2 expression by miR155 in DCs

Microarray signal intensities corresponding to *Arg2* were significantly increased relative to WT in miR155$^{-/-}$ BM-cDC and BM-pDCs after 4 and 24 h of stimulation with LPS or imiquimod, respectively (Fig. 1B, Supplemental Fig. 1A). qRT-PCR experiments confirmed that the induction of Arg2 mRNA expression was significantly elevated in miR155$^{-/-}$ BM-cDCs and BM-pDCs compared with their WT counterparts (Fig. 1C, Supplemental Fig. 1B). Maturation was controlled by measuring the induction of IL6 mRNA, IL12-p40 mRNA, and the miR155 precursor RNA (BIC).

Arg2 protein expression and arginase activity were quantified in WT and miR155$^{-/-}$ BM-cDCs and BM-pDCs following exposure to LPS or imiquimod, respectively. Arg2 protein was markedly increased in miR155$^{-/-}$ BM-cDCs and BM-pDCs compared with WT DCs (Fig. 2A, Supplemental Fig. 1C). Arginase activity also was increased strongly and significantly in miR155$^{-/-}$ BM-cDCs and BM-pDCs (Fig. 2B). This increase in arginase activity resulted from elevated Arg2 expression because Arg1 mRNA and protein levels were not deregulated in miR155$^{-/-}$ BM-cDCs and absent in BM-pDCs (Fig. 2A, Supplemental Fig. 2).

Deregulated Arg2 expression was confirmed in miR155$^{-/-}$ BM-cDCs following exposure to the TLR3 ligand poly I/C, the TLR2 ligands PGN and PAM_3CSK_4, the TLR7 ligand imiquimod, and the

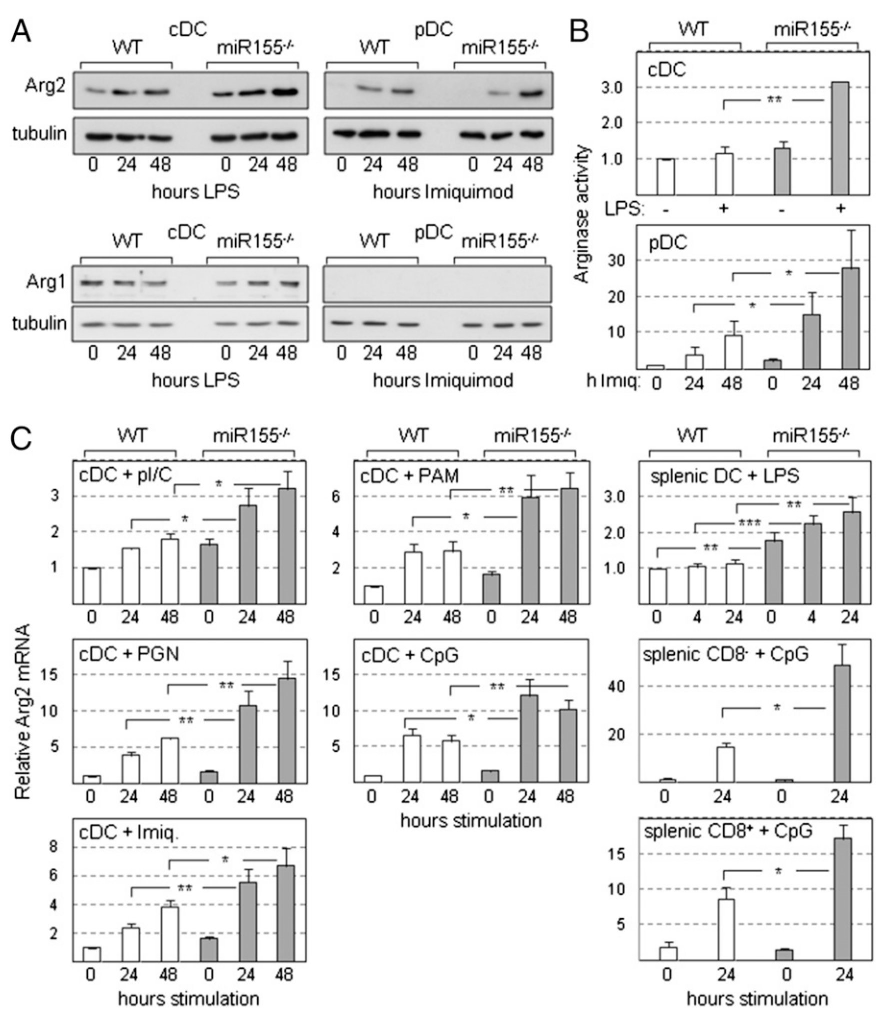

FIGURE 2. Deregulated Arg2 expression in DCs from miR155$^{-/-}$ mice. (**A**) Expression of Arg2 and Arg1 proteins was analyzed by Western blotting in WT and miR155$^{-/-}$ BM-cDCs and BM-pDCs stimulated for 0, 24, and 48 h with LPS (BM-cDCs) or imiquimod (BM-pDCs). Tubulin was used as internal control. Results are representative of two independent experiments. (**B**) Arginase activity was measured in WT and miR155$^{-/-}$ BM-cDCs and BM-pDCs stimulated for 0 and 48 h with LPS (cDCs) or 0, 24, and 48 h with imiquimod (pDCs). (**C**) Arg2 mRNA expression was analyzed by qRT-PCR in immature WT and miR155$^{-/-}$ BM-cDCs treated for 0, 24, and 48 h with poly I/C, PGN, imiquimod, PAM_3CSK_4, or CpG; total splenic DCs treated for 0, 4, and 24 h with LPS, and CD8$^-$ and CD8$^+$ splenic DCs treated for 0 and 24 h with CpG. Results in (B) and (C) are represented as fold change relative to unstimulated WT cells. Means and SDs were derived from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

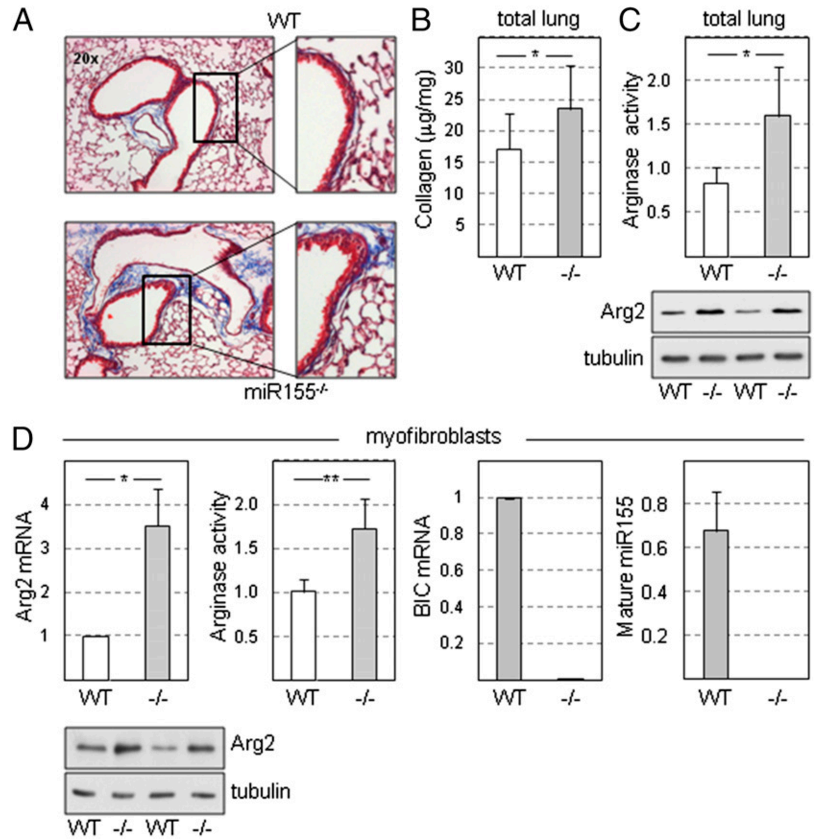

FIGURE 3. Deregulated Arg2 expression in the lungs of miR155$^{-/-}$ mice. (**A**) Masson Trichrome staining of sections showing lung bronchioles of WT and miR155$^{-/-}$ mice. Collagen is stained in blue. Results are representative of five independent experiments. (**B**) Total soluble lung collagen was quantified for WT and miR155$^{-/-}$ mice. (**C**) Arginase activity and Arg2 protein were analyzed in total lung cells from WT and miR155$^{-/-}$ mice. Means and SDs in (B) and (C) were derived from at least five mice per group. (**D**) BIC RNA (miR155 precursor), mature miR155, Arg2 mRNA, Arg2 protein, and arginase activity were measured in lung myofibroblasts from WT and miR155$^{-/-}$ mice. Tubulin was used as internal control. Means and SDs were derived from at least six mice per group. $*p < 0.05$, $**p < 0.01$.

TLR9 ligand CpG. As observed following exposure to the TLR4 ligand LPS, Arg2 expression was significantly derepressed in miR155$^{-/-}$ BM-cDCs exposed to these stimuli (Fig. 2C). Significantly increased Arg2 expression also was observed in total splenic miR155$^{-/-}$ DCs stimulated with LPS, and splenic CD8$^-$ and CD8$^+$ miR155$^{-/-}$ DCs stimulated with CpG (Fig. 2C, *right panels*).

Because arginases and NOS enzymes compete for the same substrate, increased arginase activity is expected to lead to reduced NO production. NO production was indeed significantly impaired in activated miR155$^{-/-}$ BM-cDCs (Supplemental Fig. 3). This was not because of reduced inducible NOS expression (data not shown). Collectively, these results suggest that Arg2 expression and arginase activity are repressed by miR155 in all DC subsets examined and in response to all maturation stimuli tested.

Deregulated Arg2 expression in the lungs of miR155$^{-/-}$ mice

miR155$^{-/-}$ mice develop a lung pathology characterized by increased airway remodeling and bronchiolar collagen deposition (29). This phenotype could be a consequence of deregulated Arg2 expression because arginase activity regulates the production of collagen via increased proline synthesis (44). We confirmed that a marked accumulation of collagen is evident around lung bronchioles in miR155$^{-/-}$ mice (Fig. 3A). Total collagen was significantly increased in the lungs of miR155$^{-/-}$ mice compared with WT mice (Fig. 3B). We next assessed Arg2 expression in the lungs of WT and miR155$^{-/-}$ mice. Significant increases in arginase activity and Arg2 protein were observed in total lung extracts from miR155$^{-/-}$ mice (Fig. 3C). Because collagen is mainly produced by myofibroblasts, mir155 expression, Arg2 expression, and arginase activity were assessed in myofibroblasts purified from miR155$^{-/-}$ and WT lungs. WT myofibroblasts expressed both mature miR155 and the BIC miR155 precursor RNA (Fig. 3D). Significant increases in Arg2 mRNA, Arg2 protein, and arginase activity were evident in lung myofibroblasts from miR155$^{-/-}$ mice (Fig. 3D), suggesting that collagen deposition in the lungs of miR155$^{-/-}$ mice correlates with derepressed Arg2 expression in myofibroblasts. These results indicate that the control of Arg2 expression by miR155 is not restricted to DCs.

Arg2 mRNA is a direct target of miR155

Arg2 mRNA was analyzed for the presence of potential miR155 binding sites by computational approaches relying on well-established criteria for identifying miRNA target sites (43). A likely miR155 binding site was identified in the 3′-UTR of mouse Arg2 mRNA (Fig. 4A). This site contains a seven-nucleotide sequence exhibiting high complementarity to the "seed" region situated at the 5′-end (positions 2–8) of miR155. We generated a luciferase reporter construct in which the 3′-UTR from Arg2 mRNA was inserted downstream of the luciferase gene (WT-Arg2). A matching control construct was generated by mutating the seed region of the potential miR155 binding site (Mut-Arg2). We also generated a control construct containing the 3′-UTR from Arg1 mRNA (WT-Arg1). Activities of the WT-Arg2, WT-Arg1, and Mut-Arg2 constructs were measured after transfection into 293T cells in conjunction with either the miR155 precursor or a BIC (miR155) expression vector. Luciferase activity of the WT-Arg2 construct was strongly reduced when cotransfected with the miR155 precursor and BIC expression vector (Fig. 4B). In contrast, no reductions were observed for either the Mut-Arg2 or WT-Arg1 constructs. These results confirm that miR155 targets Arg2 mRNA by binding to a specific site in its 3′-UTR.

To further confirm that Arg2 expression is controlled by miR155, we transduced WT and miR155$^{-/-}$ BM-DCs with a lentiviral vector containing the *BIC* (miR155) gene or a control vector containing a mutated *BIC* gene lacking the miR155 sequence (35). Uninduced WT BM-DCs transduced with the BIC expression

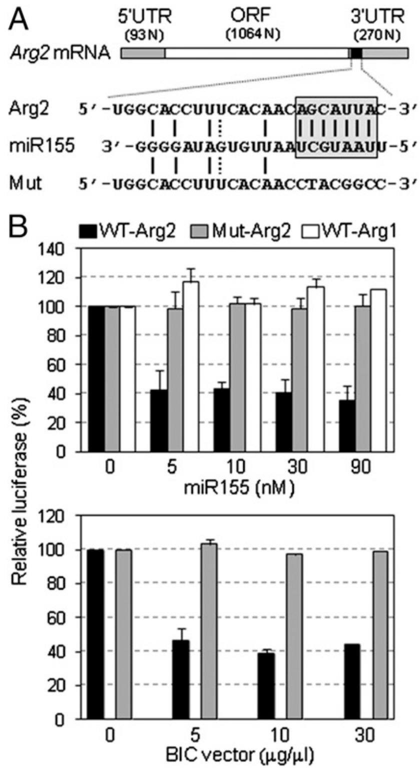

FIGURE 4. Arg2 is a direct target of miR155. (**A**) A schematic representation of mouse Arg2 mRNA is shown: sizes in nucleotides of the 5′-UTR, open reading frame (ORF), and 3′-UTR are indicated. The 3′-UTR contains one predicted binding site (black box) for miR155. The sequence of mouse miR155 is shown aligned with its predicted target site in the 3′-UTR of Arg2 mRNA. A-U and G-C bp are represented by solid lines. G-U bp are represented by dotted lines. The miR155 seed region is enclosed by a box. The sequence of the mutated (Mut) 3′-UTR of mouse Arg2 mRNA is indicated. (**B**) Luciferase reporter constructs containing the WT 3′-UTR of mouse Arg2 mRNA (Arg2), the mutated 3′-UTR of mouse Arg2 mRNA (Mut-Arg2), and the WT 3′-UTR of mouse Arg1 mRNA (Arg1) were transfected into 293T cells, alone or together with the indicated amounts of mouse miR155 precursor (*top panel*) or BIC (miR155 precursor) expression vector (*bottom panel*). Luciferase activity was measured 24 h after transfection, normalized with respect to activity obtained with the empty reporter vector and expressed as relative luciferase activity. The average and SDs were derived from three independent experiments.

vector exhibited a 5-fold increase in mature miR155, which is within a physiological range compared with the 13-fold increase observed in WT BM-DCs after 24 h of stimulation with LPS (Fig. 5A). The impact of enforced miR155 expression on Arg2 mRNA levels was assessed in the transduced DCs prior to and after exposure to LPS (Fig. 5A). Arg2 mRNA levels were significantly decreased in BIC-transduced WT and miR155$^{-/-}$ BM-DCs (Fig. 5A). The same approach was used to assess the impact of enforced BIC expression on Arg2 mRNA levels in the mouse DC^{2114} cell line (Fig. 5B). Arg2 mRNA levels were again significantly decreased by miR155 overexpression in unstimulated or CpG-treated BIC-transduced DC^{2114} cells (Fig. 5B).

Functional consequences of deregulated Arg2 expression

To study the biological relevance of appropriately-regulated Arg2 expression in DCs, we studied the consequences of deregulating Arg2 expression in these cells. Ectopic Arg2 expression was induced in DC^{2114} cells using a lentiviral vector directing the expression of Arg2 under the control of heterologous promoter and 3′-UTR regions. Transduction efficiency was assessed by analyzing vector-encoded GFP expression by flow cytometry (Fig. 6A). Immunofluorescence experiments demonstrated that the overexpressed Arg2 was correctly localized in mitochondria, as assessed by colocalization with the mitochondrial marker Tom-20 (Fig. 6B). Mitochondrial localization was not affected by stimulation with CpG. Western blot analysis confirmed the increased abundance of Arg2 protein in Arg2-transduced DC^{2114} cells (Fig. 6C). A 16-fold increase in arginase activity was observed in the Arg2-transduced DC^{2114} cells (Fig. 6D). This fold increase is within the range of that observed in miR155$^{-/-}$ cells (3- to 30-fold; Fig. 2B). Finally, overexpression of Arg2 resulted in a selective depletion of arginine from the culture medium (Fig. 6E).

Lowering arginine concentrations in the culture medium leads to impaired proliferation of WT anti–CD3/anti–CD28-activated CD4$^+$ T cells and downregulation of the CD3 ζ-chain (Supplemental Fig. 4A). We therefore investigated whether overexpression of Arg2 in DC^{2114} cells has an impact on their ability to induce T cell proliferation. Activated T cells were cultured in supernatants derived from control and Arg2-transduced DC^{2114} cells, and proliferating Ki67$^+$ T cells or CFSE-labeled T cells were quantified by flow cytometry. Proliferation was significantly reduced when T cells were cultured in supernatants from Arg2-transduced DC^{2114} cells compared with supernatants from vector-transduced DC^{2114} cells (Fig. 6F, Supplemental Fig. 4B). In contrast, no reduction in the frequency of activated CD69$^+$ T cells was observed (Fig. 6F). Reduced T cell proliferation was not associated with increased T cell apoptosis, because the frequency of AnnexinV$^+$7AAD$^+$ cells was comparable between T cells cultured in supernatants from Arg2 and vector-transduced DC^{2114} cells (Supplemental Fig. 4B). The same experimental setting was used to examine T cell proliferation in supernatants derived from WT and miR155$^{-/-}$ BM-cDCs. Proliferation was significantly impaired when the T cells were cultured in supernatants from miR155$^{-/-}$ BM-cDCs (Fig. 6G).

To determine whether arginine depletion was responsible for the impaired T cell proliferation, we supplemented supernatants from the vector- and Arg2-transduced DC^{2114} cells with exogenous L-arginine. T cell proliferation was significantly rescued, albeit only partially, by the addition of L-arginine to supernatants from the Arg2-transduced DC^{2114} cells (Fig. 6H). Furthermore, to test whether Arg2 overexpression might alter cytokine production by DCs, we compared CpG-induced IL-6, IL12-p40, IL-10, and TNF production by Arg2- and vector-transduced DC^{2114} cells. No significant difference in the induction of these cytokines was observed (Supplemental Fig. 4C).

The impact of Arg2-mediated arginine depletion on T cell proliferation was further assessed by means of in vivo Ag presentation assays, based on the adoptive transfer of OTII T cells and OVA-loaded control or Arg2-transduced DC^{2114} cells. Splenic OTII T cell proliferation and activation were examined by flow cytometry 48 and 72 h after transfer. In the absence of DC^{2114} transfer, only a basal level (1–2%) of OTII T cell proliferation was observed. Both vector- and Arg2-transduced DC^{2114} cells induced significant proliferation (Fig. 7A). However, OTII T cell proliferation was significantly impaired in the case of transfer of Arg2-transduced DC^{2114} cells (Fig. 7A, 7C). This impaired proliferation correlated with significant downregulation of the CD3 ζ-chain (Fig. 7B). No change in expression of the T cell activation markers CD69 and CD44 was observed (data not shown). In addition, no change in T cell apoptosis was observed (Fig. 7C). These results demonstrate that arginine depletion induced by deregulated Arg2 expression leads to impaired proliferation of CD4$^+$ T cells in vivo.

Discussion

We report in this paper that repression of Arg2 expression by miR155 in activated DCs is required for avoiding excessive arginine depletion in the extracellular microenvironment and is

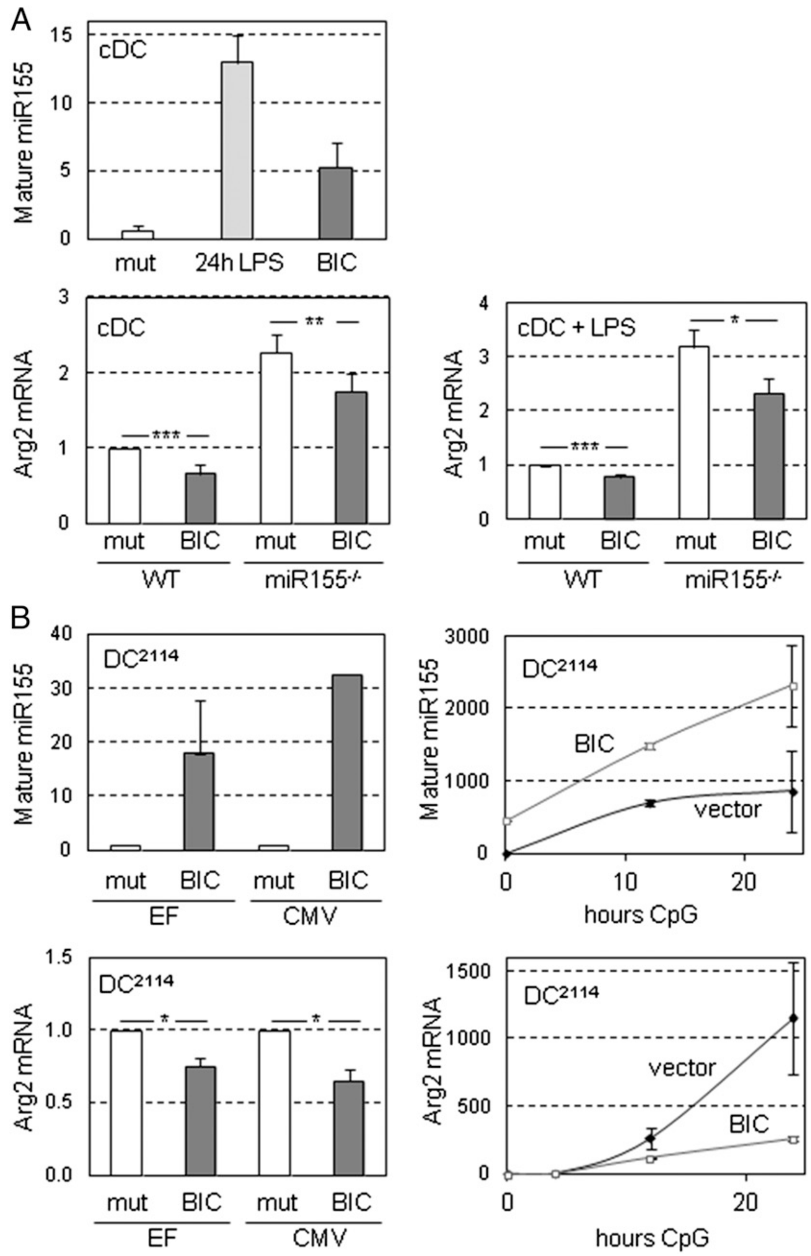

FIGURE 5. Arg2 expression is downregulated by miR155. (**A**) Expression of mature miR155 was analyzed by qRT-PCR in 24-h LPS-treated BM-cDCs and in BM-cDCs transduced with a BIC expression vector (BIC) or a mutated BIC control vector (Mut). Expression of Arg2 mRNA was analyzed by qRT-PCR in unstimulated and LPS-treated BM-cDCs from WT or miR155$^{-/-}$ mice transduced with a BIC expression vector (BIC) or the mutated control vector (Mut). Results are represented as fold change in miR155 or Arg2 mRNA expression relative to control vector–transduced WT cells. (**B**) *Left panel*, The expression of mature miR155 and Arg2 mRNA was analyzed in immature DC^{2114} cells transduced with the BIC expression vector (BIC) or mutated control vector (Mut) under the control of two different promoters (EF and CMV). The results are represented as fold change in mature miR155 or Arg2 mRNA expression relative to the control vector–transduced cells. *Right panel*, Time-course experiments were performed to analyze the expression of mature miR155 and Arg2 mRNA in immature and CpG-stimulated mouse DC^{2114} cells transduced with the BIC expression vector (BIC) or a control vector. Results are provided as fold change relative to unstimulated DC^{2114} cells. The means and SDs were derived from three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

consequently critical for the ability of mature DCs to prime T cell responses. Taken together with prior studies addressing the role of miR155 in DCs, our results establish miR155 as a central regulator of DC maturation and function.

Recent years have witnessed a growing interest in the immunomodulatory functions of amino acid metabolism. The main enzymatic pathways involved in this process of "immunomodulation by starvation" are tryptophan catabolism by IDO and arginine catabolism by arginases and NOS enzymes (6, 45). Studies on the immunoregulatory roles of arginine depletion have focused mainly on arginase expression by macrophages, myeloid-derived suppressor cells (MDSCs), and tumor cells (8, 46, 47). Our results demonstrate that the regulation of arginase-mediated arginine depletion also makes key contributions to DC function (Fig. 7).

Studies on the functional relevance of arginase expression by cells of the immune system have focused almost exclusively on Arg1, which is the major arginase expressed in macrophages, MDSCs, and granulocytes (12, 18, 20). Data on Arg2 are scarcer. It is expressed at a basal level in macrophages, where it is not modulated to a major extent by extracellular signals, such as Th1 and Th2 cytokines (15). Certain infections, such as *Helicobacter pylori*, induce Arg2 expression in macrophages (48). We show in this study that Arg2 is the dominant arginase in DCs. Arg2 mRNA and protein expression are markedly induced during maturation in all DC subtypes tested, including BM-cDCs, BM-pDCs, splenic DCs, and the CD8^{+} DC^{2114} cell line (Figs. 1, 2). In sharp contrast, Arg1 expression is not increased during maturation of BM-cDCs and is absent in BM-pDCs (Supplemental Fig. 2). Derepressed Arg2 expression in activated BM-cDCs and BM-pDCs from miR155$^{-/-}$ mice leads to a strong increase in total arginase activity (Fig. 2B). Similarly, overexpression of Arg2 in DC^{2114} cells to levels comparable to those observed in miR155$^{-/-}$ DCs strongly increases total arginase activity and arginine consumption (Fig. 6D, 6E). Collectively, these results indicate that the overall arginase activity in DCs is determined mainly by Arg2. Arg1 appears to make only a minor contribution. The role of Arg2 in modulating immune responses has therefore been underestimated and merits further investigation.

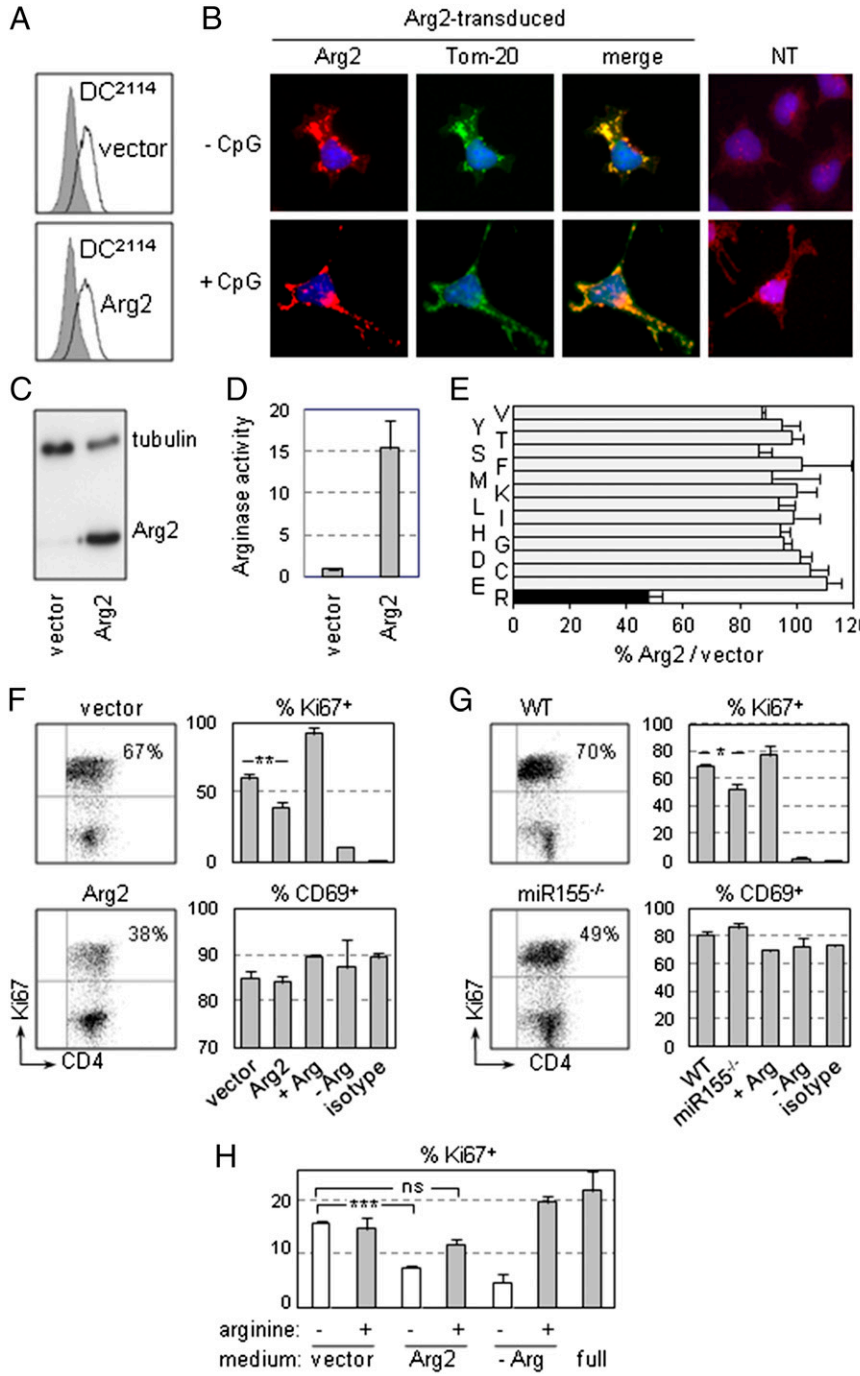

FIGURE 6. Deregulated Arg2 expression in DCs impairs T cell proliferation in vitro. (**A**) Analysis of GFP expression by flow cytometry in DC[2114] cells transduced with control or Arg2 expression vectors. (**B**) Arg2 expression was analyzed by immunofluorescence staining (red) in unstimulated (−CpG) and CpG-stimulated (+CpG) Arg2-transduced and nontransduced (NT) DC[2114] cells. Arg2-transduced cells were costained with Tom-20 (blue) to visualizing mitochondria. Merged images indicate colocalization of Arg2 and Tom-20 staining. Original magnification ×630. (**C**) The expression of Arg2 protein was analyzed in unstimulated control and Arg2-transduced DC[2114] cells. (**D**) Arginase activity was measured in unstimulated control and Arg2-transduced DC[2114] cells. (**E**) The concentrations of the indicated amino acids (single letter code) were determined in supernatants from unstimulated control and Arg2-transduced DC[2114] cells. Results are presented as percentage of the concentration in the control. The average and SD were derived from two independent experiments. (**F**) Activated $CD4^+$ T cells were cultured in supernatants from unstimulated control and Arg2-transduced (Arg2) DC[2114] cells. As controls, the cells were cultured in the presence of fresh medium (+Arg) or medium lacking arginine (−Arg). T cell activation was induced with anti-CD3/CD28 and assessed by CD69 staining. Proliferation was assessed by analyzing Ki67 expression. Representative flow cytometry profiles are shown for Ki67 staining. In control samples, the Ki67 Ab was replaced with an isotype control. The percentages of $CD69^+$ and $Ki67^+CD4^+$ T cells are indicated. Means and SDs were derived from three experiments, each with three mice per group. (**G**) Activated $CD4^+$ T cells were cultured in media from WT and $miR155^{-/-}$ BM-cDCs. Control cells were cultured in fresh medium (+Arg) or medium lacking arginine (−Arg). Proliferation was assessed by analyzing Ki67 expression. Representative flow cytometry profiles are shown: percentages of $Ki67^+CD4^+$ T cells are indicated. In control samples, the Ki67 Ab was replaced with an isotype control. Means and SDs were derived from three experiments, each with three mice per group. (**H**) Activated $CD4^+$ T cells were cultured in supernatants from unstimulated control (vector) and Arg2-transduced (Arg2) DC[2114] cells. Supernatants were supplemented (+) or not (−) with arginine. As controls, cells were cultured in the presence of fresh medium (full), medium lacking arginine (−Arg), or arginine-lacking medium supplemented (+) or not (−) with arginine. T cell activation was induced with anti-CD3/CD28. Proliferation was assessed by analyzing Ki67 expression. Means and SDs were derived from three experiments, each with three mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

With the exception of elevated plasma arginine levels, no obvious phenotypes have been reported for Arg2-deficient mice (49). However, their immune system has not been examined in detail. One report has shown that $Arg2^{-/-}$ macrophages exhibit increased NO production and decreased apoptosis induced by *H. pylori* (48). Because Arg2 is the dominant arginase in DCs, a potential immunological phenotype in $Arg2^{-/-}$ mice deserves to be investigated. It should be emphasized that a deficiency in Arg2 is not expected to result in the same DC defects observed for Arg2 overexpression, namely increased arginine consumption and an impaired ability to induce T cell responses. However, other defects could well result from strongly reduced arginase activity in DCs.

We show in this study that Arg2 expression is repressed by binding of miR155 to a specific target site localized within the 3′-UTR of its mRNA (Fig. 4). To our knowledge, this constitutes the first demonstration that arginase activity can be modulated at the posttranscription level by a miRNA. Until now, no miRNAs had been implicated in the regulation of Arg2 expression. In the case of Arg1, one study reported that hepatocyte-specific deletion of the gene encoding Dicer, the main miRNA processing enzyme, affects proper localization of Arg1 in the liver (50). The latter suggests a potential direct or indirect role for miRNAs in regulating Arg1 function, although the precise miRNAs that might be implicated have not been identified.

Functionally relevant regulation of Arg2 expression by miR155 is not restricted to DCs. We show in this paper that lung pathology exhibited by $miR155^{-/-}$ mice can be attributed to derepressed Arg2 expression in lung myofibroblasts (Fig. 3D). Perturbed control of Arg2 repression by miR155 should therefore be considered as a potential parameter contributing to the pathogenesis of lung diseases associated with deregulated collagen production. Further examination of Arg2 and miR155 expression in other healthy and diseased tissues may shed light on additional physiological functions and pathogenic mechanisms in which miR155-controlled Arg2 expression is implicated.

Arginase activity is believed to contribute to the immunosuppressive functions of tumor associated macrophages (9), DCs (51), MDSCs, and the tumor cells themselves (8, 46, 47). Increased arginase activity is thought to promote tumor development and progression by inducing arginine depletion and hence an inhibition of T cell–mediated antitumor responses. Arginase inhibitors have indeed been found to exhibit promising therapeutic effects in

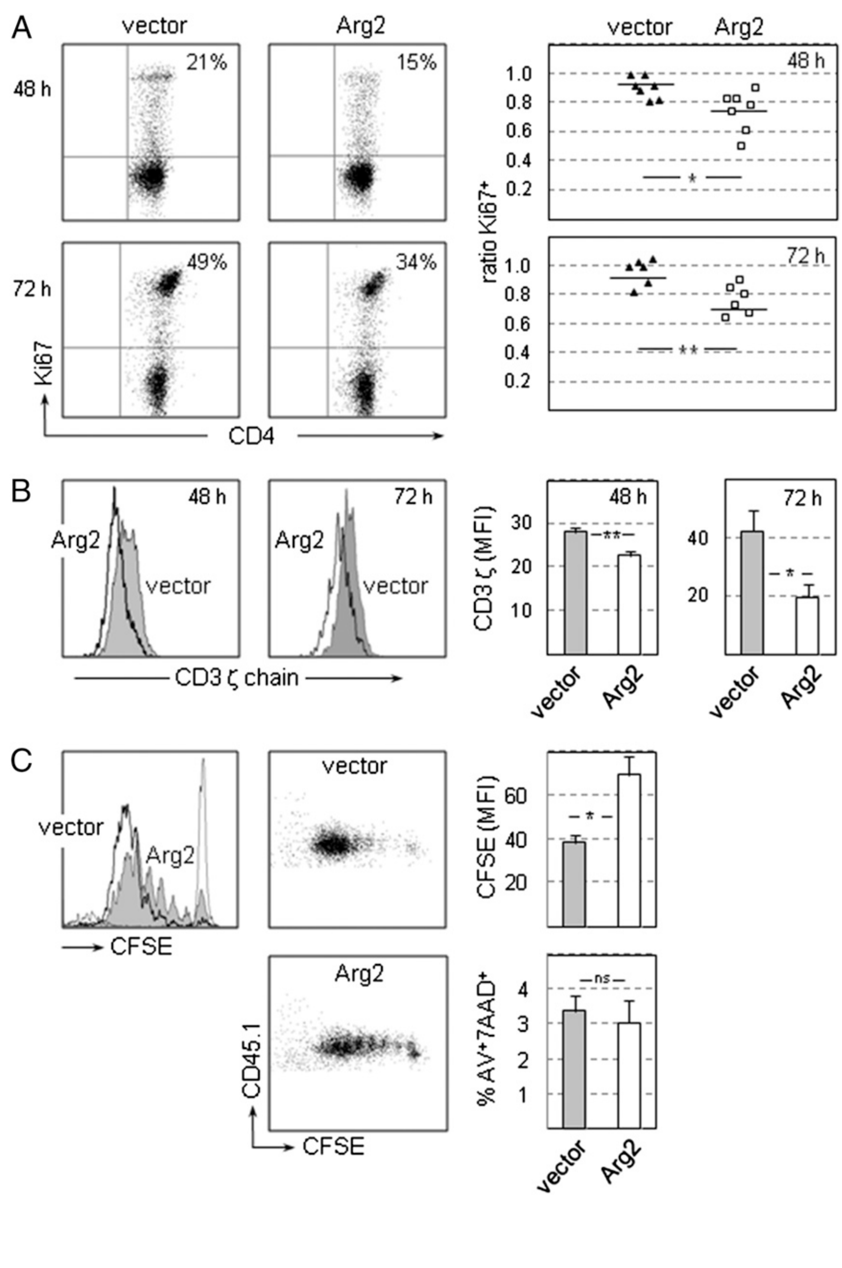

FIGURE 7. Deregulated Arg2 expression impairs T cell proliferation in vivo. $CD4^+$ T cells from OTII mice and OVA-loaded Arg2 or control (vector)-transduced DC^{2114} cells were transferred into WT recipients. (**A**) Proliferation of splenic $V\alpha2^+$ OTII $CD4^+$ T cells was assessed by analyzing Ki67 staining after 48 and 72 h. Representative flow cytometry profiles are shown: percentages of $Ki67^+V\alpha2^+CD4^+$ T cells are indicated. The means and SEM were derived from two experiments including six to seven mice per group. (**B**) Splenic $V\alpha2^+$ OTII $CD4^+$ T cells were stained with anti-CD3ζ after 48 and 72 h. Representative flow cytometry profiles are shown. Means and SDs for the mean fluorescence intensity (MFI) were derived from two experiments with six to seven mice per group. (**C**) Proliferation of splenic $CD45.1^+$ OTII $CD4^+$ T cells was assessed by analyzing CFSE dilution after 72 h. Representative flow cytometry profiles and histograms are shown. Means and SDs for CFSE mean fluorescence intensity (MFI) were derived from three experiments with three mice per group. Percentages of $AnnexinV^+7AAD^+CD4^+$ T cells are indicated. $*p < 0.05$, $**p < 0.01$.

mouse tumor models (9, 52, 53). Altered repression of Arg2 by miR155 could therefore contribute to the escape of tumors from immunosurveillance. Our results raise the hope that manipulating the expression or activity of Arg2 and/or miR155 could be of therapeutic value for the treatment of cancer.

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Disclosures

The authors have no financial conflicts of interest.

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