

Transcriptomics identified a critical role for Th2 cell-intrinsic miR-155 in mediating allergy and antihelminth immunity

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Edited by Robert L. Coffman, Dynavax Technologies, Berkeley, CA, and approved June 23, 2014 (received for review April 8, 2014)

Allergic diseases, orchestrated by hyperactive CD4⁺ Th2 cells, are some of the most common global chronic diseases. Therapeutic intervention relies upon broad-scale corticosteroids with indiscriminate impact. To identify targets in pathogenic Th2 cells, we took a comprehensive approach to identify the microRNA (miRNA) and mRNA transcriptome of highly purified cytokine-expressing Th1, Th2, Th9, Th17, and Treg cells both generated in vitro and isolated ex vivo from allergy, infection, and autoimmune disease models. We report here that distinct regulatory miRNA networks operate to regulate Th2 cells in house dust mite-allergic or helminth-infected animals and in vitro Th2 cells, which are distinguishable from other T cells. We validated several miRNA (miR) candidates (miR-15a, miR-20b, miR-146a, miR-155, and miR-200c), which targeted a suite of dynamically regulated genes in Th2 cells. Through in-depth studies using *miR-155*^{-/-} or *miR-146a*^{-/-} T cells, we identified that T-cell-intrinsic miR-155 was required for type-2 immunity, in part through regulation of *S1pr1*, whereas T-cell-intrinsic miR-146a was required to prevent overt Th1/Th17 skewing. These data identify miR-155, but not miR-146a, as a potential therapeutic target to alleviate Th2-mediated inflammation and allergy.

CD4⁺ T cells can differentiate into various Th subsets characterized by distinct molecular programs and cytokine production, e.g., IFN- γ -secreting Th1 cells, IL-4-secreting Th2 cells, and IL-17A-secreting Th17 cells. Specifically, IL-4 signaling in naive T cells activates STAT6 and GATA3 (1), promoting a Th2 differentiation pathway by activating transcription at the *Il4*, *Il5*, and *Il13* loci (2). Characteristically, Th2 cells have a detrimental role in allergies, which are becoming one of the most common global chronic diseases (3). In contrast, Th2 cells are essential for antihelminth immunity (4). Despite a good understanding of the signals required for Th2 differentiation (1), our knowledge of the molecular mechanisms involved in the posttranscriptional regulatory events that govern Th2 cell differentiation and effector function remain unclear.

microRNAs (miRNAs), encoded within the genome and cleaved by two ribonuclease-III enzymes, Dicer and Drosha (5), regulate mRNA translation by inhibiting and degrading mRNA (6). miRNAs critically shape immune cell development and function (7, 8) with targeted deletion of *Dicer* in T cells resulting in diminished peripheral CD8 and CD4 T cells (9). Among the ~2,000 identified mammalian miRNAs (miRbase v20) (10), several T-cell-associated miRNAs have been identified that regulate development (11), differentiation (12–14), and effector function (15–19). For example, miR-29 and miR-21 regulate Th1-mediated immunity (13, 17, 18), whereas miR-326 (20), miR-10a (21), miR-155 (22), and miR-132/212 (23) influence Th17 cell differentiation and effector function. Treg cells, which provide a critical brake on effector responses, also are governed by miRNAs (24, 25), with miR-182, miR-10a (21, 26), miR-155 (27), and miR-146a (16) required for proficient Treg development and suppressive capacity. Several studies have identified miRNAs, including miR-126 (28), miR-106a (29), miR-145 (32), miR-221, and miR-155/205/498/Let-7e (30), in murine (28, 29) and human

(30) allergic diseased tissue (31); however, there is a scarcity of studies specifically identifying miRNA-mediated regulation of Th2 cell differentiation and effector function. Correlations of elevated miR-181a, miR-146a, and miR-146b expression in distal, splenic CD4⁺ cells during experimental ovalbumin (OVA)-induced airway inflammation have been reported (33) but have not been tested. To date, only miR-155 has been implicated in Th2 differentiation in vitro (34), leaving a significant gap in our understanding of miRNAs involved in Th2 cell differentiation and in vivo effector function.

In this study we took a systematic and comprehensive approach to identify the miRNAome of all Th cells, using highly purified cytokine or transcription factor reporter systems to identify Th2-specific miRNAs. Using a subtractive comparative analysis, we established distinct transcriptomes, miRNAs, and their targets in Th2 cells generated in vitro and isolated ex vivo from house dust mite (HDM)- or helminth (*Heligmosomoides polygyrus*)-induced inflammation that were distinct from other Th subsets. Functionally restricting miR-155 or miR-146a deficiency to T cells by using mixed bone marrow chimeras or miRNA inhibitors and adoptive transfer systems, we identified opposing roles for miR-155 and miR-146a in respectively promoting and regulating Th2-driven immunity. Collectively these data identify critical roles for miR-155 and miR-146a in Th2-mediated immunity and suggest that targeting miR-155, but not miR-146a, may alleviate Th2-mediated inflammation and asthma.

Significance

The rising prevalence of allergic diseases throughout the world demands new approaches to treat this inflammatory disorder. CD4⁺ Th2 cells orchestrate the allergic cascade, stimulating IgE production, activating innate cells, and stimulating local tissue. This study took a comprehensive approach to identify the unique transcriptional features of pathogenic Th2 cells with the aim of identifying novel molecular regulators. Highly purified Th1, Th2, Th9, Th17, and Treg cells isolated from mice with allergy, infection, and autoimmunity identified unique mRNA and microRNAs (miRNAs) expressed in Th2 cells. Functional and mechanistic studies using miRNA-deficient mice, luciferase assays, miRNA inhibitors, and siRNA in combination with state-of-the-art adoptive transfer systems, identified a critical role for miR-155-regulated *S1pr1* in the pathogenesis of Th2-mediated allergy.

Author contributions: I.S.O. and M.S.W. designed research; I.S.O., S.C., E.K., K.R., S.M.C., V.S.P., Y.K., J.P.-L., and M.S.W. performed research; J.L.Z., D.B., and J.L. contributed new reagents/analytic tools; I.S.O., S.M.C., V.S.P., Y.K., J.L.Z., D.B., J.L., and M.S.W. analyzed data; and I.S.O. and M.S.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1406322111/-DCSupplemental.

Results

Using Purified Cytokine/Transcription Factor Reporter-Positive Cells, Rather than Whole T-Cell Cultures, Increases Sensitivity and Resolution of Transcriptional Profiling. To identify differentially regulated miRNAs in differentiated T cells, we initially used the well-studied in vitro T-cell activation and differentiation system (35) (Fig. 1*A* and *SI Appendix*, Figs. S1 and S2*A*). In the absence of supraphysiological restimulation with phorbol12-myristate13-acetate (PMA) and ionomycin, we obtained ~75% of cells committed to a Th2 phenotype as determined using *Il4^{gfp}* expression. However, the percentage of other in vitro polarized cells varied [~70% Th1, ~27% Th17, 70% Th9, and 85% induced Treg (iTreg)]. Thus, between 73–15% of cells within each bulk population were not polarized or committed (*SI Appendix*, Fig. S2*A*). Therefore we used the transcriptional reporter mice (*Ifn γ ^{gfp}*, *Il4^{gfp}*, *Il9^{Cre}R26^{eYFP}*, *Il17a^{Cre}R26^{FP635}*, and *Foxp3^{gfp}*) to

purify live, unfixed, and nonpermeabilized reporter-positive cells, eliminating the influence of nonpolarized cells and avoiding the use of PMA and ionomycin in our transcriptional analysis. Purified reporter-positive cells revealed dramatically more differentially expressed genes and miRNAs with a greater dynamic range (fold change), than the bulk cultures (*SI Appendix*, Fig. S1*A* and *B*). In particular, in *Il4gfp*⁺ Th2 cells we identified 4,644 differentially regulated genes and 137 miRNAs (an increase of more than twofold relative to naive T cells), compared with 1,926 genes and 46 miRNAs in bulk Th2 polarized cells (*SI Appendix*, Fig. S2*B* and Table 1), with many genes involved in IL-4 signaling that were undetected in bulk Th2 polarized cells (*SI Appendix*, Fig. S2*B* and *C*). In all subsets, Th cell-associated transcription factors [*Tbx21* (*Th1*), *Roryt* (*Th17*), *Foxp3* (*Treg*), and *Irf4* and *E4bp4* (*Th2*)] were up-regulated to a greater degree in purified cells than in bulk Th2 polarized cells.

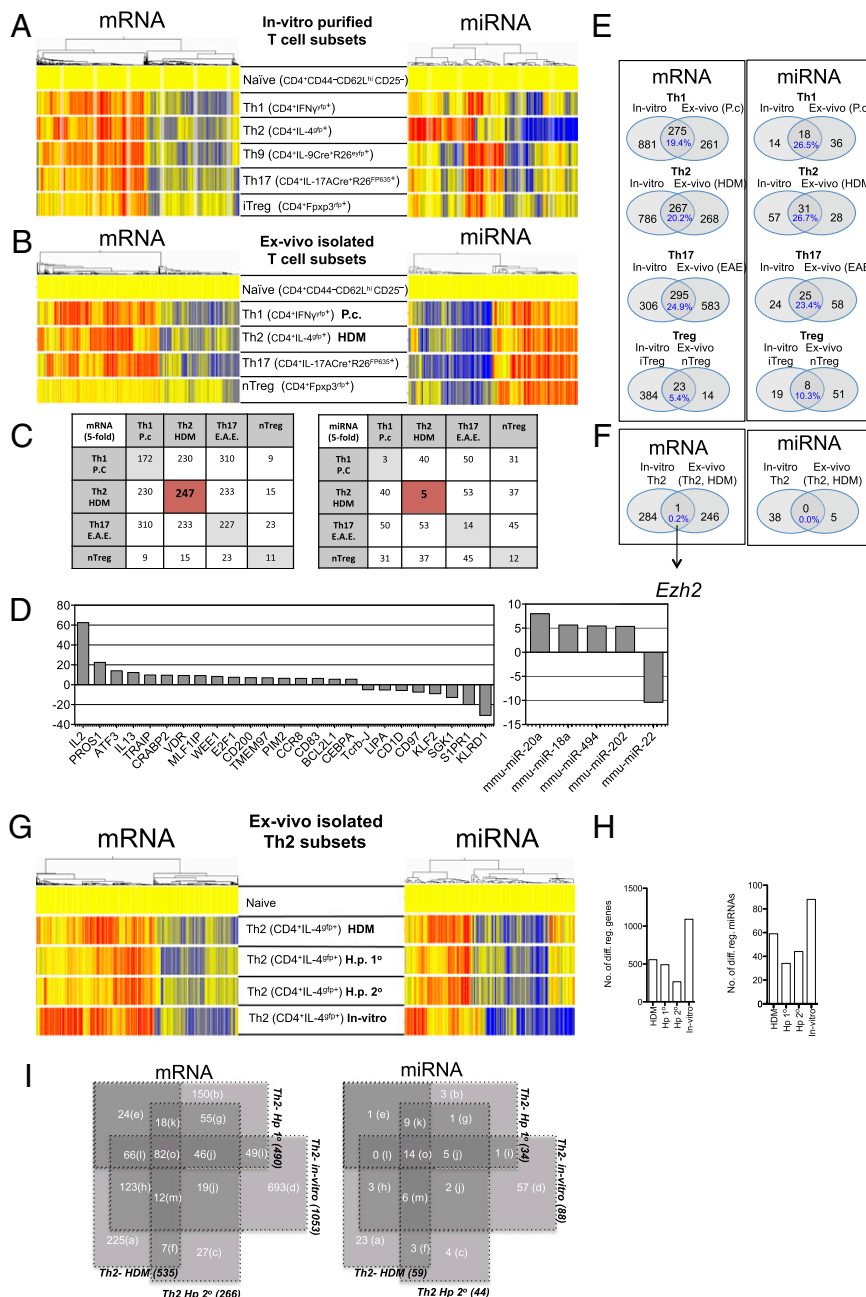


Fig. 1. Transcriptional analysis of FACS-purified reporter-positive T cells reveals overlap with ex vivo cells and identifies significant diversity between Th2 populations. Naive CD4 T cells were polarized in vitro, as described in *Materials and Methods*, with mRNA and miRNA transcriptional profiles used for comparative analysis. (A) Heat maps showing expression of mRNA (Left) and miRNAs (Right) in in vitro-derived T-cell subsets. Each heatmap lane represents the mean of three to five biological replicates per T-cell subset. (B) Heat maps showing expression of mRNA (Left) and miRNAs (Right) in ex vivo-derived T-cell subsets. Each heatmap lane represents the mean of three to five biological replicates per T-cell subset. (C) Comparative analysis showing common and unique mRNA (Left) and miRNA (Right) transcripts in each T-cell subset (greater than a fivefold change). Unique transcripts in ex vivo HDM-derived Th2 cells are highlighted in the red square. (D) Selection of HDM-derived Th2-enriched mRNAs (Left) and miRNAs (Right). (E) Venn diagrams showing comparative analysis of common and unique mRNA (Left) and miRNA (Right) transcripts in the in vitro and ex vivo T-cell subsets (greater than a fivefold change). The percentage of overlap is shown in blue. (F) Venn diagrams showing comparative analysis of common and unique Th2-enriched mRNA (Left) and miRNA (Right) transcripts in the in vitro and ex vivo T-cell subsets (greater than a fivefold change). The percentage of overlap is shown in blue. (G) Heat maps showing expression of mRNA (Left) and miRNAs (Right) in Th2 cell subsets from allergic HDM-challenged mice and mice susceptible (*H.p.* 1°) and resistant (*H.p.* 2°) to *H. polygyrus*. (H) (Left) Total number of differentially regulated mRNA transcripts (greater than a fivefold change) in different Th2 samples. (Right) Number of differentially regulated miRNA transcripts (greater than a fivefold change) in different Th2 samples. Each heatmap lane represents the mean of three to five biological replicates per T-cell subset. (I) Four-way Venn diagrams showing comparative analysis of common and unique mRNA and miRNA transcripts in Th2 subsets. Numbers on Venn diagram are the number of transcripts; letters refer to the bar chart in *SI Appendix*, Fig. S7.

An miRNA and mRNA Signature Distinguishes Th2 Cells from Other Th and Treg Subsets. Comparing the transcriptomes across all five purified reporter-positive T-cell subsets generated in vitro (Th1, Th2, Th9, Th17, and iTreg), we identified a suite of 285 Th2-enriched genes including *Ccr1*, *Fosb*, *Ccr2*, *Socs1*, and *Il10ra*, that were unchanged in other T-cell subsets (SI Appendix, Fig. S3 A and B and Table 2). We also applied an increasing fold-change filter (a two-, five-, or 10-fold change, relative to naive T cells) to identify highly abundant transcripts that distinguished Th2 cells from the other T-cell subsets (SI Appendix, Fig. S4). We identified 38 Th2-enriched miRNAs, including miR-146a, miR-200c, miR-15a, and miR-1931, that were unchanged in other T-cell subsets (Fig. 1A and SI Appendix, Fig. S3 A and B and Table 3). It was reported recently that upon T-cell activation in vitro there is a down-regulation in global miRNA and miRNA biogenesis pathways (36). We observed a similar down-regulation (SI Appendix, Fig. S5A) along with reduced *Argonaute*, *Dicer*, and *Dgcr8* transcripts (SI Appendix, Fig. S5B). Taken together, this strategy allowed us to identify more accurately transcripts that were differentially regulated in highly purified Th2 cells, and the broad, comprehensive approach allowed us to distinguish more accurately unique features of Th2 cells.

Transcriptional Diversity Between in Vitro-Differentiated and ex Vivo Th2 (*IL4^{gfp+}*) Cells from Helminth-Infected and HDM-Challenged Mice. To determine how similar in vitro-generated T cells were to their in vivo counterparts, we purified reporter-positive cells from various inflammatory settings: Th1 cells (CD4⁺*Ifn γ ^{gfp+}*) from *Plasmodium chabaudi*-infected mice, Th2 cells (CD4⁺*IL4^{gfp+}*) from mice with HDM-induced airway inflammation, Th17 cells (CD4⁺*IL17a^{Cre};R26e^{FP635+}*) from mice with experimental autoimmune encephalomyelitis (EAE), and natural Treg cells (nTregs) (CD4⁺*Foxp3^{gfp+}*) from naive mice (Fig. 1B). We could not isolate sufficient Th9 cells ex vivo under any conditions. Using this suite of highly purified ex vivo T cells, we performed genomewide transcriptional analysis and identified distinct mRNA and miRNA expression profiles in ex vivo Th2 cells with 247 Th2-enriched genes, including *Il2*, *Prosl*, *Il13*, *Traip*, *S1pr1*, and five miRNAs (miR-22, -202, -494, -18a, and -20a) (Fig. 1C and SI Appendix, Fig. S6 and Table 4).

Strikingly, when we compared purified in vitro-generated Th2 cells with ex vivo HDM-elicited Th2 cells, we observed very little overlap, with only 20% (267 transcripts) of differentially regulated genes in common (Fig. 1E). Similar percentages were observed for other Th subsets, with even lower concordance (5.4%) between iTreg and nTreg cells (Fig. 1E). When we compared Th2-enriched genes (i.e., genes that were differentially regulated in Th2 cells but not in other T cells) in the in vitro-generated (285 genes) and in the ex vivo HDM-elicited (247 genes) Th2 cells, we observed an overlap of less than 1%, with only one gene in common, the polycomb family histone methyltransferase, *Ezh2* (Fig. 1F), previously implicated in *Il4* and *Il13* gene regulation (37). We did not find any overlap in Th2-enriched miRNAs between the in vitro and ex vivo Th2 cells. These data highlight the great discrepancy between in vitro-generated and ex vivo-isolated T-cell subsets and particularly the poor concordance between in vitro and ex vivo Th2 cells.

To investigate Th2 cell diversity further and to determine how transcriptionally different other Th2 cells were, we isolated Th2 cells from mice susceptible (primary infection, *H.p.* 1°) or resistant (drug-cured and reinfected, *H.p.* 2°) to infection with the intestinal helminth *H. polygyrus*. Genomewide transcriptional analysis showed that mRNA and miRNA transcripts in these ex vivo samples were distinct from in vitro Th2 cells and also were distinct from HDM-elicited Th2 cells (Fig. 1G). A four-way comparison of all Th2 populations (in vitro, HDM-elicited, *H.p.* 1°, and *H.p.* 2°) using all differentially regulated genes identified only 82 genes and 14 miRNAs in common (Fig. 1I and SI Appendix, Figs. S7–S10). Together, these data indicate that *IL4^{gfp+}* Th2 cells generated in vitro or isolated ex vivo are largely hetero-

geneous, with more unique than overlapping mRNA and miRNA transcripts.

miRNA Target Prediction Identifies Putative miRNA-Mediated Regulation of Metabolic, Signaling, and Migration Pathways in Th2 Cells. Given the diversity in each Th2 population, we analyzed each Th2 population separately to identify Th2-associated miRNAs and their mRNA targets. We used several *in silico* analyses to screen for putative functional miRNA–mRNA interactions. First we used an expression-pairing analysis (asking specifically whether up-regulated miRNAs were predicted to target down-regulated mRNAs, and vice versa). We then combined this analysis with a series of fold-change filters (two-, five-, or 10-fold change) in each Th2 population to identify transcripts that were more prominently changed in each Th2 subset. Finally, for each putative miRNA, we assessed the target abundance (i.e., the number of mRNAs targeted by any given miRNA, which would indicate an miRNA regulatory hub). For example, using in vitro-generated Th2 cells with a twofold filter, we identified seven miRNAs (SI Appendix, Fig. S11A, Left) that both were predicted to target and had expression patterns opposing 46 mRNAs (SI Appendix, Fig. S11, Top Right). Of these seven miRNAs, miR-214-3p (which was increased 3.6-fold from the level in naive T cells) was predicted to target 21 mRNAs. By increasing the fold-change filter to five- and 10-fold change, we lost miR-214-3p but identified several other miRNAs, including miR-15a, which was down-regulated 70-fold from levels in naive T cells and was predicted to target 22 (fivefold) and 14 (10-fold) Th2-enriched mRNAs, respectively (boldface in SI Appendix, Fig. S11A). Of note, miR-15a was down-regulated by 2.3-fold in both Th1 and iTreg cells, explaining why we did not identify miR-15a in the twofold-cutoff filter.

Combining these analyses from the in vitro-generated Th2 cells (SI Appendix, Fig. S11A), HDM-induced Th2 cells (SI Appendix, Fig. S11B), and helminth-elicited Th2 cells (SI Appendix, Fig. S11C), we selected five candidate miRNAs for further analysis: miR-15a, miR-20b, miR-146a, miR-155, and miR-200c (SI Appendix, Fig. S11E). In line with the diversity of in vitro and ex vivo Th2 populations, not all candidate miRNAs were regulated in a similar manner. For example, both miR-146a and miR-15a were down-regulated in Th2 cells generated in vitro but were up-regulated in ex vivo HDM- or helminth-elicited Th2 cells (SI Appendix, Fig. S11F), again highlighting the disparity between in vitro and ex vivo Th2 populations.

We identified several mRNA targets of the candidate miRNAs that have been implicated in Th2 cell biology. For example, miR-146a was predicted to target the IL-4-regulated genes *Abl2*, *Kdm6b*, and *Mtdh* (38) in both helminth-derived (*H.p.* 1°) and in vitro-derived Th2 samples. miR-146a also was predicted to target *Itch* (39), *Stat1* (40), and *Smad4* (41) in all ex vivo Th2 cells (SI Appendix, Fig. S13). miR-15a was predicted to target the antiapoptotic gene *Bcl2* and TGF- β -associated *Smad5* in all Th2 samples and, along with miR-155, was predicted to target the metabolic regulators *Sgk3* (42) and *Rictor* (43), suggesting that these genes are tightly controlled in Th2 cells by two independent miRNAs (SI Appendix, Fig. S13B). miR-155 also was predicted to target *Il6r*, *S1pr1*, and *Tia-1*, all of which have critical roles in Th2 cell biology (44–46). For the down-regulated miRNA candidates (miR-200c and miR-20b), we identified a significant number of common mRNA targets, including *Gfi1*, *Maf*, *Egr2*, *Hif1 α* , *Lif*, *Map3k8*, *Rora*, and *Tnfrsf11* (SI Appendix, Fig. S13C), all of which have reported roles in Th2 cells.

Spatial and Temporal Expression of miRNAs and Their mRNA Targets in Th2 Cells. We hypothesized that the discrepancy between miRNA expression patterns in the in vitro-generated and ex vivo Th2 cells reflected different stages of differentiation or maturation. Therefore we analyzed the temporal expression of the five candidate miRNAs in FACS-purified, Th2-committed *IL4^{gfp+}* cells at early (day 2, 3, and 7) and late (day 10 and 14) stages of Th2 differentiation in vitro (SI Appendix, Fig. S12A). At day 2, the expression of miR-155 was elevated, but the expression of

miR-146a, miR-15a, miR-20b, and miR-200c was down-regulated relative to naive T cells (*SI Appendix, Fig. S12B*). By day 7, miR-155 declined, but other miRNAs increased, peaking at day 10. Th2-associated genes (*Gata3*, *Stat6*, *Il4*, *Il5*, and *Il13*) were prominent from day 2 onwards. Concomitantly, several predicted targets of miR-155 (*Sgk3*, *Il6r*, *Rictor*, and *S1pr1*), miR-15a (*Tia1*), miR-146a (*Itch*, *Stat1*, and *Smad4*), and miR-20b (*Egr2*, *Lif*, *Rora*, and *Tnfrsf3*) had an inverse expression pattern at several time points (*SI Appendix, Fig. S12B*), supporting a functional interaction. Focusing on miR-155 and miR-146a, we also observed a temporal relationship between miRNA and mRNA target expression in purified ex vivo Th2 cells from the inflammatory site [airspaces, obtained by bronchoalveolar lavage (BAL)] and local lung-draining lymph nodes (tLN) of mice with HDM-induced airway inflammation. Higher relative levels of miR-155 in the tLN correlated with lower *S1pr1* and *Rictor* levels relative to Th2 cells from the BAL, whereas higher miR-146a in BAL Th2 cells correlated with lower *Stat1* and *Itch* levels than found in tLN Th2 cells (*SI Appendix, Fig. S12C*).

To test formally whether miR-155 or miR-146a regulate their predicted targets in Th2 cells, we transfected FACS-purified 7-d polarized *IL4^{gfp+}* Th2 cells (Fig. 2A) either with miR-155 hairpin inhibitors to reduce the elevated levels of miR-155 or with miR-146a miRNA mimics to increase the down-regulated levels of miR-146a (*SI Appendix, Fig. S12A*). miR-155 inhibitors reduced miR-155 expression without compromising cell viability (Fig. 2B) and resulted in elevated *S1pr1* and *Rictor* expression (Fig. 2C). Furthermore, miR-155 inhibitors also reduced IL-13 and IL-5 protein secretion from Th2 cells (Fig. 2E), suggesting that miR-155 contributes to Th2 effector function. miR-146a mimicked elevated miR-146a expression and reduced *Itch* and *Stat1* (Fig. 2D) and slightly increased *Il5* and *Il13* mRNA ex-

pression (Fig. 2E and F) but did not impact IL-5 or IL-13 secretion significantly. This discrepancy in mRNA and protein secretion may be caused by the timing of miRNA treatment and differential impact on translation and mRNA decay (47). Taken together, these data reveal the dynamic expression pattern of several miRNAs and their mRNA targets in vitro and from different tissues ex vivo and suggest that miR-155 and miR-146a promote and regulate Th2 cells, respectively.

T-Cell-Intrinsic miR-146a Regulates Th2-Mediated Immunity and Allergy. miR-146a was regulated dynamically in Th2 cells in vitro (*SI Appendix, Fig. S12A*) but was elevated consistently in ex vivo Th2 cells (*SI Appendix, Fig. S11F*). To test formally whether miR-146a is required for Th2-mediated immunity in vivo, we generated T-cell chimeras using miR-146a-deficient bone marrow, restricting miR-146a deficiency to T cells (Fig. 3A). miR-146a has been implicated in Treg cells (16); however, in our T-cell chimeras a normal frequency of Treg and Th cells was observed (*SI Appendix, Fig. S14B*), without any signs of autoimmunity. Following HDM airway challenge, mice with *miR-146a^{-/-}* T cells had increased airway infiltrates, with more eosinophils and significantly more neutrophils (Fig. 3B). Elevated eosinophils and neutrophils were accompanied by elevated *Il5*, *Il13*, *Il17a*, and *Ifn γ* mRNA in the lung (Fig. 3C). Mice with *miR-146a^{-/-}* T cells also had elevated *Muc5ac* expression (Fig. 3C) and greater interstitial and per-vascular inflammation following HDM challenge (Fig. 3D), suggesting that T-cell-intrinsic miR-146a is required to regulate a polarized Th2 response. To determine the phenotype of HDM-elicited *miR-146a^{-/-}* T cells, we isolated CD4⁺CD44⁺ *miR-146a^{-/-}* and CD4⁺CD44⁺ *miR-146a^{+/+}* T cells from HDM-allergic mice. As predicted, *miR-146a^{-/-}* T cells had elevated miR-146a-targeted genes, i.e., *Itch*, *Smad4*, and *Stat1*, relative to their miR-146a-sufficient counterparts (Fig. 3E), again indicating that these genes are miR-146a targets and suggesting that these dysregulated miR-146a targets may contribute to the increased inflammation.

Unlike *H. polygyrus*, expulsion of the intestinal whip worm *Trichuris muris* is critically dependent on a highly polarized Th2 response (48), and a mixed Th1/2/17 response leads to chronic infection (49). Therefore we tested whether mice with *miR-146a^{-/-}* T cells could mount the polarized Th2 response required for proficient antihelminth immunity. In line with observations made in the HDM-allergy model, mice with miR-146a-deficient T cells had a compromised Th2 response following infection with *T. muris*; reduced IL-13⁺ but elevated IL-17A⁺ T cells resulted in significantly more worms present in the cecum (Fig. 3F). Similarly, mice with *miR-146a^{-/-}* T cells had elevated IFN- γ ⁺ and IL-17A⁺ and compromised IL-5⁺ CD4⁺ T cells following infection with *H. polygyrus* (Fig. 3G), resulting in greater intramuscular inflammation and mucus secretion (Fig. 3H). In summary, after HDM challenge or helminth infection the deletion of miR-146a in T cells led to a mixed Th1/Th2/Th17 response, suggesting that miR-146a regulates the polarized differentiation of cells and, importantly, regulates the differentiation of Th1 and Th17 cells in otherwise Th2-dominated environments.

T-Cell-Intrinsic miR-155 Is Required for Th2-Mediated Immunity and Allergy. miR-155 was elevated in whole-lung tissue from HDM-allergic mice (Fig. 4A) and also was elevated in HDM-elicited and in vitro-generated Th2 cells (*SI Appendix, Fig. S11F*). Therefore we tested the function of miR-155 in vivo using *miR-155^{-/-}* mice and observed dramatically reduced airway inflammation, with reduced eosinophilia (Fig. 4B) and IL-13 production (Fig. 4C). Consequently, IL-13-regulated Gob5 expression (Fig. 4D) and airway mucus production (Fig. 4E) were reduced in *miR-155^{-/-}* mice, indicating that miR-155 is essential for Th2-mediated airway allergy. miR-155 also was elevated in the intestinal tissue of *H. polygyrus*-infected mice (Fig. 4F), specifically within the intestinal lesions around larvae (Fig. 4G), and in *H. polygyrus*-elicited Th2 cells (*SI Appendix, Fig. S11F*). In contrast to miR-155-sufficient mice, *miR-155^{-/-}* mice were completely susceptible to a challenge

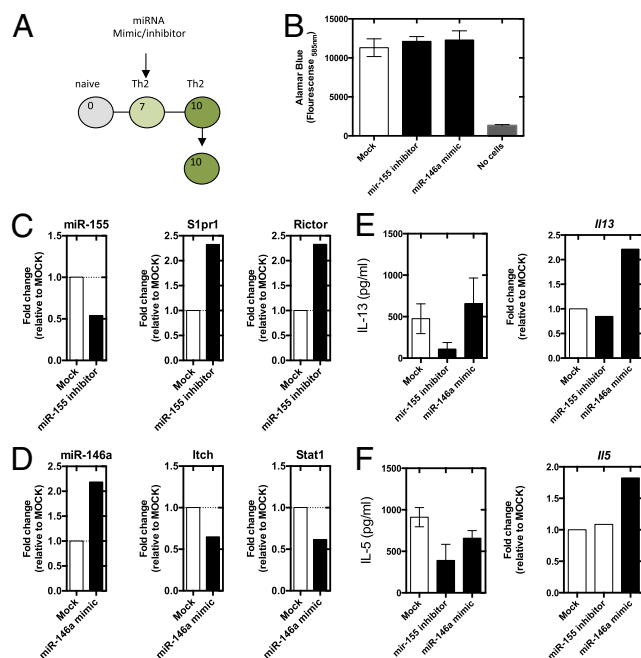


Fig. 2. miR-155 and miR-146a regulate discrete targets in differentiated Th2 cells. (A) In vitro-generated *IL4^{gfp+}* Th2 cells were FACS purified at day 7 of culture in vitro and were transfected with miR-155 short hairpin inhibitors or miR-146a mimics (100 nM) to reverse the expression profile observed at day 7. (B) Cell viability at day 10 of culture in vitro, determined by AlamarBlue fluorescence. (C) Expression of miR-155 and miR-155 target genes at day 10, expressed relative to mock-treated cells. (D) Expression of miR-146a and miR-146a-target genes at day 10, expressed relative to mock-treated cells. (E) IL-13 secretion (Left) and *Il13* mRNA (Right) at day 10. (F) IL-5 secretion (Left) and *Il5* mRNA (Right) at day 10. Results from one of three independent experiments are shown.

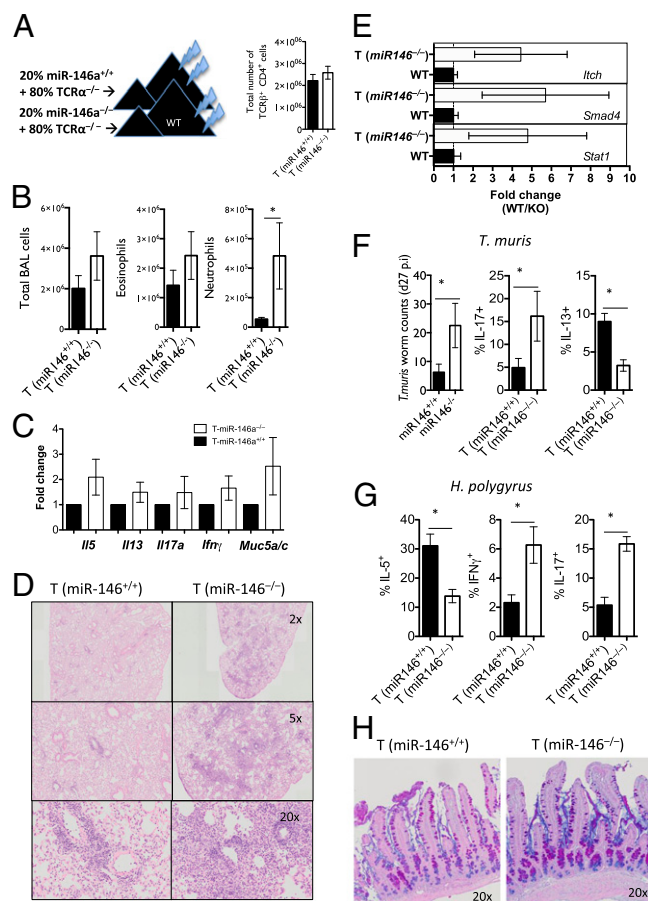


Fig. 3. T-cell-intrinsic miR-146a regulates the phenotype and magnitude of Th2-driven immunity and inflammation. (A) Schematic representation of the generation of miR-146a mixed T-cell bone marrow chimeric mice. (B) Total number (Left) and composition (Center and Right) of BAL cells following HDM challenge. (C) Expression of *IL5*, *IL13*, *IL17a*, *Ifn γ* , and *Muc5a/c* in the lung of HDM-challenged mice. (D) H&E-stained lung of HDM-challenged mice. (E) Expression of miR-146a target genes *Itch*, *Smad4*, and *Stat1* in WT and miR-146a^{-/-} CD4⁺CD44⁺ T cells from the lungs of HDM-challenged mice, expressed relative to WT T cells. (F) (Left) Total *T. muris* worm counts in the colon and large intestine on day 27. (Center and Right) Frequency of IL-17⁺ and IL-13⁺ CD4⁺ T cells in the local lymph nodes of mice infected with *T. muris*. (G) Frequency of IL-5⁺, IFN- γ ⁺, and IL-17⁺ CD4⁺ T cells in the local lymph nodes of mice infected with *H. polygyrus*. (H) Mucus staining (AB-PAS) of small intestine of mice infected with *H. polygyrus*. (Magnification: 20 $\times$.) Five mice per group were used; results of one of three to five experiments are shown. **P* < 0.05, using a nonparametric Mann–Whitney test.

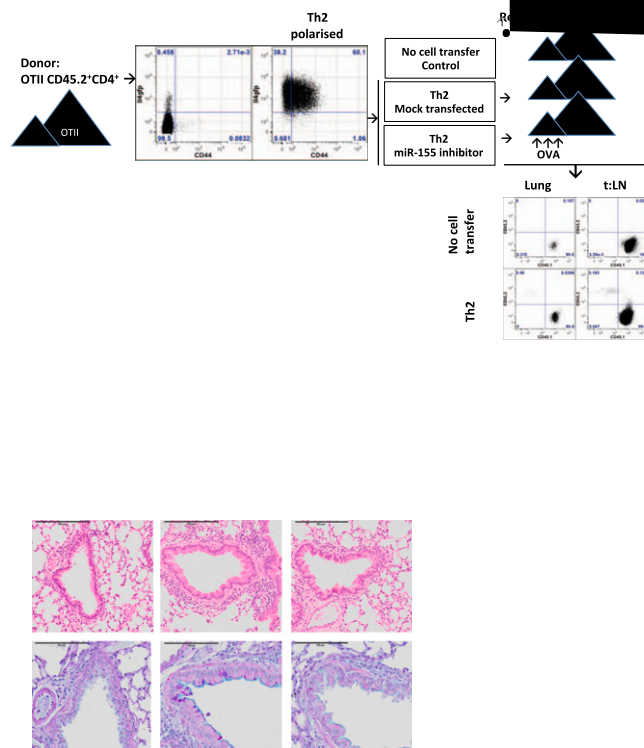
infection with *H. polygyrus* (Fig. 4H) and failed to develop Th2-dependent immunity (IL-13 and IL-5) (Fig. 4I) with reduced goblet cell hyperplasia (Fig. 4J). miR-155^{-/-} mice were fully capable of mounting a protective Th1 response following blood-stage *P. chabaudi* infection, indicating that miR-155 is not required for a proficient Th1 response (SI Appendix, Fig. S15). Together, these data indicate that miR-155 is critical for mucosal type-2 immunity in the lung after HDM challenge and in the gut after *H. polygyrus* infection but is not required for tissue Th1 responses after *Plasmodium* infection.

To test whether T-cell-intrinsic miR-155 is required for type-2 immunity, we generated T-cell chimeras restricting miR-155 deficiency to T cells (Fig. 4K), similar to the miR-146a studies described above (Fig. 3). Despite comparable T-cell chimerism (Fig. 4L) without any appreciable change in baseline T-cell populations (SI Appendix, Fig. S14), miR-155^{-/-} T cells failed to orchestrate HDM-induced airway inflammation with reduced airway infiltrates (Fig. 4M), eosinophilia (Fig. 4N), and IL-13

production in the lung (Fig. 4O). Mice with miR-155^{-/-} T cells also had reduced *il5*, *il13*, and *Gob5* mRNA in the lung (Fig. 4P) and reduced goblet cell hyperplasia, phenocopying complete miR-155^{-/-} mice (Fig. 4A–E). T-cell-intrinsic miR-155 also was required for fully proficient Th2-dependent immunity in the gut, because mice with miR-155^{-/-} T cells had compromised worm expulsion (Fig. 4R) and goblet cell hyperplasia (Fig. 4S) after *H.p.* 2^o infection [albeit to a much lesser extent than complete miR-155^{-/-} mice (Fig. 4B)], indicating that non-T-cell-expressed miR-155 also contributes significantly to type-2 immunity. Finally, miR-155^{-/-} T cells isolated from helminth-infected mice had elevated miR-155 targets, *S1pr1* and *Rictor*, relative to miR-155-sufficient T cells (Fig. 4T), suggesting that dysregulated *S1pr*–*S1pr1* interactions (45) or Rictor-associated metabolic pathways (50) in miR-155^{-/-} T cells may contribute to the compromised Th2 response.

Directly Targeting miR-155 in Th2 Cells Prevents Th2-Mediated Airway Allergy. Following the observation that miR-155-deficient mice and more specifically mice with miR-155-deficient T cells had a reduced capacity to mount Th2 responses in vivo, we next tested whether miR-155 could be targeted specifically within differentiated Th2 cells and whether such targeting would compromise Th2 effector function. For these tests, we polarized and FACS purified OVA-reactive *IL4*^{gfp+} Th2 cells using CD45.2 C57BL/6 OTII mice backcrossed onto the *IL4*^{gfp} background (Fig. 5A). We then knocked down miR-155 in OTII Th2 cells using miR-155 hairpin inhibitors and adoptively transferred these cells into congenic CD45.1 C57BL/6 mice (Fig. 5A). Targeting miR-155 in Th2 cells reduced the capacity of Th2 cells to orchestrate airway inflammation with reduced airway infiltrates (Fig. 5B), airway eosinophilia (Fig. 5C), and IL-13 production (Fig. 5D), thus confirming that miR-155 is required for Th2 effector function in vivo. miR-155-sufficient Th2 cells caused peribronchial inflammation with airway mucus production (Fig. 5E) after OVA challenge. However, OTII Th2 cells with reduced miR-155 caused much less tissue inflammation and mucus secretion (Fig. 5E), with reduced *Gob5* and *Muc5a/c* expression in the lung tissue. Similarly, the IL-4- and IL-13-regulated genes *Fizz1* and *Arg1* were reduced significantly when miR-155 was targeted in Th2 cells (Fig. 5F).

miR-155 Regulates *S1pr1* in Th2 Cells, Preventing Th2-Mediated Airway Allergy. Ex vivo and in vitro-generated Th2 cells had an inverse relationship between miR-155 and several mRNA targets, including *S1pr1* (SI Appendix, Fig. S12). Similarly, miR-155 inhibition in WT Th2 cells dysregulated *S1pr1* expression (Fig. 2), suggesting that miR-155 regulates *S1pr1*. We confirmed that miR-155 regulates *S1pr1* directly using miR-155 miRNA mimics and a dual luciferase reporter assay with the 3' UTR of *S1pr1* (Fig. 6A). Furthermore, unlike WT Th2 cells which down-regulated *S1pr1* upon activation in vitro, miR-155^{-/-} Th2 cells failed to down-regulate *S1pr1* following activation (Fig. 6B and C). These observations all indicate that miR-155 regulates *S1pr1* in Th2 cells. To test whether miR-155-regulated *S1pr1* contributed to the compromised Th2 responses in vivo, we treated OVA-reactive Th2 cells in vitro with miR-155 inhibitors or with siRNA directed against *S1pr1* (SI Appendix, Fig. S16) or with both miR-155 inhibitors and *S1pr1* siRNA. Cell viability was not compromised (Fig. 6D) for up to 48 h after treatment. Inhibition of miR-155 in Th2 cells (Fig. 6E) led to up-regulated *S1pr1* (Fig. 6F), as observed earlier (Fig. 2). *S1pr1* siRNA combined with miR-155 inhibition reduced the elevated levels of *S1pr1* (Fig. 6F). We used this strategy to test whether miR-155-regulated *S1pr1* is an important downstream target of miR-155 contributing to the reduced airway inflammation that we observed in miR-155^{-/-} mice (Fig. 4) and more specifically in miR-155-depleted Th2 cells (Fig. 5). We adoptively transferred OVA-reactive Th2 cells into WT mice following inhibition of either miR-155 or *S1pr1* siRNA or inhibition of both miR-155 and *S1pr1* siRNA. Mice were challenged subsequently by intratracheal (i.t.) delivery of



more previously unidentified miRNAs in all Th subsets in vitro and ex vivo. Strikingly, in vitro-generated cells had little transcriptional resemblance to ex vivo-isolated cells, which may be influenced by tissue- (gut or lung) or stimuli- (allergen or helminth) specific signals. Thus, therapies targeting Th2 or other T cells may require disease-specific approaches. Despite such heterogeneity, this approach also identified a core set of 82 Th2-associated genes (fivefold change, 5.14%) in common, including genes involved in IL-5 signaling and immune cell trafficking [*Egln3*, *IL1r2* (38), *Il2*, *IL1r1* (*Il33r*), *Ccl1*, *Rgs1*, and *Socs2*], and metabolic and environmental sensing (*Cst7*, *stx11*, *Ahr*, *Hsp70*); IL-4 signaling [*Ctla2*, *Nfil3* (51)] and genes that regulate IL-4, IL-10, and IL-2 secretion [*Tnfrsf14* (Light), *Nfil3*, and *Socs2* (52)].

miR-146a regulates many functions in innate and adaptive immune cells (53), with *miR-146a*^{-/-} mice suffering from a myeloproliferative disorder (54). In vitro-differentiated Th2 cells had decreased miR-146a at day 7, after an initial spike at day 2 (SI Appendix, Fig. S12), similar to a previous report (55). However, all ex vivo Th2 cells had elevated miR-146a, again highlighting the disparity in miRNA expression between in vitro and ex vivo Th2 cells.

Importantly, in vivo we observed a regulatory role for T-cell-intrinsic miR-146a, with *miR-146a*^{-/-} T cells giving rise to a mixed and more aggressive type-1/2/17 response following allergen challenge or helminth infection, an observation in line with previous studies (15, 56). Of note, when miR-146a deficiency was restricted to Treg cells, but not Th cells, miR-146a was required for Treg function (16). In our experiments, mice with *miR-146a*^{-/-} T cells had a normal frequency of Treg cells and did not display any autoimmunity-associated pathology before infection or allergen challenge. Our in vivo data therefore support a Th cell-intrinsic role for miR-146a, similar to other reports (15), rather than a Treg defect (16).

Several miR-146a targets were inversely expressed with miR-146a in Th2 cells, including *Irak2*, *Traf6* (57), and *Stat1* (16). We also identified *Itch*, an E3 ubiquitin ligase, as a common target of miR-146a in all ex vivo Th2 cells. *Itch* also was inversely expressed with miR-146a at day 2 and day 7 of Th2 polarization in vitro (SI Appendix, Fig. S12) and was elevated in *miR-146a*^{-/-} T cells ex vivo. *Itch* regulates cytokine production and activation of Th2 cells (39) and is required for Th2 tolerance (58). Here, we propose an additional mechanistic pathway of miR-146a-mediated regulation of *Itch*, preventing an aggressive Th2 and Th1/17-mediated response. Elevated miR-146a has been observed in T cells from patients with arthritis (59) and T-cell leukemia (60), and polymorphisms in miR-146a are associated with asthma (61). However, as we report here, elevated miR-146a may be regulating rather than contributing to T-cell malignancies and should be therapeutically targeted with caution.

As mentioned above, we observed dynamic miRNA expression in Th2 cells in vitro. These observations, combined with little overlap between in vitro and ex vivo cells, indicate that caution must be taken when translating in vitro miRNA observations to in vivo settings. Indeed, it has been reported that miR-155 regulates Th2 cell differentiation in vitro, with slightly enhanced Th2 differentiation in *miR-155*^{-/-} T cells in vitro (34, 62). These observations predicted that miR-155 functions as a negative regulator of Th2-differentiation and Th2 immunity. However, as we report here, in vitro observations of miR-155 in T cells did not predict in vivo function, because miR-155 was required for type-2 responses, and, more specifically, T-cell-intrinsic miR-155 was essential for competent type-2 immunity. Thus, contrary to in vitro predictions, specifically targeting miR-155 may block rather than exacerbate the progression of T-cell-mediated airway allergy and asthma.

Although miR-155 regulates both innate and adaptive responses (63), miR-155 may be a promising target for other hyper-inflammatory T-cell-mediated diseases including colitis (64) and autoimmunity (65), with the potential caveat that intestinal Th1/17-mediated immunosurveillance may be compromised (64). miR-155 also is required for optimal Treg proliferation in vivo (66), suggesting that miR-155 may function as a broad regulator of T-cell expansion. This notion is supported by transgenic miR-155 overexpression studies, which led to aggressive and disseminated lymphomas (67), underlining the oncogenic properties of dysregulated miR-155 (68). However, *miR-155*^{-/-} mice could mount a sufficient and protective splenic Th1 response after *Plasmodium* infection (SI Appendix, Fig. S15), suggesting that miR-155 may critically regulate homing, migration, or mucosal T-cell responses, irrespective of T-cell phenotype.

Allergen-exposed peripheral blood mononuclear cells from patients with atopic dermatitis also up-regulated miR-155 (69), which correlated with reduced CTLA-4 and increased proliferation. Whether a miR-155–CTLA-4 axis is responsible for the hyperproliferative role of miR-155 is unclear. We identified several putative targets of miR-155, including the metabolic regulators *Sgk3* (42) and *Rictor* (43) and also *Il6r*, *S1pr1*, and *Tia-1*. Although *Rictor* is critical for T-cell development (70) and Th2 differentiation in vitro (50), we observed a down-regulation of *Rictor* in Th2 cells in vitro with a concomitant increase in miR-155. In line with this observation, *Rictor* was elevated in ex vivo *miR-155*^{-/-} T cells from helminth-infected mice (Fig. 4T). These data

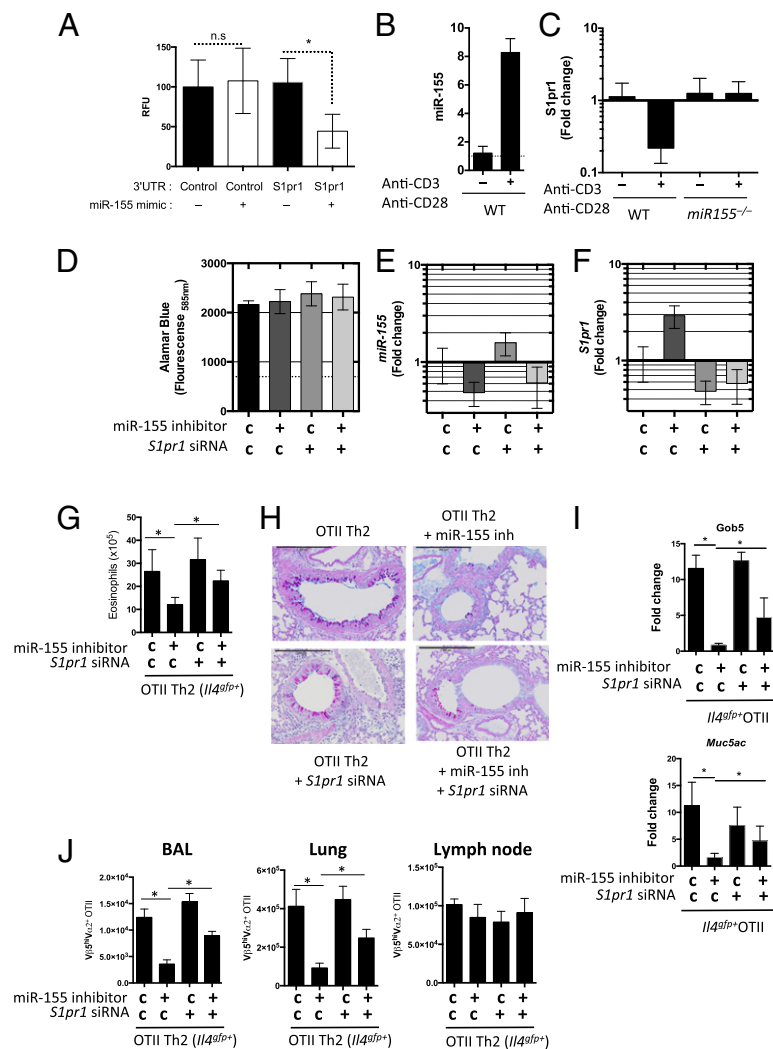


Fig. 6. miR-155 regulates *S1pr1* in Th2 cells, preventing Th2-mediated airway allergy. (A) Jurkat T cells were transfected with dual luciferase reporter clones containing 1 nM of either the 3' UTR of *S1pr1* or a control 3' UTR. Cells also were cotransfected with either miR-155 mimics (100 nM) or a scrambled miRNA control (–). Relative fluorescent units (RFU) were determined after 24 h. (B and C) Seven-day Th2 cells were generated from WT or *mir-155*^{–/–} mice and left to rest for a further 48 h. Cells then were stimulated with plate-bound anti-CD3 and anti-CD28 for 12 h before miR-155 and *S1pr1* levels were measured by qRT-PCR. (D–F) Naive CD4⁺ cells from IL-4^{gfp}CD45.2 C57BL/6 OTII mice were polarized under Th2 conditions, as described in *Materials and Methods*, and were FACS purified using IL4^{gfp}. Th2 cells then were transfected with either miR-155 inhibitors (+; 100 nM) or scrambled control miRNA (c; 1 nM) and with *S1pr1* siRNA (+; 1 nM) or control siRNA (100 nM), as indicated. Cells were cultured for 48 h to determine the impact of transfection on cell viability (D) or for 24 h to determine miR-155 (E) or *S1pr1* (F) expression. (G–I) Th2 (IL4^{gfp}CD45.2 C57BL/6 OTII) cells transfected with either miR-155 inhibitors (+; 100 nM) or scrambled control miRNA (c; 1 nM) and with *S1pr1* siRNA (+; 1 nM) or control siRNA (100 nM), as indicated, were adoptively transferred into the tail vein of WT mice (day 1), 1 d after an intratracheal delivery of OVA (day –1). Mice received two further OVA airway challenges on day 2 and day 4. Airway pathology was assessed on day 5. (G) Airway eosinophilia was determined in BAL fluid. (H and I) Lungs were stained with AB-PAS to determine airway goblet cell and mucus secretion (H), and results were quantified using qRT-PCR (I). (J) Single-cell suspensions of lungs and local lung-draining lymph nodes were stained for congenic (CD45.2) and T-cell receptor markers (Vβ5 and Vα2) to identify Th2 OTII cells in the BAL, lung, and lymph nodes.

suggest that low, but not absent, levels of *Rictor* are sufficient for Th2 cell differentiation and that miR-155-regulated *Rictor* may be required for proficient Th2 responses.

Following activation, lymphocyte egress from lymphoid tissue requires the down-regulation of *S1pr1* (45). We observed a rapid down-regulation of *S1pr1* in activated Th2 cells in vitro with a concomitant increase in miR-155 (SI Appendix, Fig. S12), suggesting that miR-155 targets *S1pr1*. Indeed, *mir-155*^{–/–} T cells isolated ex vivo had elevated *S1pr1* (Fig. 4), further supporting the notion that miR-155-regulates *S1pr1*. In vitro, dual luciferase reporter assays confirmed that miR-155 directly regulates *S1pr1*, whereas in vivo studies using purified Th2 cells confirmed that, although miR-155 targets several genes in Th2 cells, miR-155-regulated *S1pr1* is required for Th2-mediated airway inflammation, possibly via controlling Th2 migration to the lung. These data identify a previously unknown function of miR-155 in regulating Th2 cell migration through control of *S1pr1* expression and provide an miRNA-mediated upstream mechanistic pathway explaining the importance of *S1pr1* in Th2 cell biology (71, 72).

In conclusion, this study provides a comprehensive analysis of miRNA expression in highly purified reporter-positive T cells, highlighting the disparities between in vitro and ex vivo T cells, and functionally demonstrates the opposing roles of miR-155 and miR-146a in Th2-mediated allergic and helminth-induced immunity. The miR-155-mediated pathways regulating Th2 cell activation and egress and fulminant type-2 immunity suggest that

targeting miR-155, but not miR-146a, may help shape new strategies targeting specific miRNAs to treat allergic asthma and other Th2-mediated diseases.

Materials and Methods

Animals. C57BL/6, 129S8.B6 (F1), C57BL/6.Tcr^{tm1}Phi, *Rag2*^{–/–}, IL4^{gfp} (IL4/GFP-enhanced transcript; 4get) (73), *Il9*^{Cre}*R26*^{eyfp}, *Il17a*^{Cre}*R26*^{FP635} [kindly provided by Brigitta Stockinger, Medical Research Council, National Institute for Medical Research (NIMR), London], *Ifn*^{eyfp} (YETI), *Foxp3*^{rfp}, and *mir-155*^{–/–} and *mir-155*^{+/+} littermates were bred and kept in the specific pathogen-free facility at the NIMR. *mir-146a*^{–/–} bone marrow was kindly provided by David Baltimore (Braun Laboratories, California Institute of Technology, Pasadena, CA). Mice were randomly separated into cages containing five mice before the experimental procedure. All animal experiments were approved by local and national ethical review panels at NIMR and were carried out under UK Home Office regulations (Project License no. 80/2506). All mice were 6–8 wk old at the onset of experiments. A minimum of five mice per group was used for each experiment, unless otherwise indicated.

Generation of Mixed T-Cell Bone Marrow Chimeric Mice. Six- to eight-week-old mice were sublethally irradiated (2×, 450 rad) followed by adoptive transfer of 2–5 × 10⁶ bone marrow cells (20% bone marrow from *mir-146a*^{–/–}, *mir-155*^{–/–}, or WT donor mice with 80% bone marrow from C57BL/6.Tcr^{tm1}Phi mice). Mice were given water ad libitum supplemented with Baytril (Bayer) for 3 wk after radiation.

CD4 T-Cell Isolation and Flow Cytometry. CD4⁺ T cells were isolated from tissues by mechanical disruption followed by red blood cell lysis and Percoll

(Sigma Aldrich) gradient separation followed by positive or negative cell enrichment using magnetic beads (L3T4; Miltenyi Biotec). Cells then were stained with anti-mouse CD4 (clone RM4-5), CD44 (clone IM7), CD25 (clone PC61), TCR- β (clone H57-597), V β 5 (clone MR9-4), and V α 2 (clone B20.1). Dead cells were excluded from sorting and analysis with propidium iodide or Live/Dead stain (Invitrogen). For intracellular cytokine staining, anti-IFN- γ (clone XMG1.2), IL-4 (clone 11B11), IL-5 (clone TRFK5), IL-13 (clone eBio 13A), IL-17 (clone TC11-18H10), and anti-Foxp3 (clone FKJ-16s) (BD and eBioscience) were used after 6 h of stimulation with PMA (50 ng/mL), ionomycin (1 μ g/mL), and GolgiStop/Golgi plug (10 μ g/mL) (BD Biosciences) with Fix/Perm buffer (eBioscience) and Perm/Wash buffer (eBioscience) used according to the manufacturers' instructions. Anti-CD16/32 was used in all staining. Cells were acquired using a BD LSRII and analyzed with FlowJo software (Treestar). For cell sorting, BD Influx, FACSARIA (BD), or MoFlo XDP (Beckman Coulter) sorters were used; the purity of sorted cells was >95%.

In Vitro Th Cell Polarization. T cells were polarized under the following conditions: Th1: IL-12 (10 ng/mL), anti-IL-4 (10 μ g/mL); Th2: IL-4 (10 ng/mL), IL-2 (5 ng/mL), anti-IFN- γ (10 μ g/mL); Th17: IL-6 (10 ng/mL), TGF- β (1 ng/mL), IL-1 β (10 ng/mL), 6-formylmethyl-dolo (3,2-b) carbazole (250 nM), anti-IL-4, anti-IFN- γ , anti-IL-2 (10 μ g/mL); Th9: IL-6 (10 ng/mL), IL-4 (10 ng/mL), TGF- β (5 ng/mL), IL-1 β (10 ng/mL), anti-IFN- γ (10 μ g/mL); iTreg: TGF- β (5 ng/mL), anti-IL-4, anti-IFN- γ (10 μ g/mL). Bulk or FACS purified reporter-positive in vitro Th cells were harvested on day 7, or as indicated. All recombinant cytokines and antibodies were purchased from R&D Research, BioXcell, or Peprotech.

Airway Allergy, EAE, and Infection Models. *H. polygyrus*. Mice were infected with 200 *H. polygyrus* infective larvae (stage 3) by oral gavage. Primary (*H.p.* 1 $^{\circ}$) *IL4^{gfp}*CD4 $^{+}$ T cells were FACS sorted on day 14 from mesenteric lymph nodes. Secondary (*H.p.* 2 $^{\circ}$) *IL4^{gfp}*CD4 $^{+}$ T cells were recovered from mice on day 42, following drug cure (day 14) and challenge infection (day 28). Th2-mediated immunity to *H. polygyrus* was determined 14 d after secondary infection, with luminal worms counted in the small intestine.

T. muris. Mice were infected with 200 *T. muris* eggs (kindly provided by Richard Grencis, University of Manchester, Manchester, United Kingdom). Worms in the cecum and large intestine were counted on day 27 postinfection.

HDM-induced airway allergy. For HDM-induced airway inflammation, mice were sensitized by i.p. injection of 100 μ g HDM (Greer) with 2 mg of Imject Alum (Pearce) followed by i.t. challenge with 100 μ g HDM in PBS on day 21 and day 24. *IL4^{gfp}*CD4 $^{+}$ T cells were purified from the lung and local lymph nodes by FACS sorting on day 25. Airway infiltrates were determined on stained cytopins of BAL recoveries, performed with 1.5 mL of cold PBS.

OTII-Th2-mediated airway allergy. For OVA-induced OTII reactivation in the airways, 10^6 *IL4^{gfp}* OTII cells were FACS purified following in vitro Th2 differentiation and were mock transfected or were transfected with 100 nm of miR-155 short hairpin inhibitors, *S1pr1* siRNA, or control scrambled miRNA or siRNA, as indicated, and were adoptively transferred into the tail vein of recipient mice. Recipient mice were given an intratracheal delivery of OVA (Sigma Grade V), 1 d before cell transfer (day -1) and 1 and 3 d after cell transfer (day 2 and day 4). Mice were analyzed for evidence of Th2-mediated disease on day 5.

P. chabaudi. Th1 cells were isolated from *Ifn γ ^{yfp}* mice that had received 10^5 *P. chabaudi*-infected red blood cells i.p. *Ifn γ ^{yfp}*CD4 $^{+}$ T cells were FACS purified from spleens on day 7 postinfection. In experiments with miR-155 $^{-/-}$ mice, animals were infected with 10^5 *P. chabaudi*-infected red blood cells and analyzed for their ability to mount protective Th1 responses on day 8–9 postinfection.

EAE. *IL17a^{Cre}Rosa26^{FP635+}* CD4 $^{+}$ T cells were FACS purified from the spinal cord and lymph nodes of mice with EAE on days 9–12 as described previously (74). nTregs (CD4 $^{+}$ CD25^{hi}Foxp3^{hi}) were purified from the spleen of naive Foxp3^{hi} mice. For histopathology, lung or gut tissue was fixed in 10% neutral-buffered formalin [4% (wt/vol) formaldehyde] for at least 24 h before being transferred to 70% (wt/vol) ethanol. Tissues were stained with Alcian blue periodic acid-Schiff (AB-PAS) stain and H&E stain for detection of mucus production and leukocyte infiltration, respectively. For FISH, tissue was first perfused with 4% paraformaldehyde (PFA) and stored in 30% (wt/vol) sucrose/PBS solution at 4 $^{\circ}$ C overnight before snap freezing and sectioning (10 μ m). Tissue was refixed with 4% (wt/vol) formaldehyde and treated with proteinase K before incubation

with digoxigenin-labeled LNA (Exiqon) miRNA probes (miR-155 sequence ACCCTATCACAAATTAGCATTA; control sequence, GTGTAACACGTCTATACGCCCA) in hybridization buffer.

Transfection and Dual Luciferase Reporter Assays. T cells were washed, counted, and resuspended in Nucleofector solution (Lonza) with supplements, according to the manufacturer's recommendations. miRNA mimics or inhibitors (100 nM) (Thermo Scientific, Dharmacon) or *S1pr1* siRNA or control siRNA were added to cell suspension, and cells were plated at a density of $2-3 \times 10^5$ cells per well in a 96-well Nucleocuvette Plate. Cells were transfected in a 96-well-Shuttle System and left to rest for 3–20 h at 37 $^{\circ}$ C before analysis or adoptive transfer, as indicated in figure legends. For dual luciferase reporter assays, Jurkat T cells were transfected with firefly/Renilla Duo-Luciferase reporter clones containing 1 nM of either the 3' UTR of *S1pr1* or a control 3' UTR, using the Nucleofector system (Genecopeia), as above, according to the manufacturer's instructions. Cells were cotransfected with 100 nM of either miR-155 mimics or a scrambled miRNA control, as indicated. Relative fluorescent units were determined 24 h later.

RNA, Quantitative RT-PCR, and Microarray. RNA was isolated from tissues and cells using RNeasy mini-spin columns (Qiagen) according to the manufacturer's instructions. cDNA was generated from 5 ng of total RNA using the WT-Ovation Pico system (version 1) RNA Amplification System followed by double-stranded cDNA synthesis using the WT-Ovation Exon Module. cDNA quality was determined by the Systems Biology Unit at the NIMR using an Agilent BioAnalyzer and through hybridization performance on Affymetrix GeneChip mouse Genome 430A 2.0 microarray (Affymetrix) and miRNA 3.0 arrays (Affymetrix). Microarray data were quantile-normalized and analyzed using GeneSpring software (Agilent). Differentially expressed genes were determined using ANOVA and *t* tests. Genes with false discovery rate-corrected *P* values <0.1 and fold change values ≥ 1.5 were considered significant, as indicated in figure legends. Three to five biological replicates of each T-cell subset were used. Four-way comparative analyses, expression-pairing analyses, target predictions (miRecords, Tarbase, Targetscan), and target abundance were determined using Ingenuity Pathways Analysis (Ingenuity Systems; www.ingenuity.com). For quantitative RT-PCR (qRT-PCR), RNA was reverse-transcribed using the miScript II RT Kit (Qiagen). Real-time RT-PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems); relative quantities of mRNA or miRNAs were determined using SYBR Green PCR Master Mix (Applied Biosystems) and by the comparative threshold cycle method as described by Applied Biosystems for the ABI Prism 7700/7900HT Sequence Detection Systems. mRNA levels were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT), and miRNA levels were normalized to the small nucleolar RNA, RNU6b, and were expressed as a relative increase or decrease compared with levels in controls or relative to HPRT or RNU6b, as indicated.

ELISA. Cytokines and IgE were measured by ELISA. Capture and biotinylated detection antibodies for IL-4, IL-5, IL-13, IFN- γ , IL-17A, and IgE were from R&D Systems. The concentration of analytes in the sample was determined from a serial-fold diluted standard curve with OD read at 405 nm in an ELISA reader (Tecan II Safire).

Statistical Analysis. Datasets were compared by Mann-Whitney test or one-way ANOVA as specified in the figure legends using GraphPad Prism (V.5.0). Differences were considered significant at *P* $\leq$ 0.05.

ACKNOWLEDGMENTS. We thank Abdul Sesay, Harsha Jani, and Leena Bhaw-Rosun [Systems Biology Department, National Institute for Medical Research (NIMR)] for help with microarray experiments; Radma Mahmood and Radika Anand for help with histology; Samir Kelada (University of North Carolina at Chapel Hill) for help with transcriptional analysis; Natalia Dinischitau (Immune Cell Biology, NIMR), for help with expanding and purifying plasmids; Brigitta Stockinger and Alexandre Potocnik for *IL17a-Cre*, *IL13Cre*, *R26^{FP635}* mice; Joao Duarte for help with experimental autoimmune encephalomyelitis experiments; Graham Preece, Wayne Turnbull, and Bhavik Patel for assistance with flow cytometry-related sorting and analysis; and Trisha Norton, Keith Williams, Adebambo Adekoya, and the B2 and Building C staff for animal husbandry. This work was supported by the Medical Research Council (file reference nos. MC_UP_A253_1028 and U117584248).

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