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Cutting Edge

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Cutting Edge: The Foxp3 Target miR-155 Contributes to the Development of Regulatory T Cells[1]

Susan Kohlhaas,[2*] Oliver A. Garden,[2†‡] Cheryl Scudamore,[§] Martin Turner,[*] Klaus Okkenhaug,[*] and Elena Vigorito[3*]

Foxp3 is a transcription factor that is essential for the normal development of regulatory T cells (Tregs). In the absence of microRNAs (miRNAs), Foxp3$^+$ Tregs develop but fail to maintain immune homeostasis, leading to a scurfy-like disease. Global analysis of the network of genes regulated by Foxp3 has identified the miRNA miR-155, which is highly expressed in Tregs, as a direct target of Foxp3. In this study we report that miR-155-deficient mice have reduced numbers of Tregs, both in the thymus and periphery, due to impaired development. However, we found no evidence for defective suppressor activity of miR-155-deficient Tregs, either in vitro or in vivo. Our results indicate that miR-155 contributes to Treg development, but that additional miRNAs control Treg function. ***The Journal of Immunology,*** **2009, 182: 2578–2582.**

Regulatory T cells (Tregs)[4] constitute a subset of CD4$^+$ T cells essential for the maintenance of self-tolerance. The transcription factor Foxp3 is specifically expressed in Tregs and is a key regulator of their differentiation and suppressor function. Deficiency in Foxp3 results in severe autoimmune disease in both mice and humans (1). Expression of Foxp3 is needed to establish and maintain a particular transcriptional profile of protein-coding genes and microRNAs (miRNAs) that distinguishes Tregs from other T cell lineages (2–4).

miRNAs are 21- to 23-nt RNA molecules that regulate the expression of protein-coding genes. More than 700 miRNAs have been identified in mammals and have been implicated in a wide range of biological functions (5, 6). The biogenesis of miRNAs involves two processing steps, respectively catalyzed by the RNases Drosha and Dicer (reviewed by Bartel in Ref. 5). Conditional ablation of Dicer has uncovered key roles for miRNAs in the development of B and T cells (7–9). More recently, conditional deletion of Drosha or Dicer in Foxp3$^+$ regulatory T cell lineages has implicated miRNAs as being essential for Treg function, because these mice develop a similar wasting disease to that observed in Foxp3-knockout mice (10–12). However, the specific miRNAs that are important for Treg function remain unidentified. miR-155 is an attractive candidate because Foxp3 binds to the promoter region of its host gene *bic*, and miR-155 is highly expressed in Tregs (2, 4, 13). Moreover, miR-155 is an important regulator of lymphocyte function and homeostasis (14, 15). However, the functional significance of miR-155 expression in Tregs has not been reported to date.

In this report, we investigated the contribution of miR-155 to Treg development and function. Mice deficient in bic/miR-155 showed reduced numbers of Tregs in the thymus and spleen. We found that Treg development required bic/miR-155, but that it was dispensable for Treg proliferation or survival in the periphery. Despite the lower number of Tregs, their suppressor function in vitro remained intact. Furthermore, no signs of spontaneous inflammatory bowel disease (IBD) were observed in young or aged miR-155-deficient mice, and miR-155-deficient CD4$^+$CD25$^+$ T cells were able to prevent colitis induced by the adoptive transfer of CD4$^+$CD45RB^{high} T cells into lymphopenic hosts. Thus, miR-155 is required for development of the Treg lineage, but its absence does not overtly compromise Treg function in vitro or in vivo.

Materials and Methods

Mice

bic/miR-155-deficient mice, *bic*$^{m2/m2}$ (15), were backcrossed five times to the C57BL/6 background. Rag2$^{-/-}$ mice (16) on the C57BL/6 background were provided by F. Colucci (Babraham Institute, Cambridge, U.K.). All experiments were performed according to U.K. Home Office regulations.

*Laboratory of Lymphocyte Signalling and Development, Babraham Institute, Cambridge, United Kingdom; †Regulatory T Cell Laboratory, Infection and Immunity Research Group, Department of Veterinary Clinical Sciences, Royal Veterinary College, Camden Campus, London, United Kingdom; ‡Department of Immunology, Imperial College London, Hammersmith Campus, London, United Kingdom; and §Department of Pathology and Infectious Diseases, Royal Veterinary College, North Mymms, Hatfield, Hertfordshire, United Kingdom

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[2] S.K. and O.A.G. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Elena Vigorito, Laboratory of Lymphocyte Signalling and Development, Babraham Institute, Cambridge, CB22 3AT, U.K. E-mail address: elena.vigorito@bbsrc.ac.uk

[4] Abbreviations used in this paper: Treg, T regulatory cell; IBD, inflammatory bowel disease; miRNA, microRNA.

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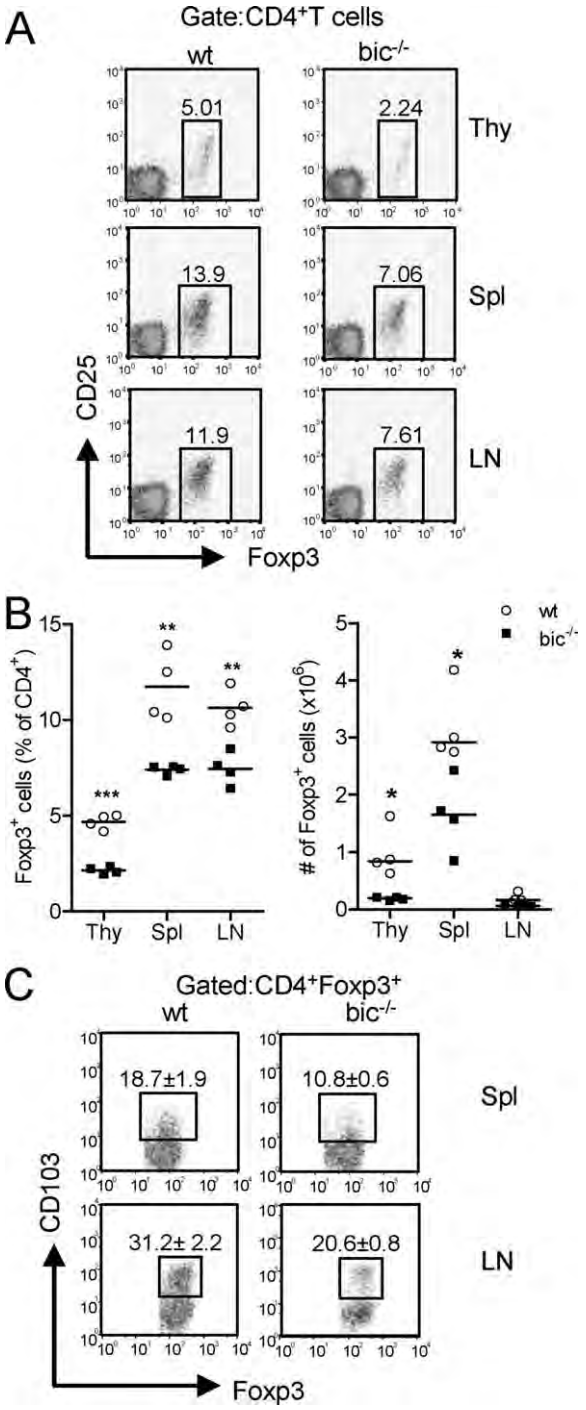

FIGURE 1. Reduced number of Tregs in the absence of bic/miR-155. *A*, The percentage of $CD4^+Foxp3^+$ cells in the thymus (Thy; *top panels*), spleen (Spl; *middle panels*), and inguinal lymph nodes (LN; *bottom panels*). Density plots are gated on CD4 single positive cells. In spleen and lymph node, most $Foxp3^+$ cells were also $CD25^+$. *B*, The percentage of $Foxp3^+$ cells that are also $CD4^+$ cells in thymus (Thy), spleen (Spl), and lymph nodes (LN) (*left panel*) or the absolute number of $Foxp3^+$ cells in the same tissues (*right panel*) in wild-type mice (wt; open circles) and bic/miR-155-deficient mice ($bic^{-/-}$; filled squares) is shown. Statistical differences between the two populations were determined by two-tailed student's *t* test. ***, $p < 0.001$, **, < 0.01; and *, $p < 0.05$. *C*, Frequency of $CD103^+Foxp3^+$ among $CD4^+$ cells in spleen (Spl) and lymph node (LN) of wild-type (wt) or bic/miR-155-deficient mice ($bic^{-/-}$). Numbers indicate the percentage of cells in the designated gate as the mean and SD for three mice.

Flow cytometry

Cells were stained for surface markers (antibodies from BD Biosciences or eBioscience) followed by Foxp3, according to instructions from eBioscience. Data were acquired with a FACSCalibur or LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star).

Foxp3 induction

Naive $CD4^+$ T cells purified by negative selection (Miltenyi Biotec) were stimulated by plate-bound anti-CD3 and anti-CD28 mAbs in the presence of 1 ng/ml human TGF-β (Peprotech). After 4 days, cells were stained for CD4 and Foxp3.

Radiation chimeras

Bone marrow cells from wild-type or bic/miR-155-deficient mice were mixed at a 1:1 ratio and 3×10^6 cells were injected into sublethally irradiated (500 rad) $Rag2^{-/-}$ mice. Chimeric mice were analyzed 7 wk after reconstitution.

BrdU incorporation

Mice were injected daily with 0.8 mg of BrdU for 3 days. On day 4, spleen and lymph node cells were analyzed for BrdU incorporation by flow cytometry.

$CD4^+CD25^-$ suppression assay in vitro

$CD25^+$ and $CD25^-$ $CD4^+$ T cells were purified by positive and negative selection, respectively (Miltenyi); the purity of each population was >90% as determined by flow cytometry. A total of 5×10^4 $CD4^+CD25^-$ T cells were cultured with the indicated ratios of either wild-type or bic/miR-155-deficient $CD4^+CD25^+$ T cells with 1 μg/ml anti-CD3 and irradiated APCs for 72 h. The incorporation of [^{3}H]TdR was measured over the last 6 h.

$CD4^+CD25^-$ suppression assay in vivo

$CD4^+CD25^+$ and $CD4^+CD45RB^{high}$ cells were isolated by cell sorting on a FACSAria flow cytometer, yielding populations of >99% purity. A published adoptive transfer model of colitis was used, injecting 5×10^5 $CD45RB^{high}$ T cells alone or together with 1×10^5 wild-type or bic/miR-155-deficient $CD4^+CD25^+$ cells into $Rag2^{-/-}$ recipients (17). The severity of colitis up to 12 wk after injection was independently scored by O.A.G. and C.S., according to previously published criteria (18), and consensus scores are presented.

Results and Discussion

Reduced number of Tregs in the absence of bic/miR-155

Tregs were identified as CD4 single positive and $Foxp3^+$ in the thymus, spleen, and lymph nodes of adult mice. We found a 2- to 3-fold reduction in the frequency and absolute number of Tregs in the thymi and spleens from miR-$155^{-/-}$ mice (Fig. 1, *A* and *B*). A similar trend was observed in lymph nodes, though the results were more variable (Fig. 1, *A* and *B*). Similar results were observed in 3-wk-old mice (data not shown). In 1-year-old mice the phenotype was milder, as the number of Tregs was only marginally reduced in the thymus and spleen (data not shown). Despite a reduced number of Tregs, phenotypic analysis revealed normal expression of surface markers such as CD25, CTLA-4, GITR, or CD44 by Tregs from bic/miR-$155^{-/-}$ mice (Fig. 1*A* and data not shown). However, we observed a decrease in the proportion of miR-155-deficient Tregs expressing the $\alpha_E\beta_7$ integrin CD103 (Fig. 1*C*).

The lower proportions and numbers of Tregs in the absence of miR-155 prompted us to investigate the requirement of miR-155 for development, proliferation, or survival of Tregs. To address the requirement of miR-155 for Treg development, we generated bone marrow chimeric mice by reconstituting sublethally irradiated $Rag2^{-/-}$ mice with a 1:1 mixture of wild-type (B6SJL) CD45.1 bone marrow cells with either wild-type (B6) or bic/miR-155 (bic)-deficient bone marrow cells expressing the allotypic marker CD45.2. Analysis after 7 wk showed that bone marrow precursors from wild-type CD45.2 cells were able to competitively reconstitute all lymphoid subsets. As expected, the ratio of CD45.2 to CD45.1 was ~1:1 (Fig. 2*A*;

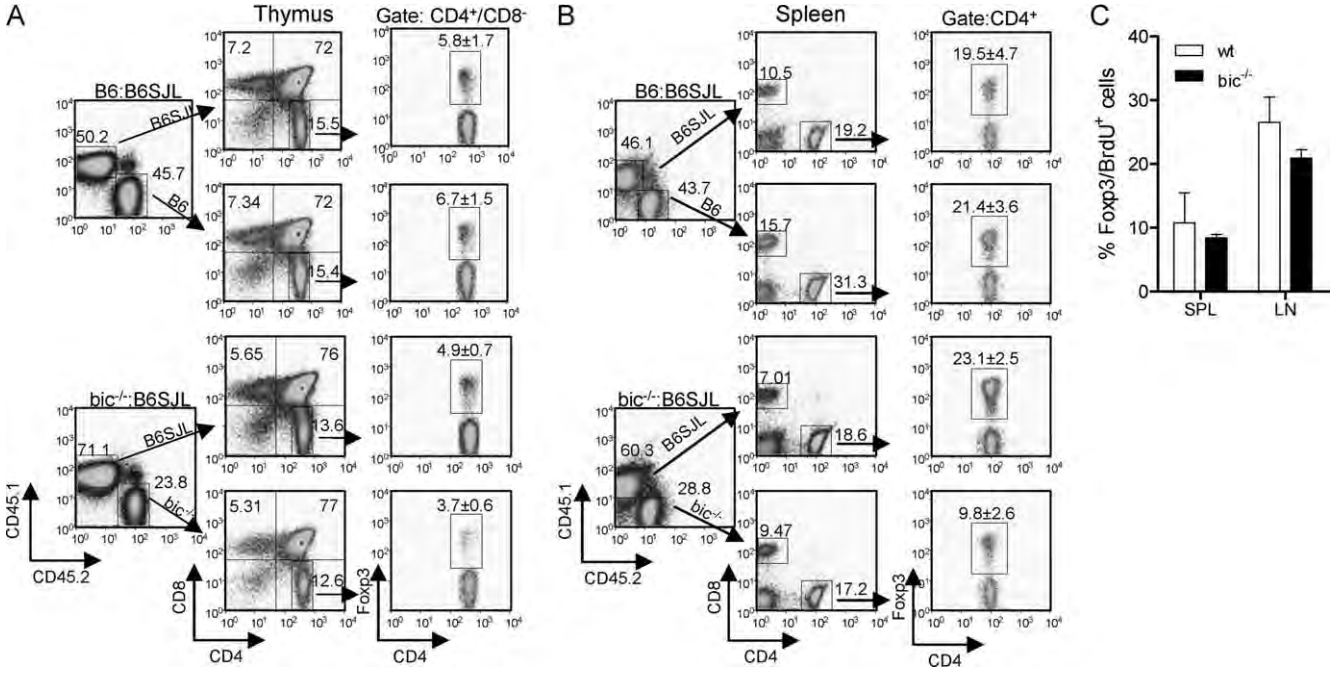

FIGURE 2. miR-155 is required for the development of Tregs in an intrinsic manner. *A* and *B*, Representative example of flow cytometric analysis of thymocytes (*A*) and spleen (*B*) from 1:1 mixed chimeras 7 wk after bone marrow reconstitution. B6:B6SJL corresponds to the 1:1 mixture of wild-type CD45.2 and wild-type CD45.1 cells ($n = 5$). bic$^{-/-}$:B6SJL corresponds to the mixture of bic/miR-155-deficient precursors (CD45.2) with wild-type (CD45.1) cells ($n = 4$). Density plots are gated on live lymphocytes based on forward and side scatter (*left panels*). *Middle panels* show CD4 vs CD8 profiles gated on CD45.1$^+$ (*top*) and CD45.2$^+$ (*bottom*) cells. *Right panels* show CD4 vs Foxp3 stain gated on CD45.1$^+$CD4$^+$ (*top*) or CD45.2$^+$CD4$^+$ (*bottom*) cells. In the CD4 vs Foxp3 density plots, the numbers indicate the percentage of cells in the designated gate as the mean and SD for five chimeric mice. *C*, Normal proliferation of bic/miR-155-deficient Tregs. The graph shows the proportion of BrdU$^+$ Tregs from spleen (SPL) and lymph nodes (LN) for three mice per group. In this experiment, Tregs were identified as CD4$^+$CD25$^+$ owing to incompatibility of the Foxp3 and BrdU staining procedures. wt, Wild type.

CD45.1 vs CD45.2 stain and data not shown). Analysis of the wild-type CD45.1 and bic/miR-155$^{-/-}$ CD45.2 chimeras revealed a decreased proportion of bic/miR-155-deficient thymocytes and splenocytes (Fig. 2, *A* and *B*; CD45.1 vs CD45.2 stain). The decreased representation of miR-155$^{-/-}$ thymocytes was likely to be referable to a defect occurring at an early stage of development, as double-negative, double-positive, and CD4 and CD8 single-positive cells were all similarly under-represented (data not shown). In addition, the proportions of bic/miR-155-deficient double-negative, double-positive, CD4 or CD8 single-positive thymocytes were normal, arguing against a block at a later stage of development (Fig. 2*A*; CD4 vs CD8 stain). However, we found a relative reduction in the proportion of Foxp3$^+$ cells within the bic/miR-155$^{-/-}$ CD4$^+$ T cell subset (Fig. 2*A*; CD4 vs Foxp3 stain for bic:B6SJL chimeras) that indicated an intrinsic developmental defect. Similarly, the CD4 compartment in the spleen showed a reduced proportion of bic/miR-155-deficient Tregs compared with their wild-type counterparts (Fig. 2*B*; CD4 vs Foxp3 stain). Taken together, our results showed that bic/miR-155 is required for optimal Treg development in a T cell-intrinsic manner.

To test whether bic/miR-155 is required for Treg proliferation in the periphery, we measured the ability of CD4$^+$CD25$^+$ T cells from spleen or lymph node to incorporate BrdU after continuous labeling for 3 days. The bic/miR-155$^{-/-}$ Tregs showed similar levels of BrdU incorporation, suggesting that miR-155 is not required for mature Treg cell cycling or survival in vivo.

To determine whether bic/miR-155 is required for Treg homeostatic proliferation and survival, we adoptively transferred either wild-type or bic/miR-155-deficient Tregs (CD45.2) in combination with wild-type CD45RB^{high}CD4$^+$ T cells (CD45.1) into lymphopenic mice and, after 5 wk, counted Tregs in both the spleen and mesenteric lymph nodes. We recovered similar numbers of wild-type or bic/miR-155-deficient Tregs from spleen and lymph nodes, indicating that bic/miR-155 is dispensable for homeostatic proliferation or survival of Tregs (data not shown).

In summary, our results support a requirement for miR-155 during thymic development of Tregs, but not for their maintenance or proliferation in the periphery.

bic/miR-155 deficiency does not affect Foxp3 up-regulation or Treg-suppressive activity in vitro

Peripheral T cells can be induced to express Foxp3 upon TCR stimulation in the presence of TGF-β, providing a model for post-thymic Treg differentiation. As miR-155 is induced upon TCR activation, we wished to test whether it is required for Foxp3 induction. However, no impairment was observed in the ability of bic/miR-155-deficient T cells to up-regulate Foxp3 (Fig. 3*A*). We next assessed the ability of CD4$^+$CD25$^+$ bic/miR-155-deficient cells to suppress the proliferation of wild-type CD4$^+$CD25$^-$ cells stimulated by anti-CD3 in the presence of wild-type APCs. The bic/miR-155-deficient Tregs suppressed as effectively as wild-type cells in this assay (Fig. 3*B*). Similar results were observed when wild-type CD4$^+$CD25$^-$ cells were stimulated by anti-CD3- and anti-CD28-coated beads (data not shown).

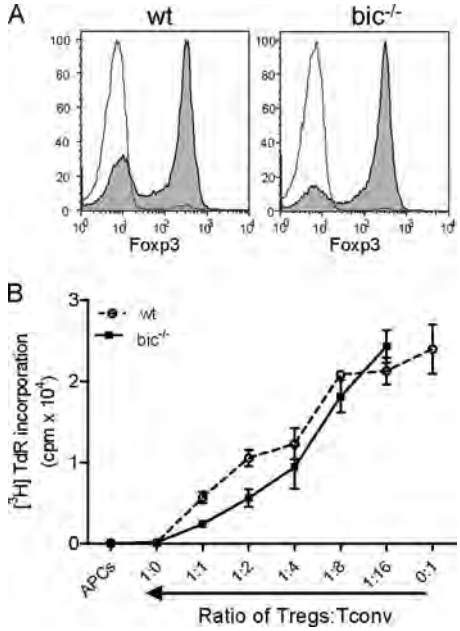

FIGURE 3. Normal Foxp3 induction and suppressor function of bic/miR-155-deficient Tregs in vitro. *A*, Foxp3 induction in vitro of wild-type (wt) or bic/miR-155-deficient ($bic^{-/-}$) T cells. Unfilled lines indicate the isotype control and gray-filled lines indicate the Foxp3-stained cells. The mean and SD for three independent cultures was 78.5% ± 8.5% for wild-type cells and 81.4% ± 0.4% for bic/miR-155-deficient cells. *B*, Wild-type (wt) $CD4^+CD25^-$ conventional T cells (Tconv) were stimulated with anti-CD3 in the presence of irradiated APCs at the indicated ratio of either bic/miR-155-deficient ($bic^{-/-}$; closed squares) $CD4^+CD25^+$ T cells (Tregs) or their wild-type counterparts (open circles). Data points represent the mean ± SEM of triplicate measures. One representative experiment of three is shown.

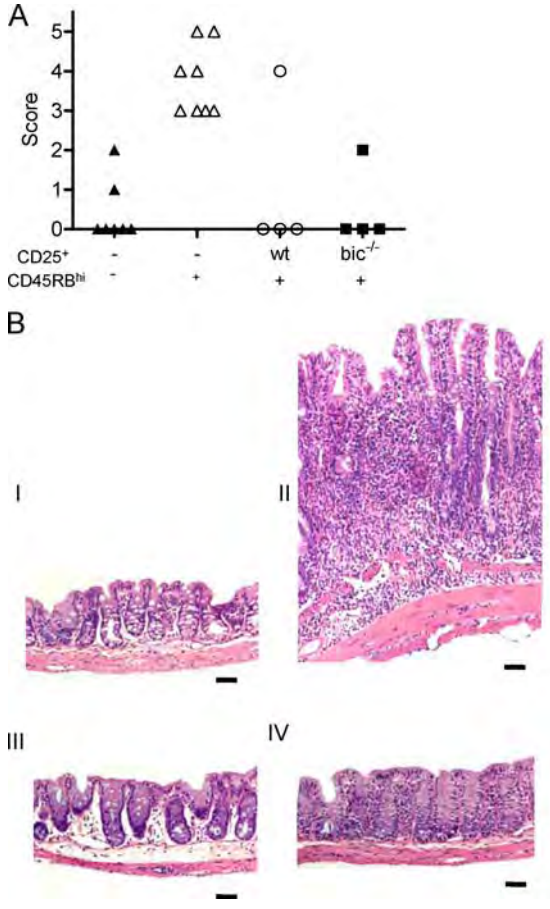

FIGURE 4. bic/miR-155 is dispensable for Treg function in vivo. *A*, $Rag2^{-/-}$ mice were respectively injected with PBS (filled triangles), $CD4^+CD45RB^{high}$ T cells (open triangles), or $CD4^+CD45RB^{high}$ cells with wild-type (wt; open circles) or $bic^{-/-}$ Tregs (closed squares). Large intestine was collected 12 wk after injection and colonic inflammation was scored from 0 (normal) to 5 (severe colitis) using previously published criteria (18). *B*, Representative photomicrographs showing absence of inflammation in mice injected with PBS (*I*) but severe inflammation in mice injected with $CD45RB^{high}$ T cells (*II*) characterized by heavy infiltrates of predominantly mononuclear cells in the mucosa and submucosa, scattered neutrophils with microabscessation, crypt hyperplasia, and epithelial metaplasia and occasional ulceration. When either wild-type (*III*) or bic/miR-155-deficient (*IV*) Tregs were cotransferred with the $CD45RB^{high}$ T cells, colitis was not observed in the majority of mice. H&E staining was used; scale bar represents ~250 μm (original magnification: ×200).

bic/miR-155-deficient Tregs show competent suppressor activity in vivo

We previously reported that bic/miR-155-deficient mice, $bic^{m1/m1}$, displayed airway remodeling and developed enteric inflammation on a mixed 129/C57BL6 background (15). As impaired Treg function can lead to spontaneous IBD, we examined mice of 6 and 12 mo of age for signs of gastrointestinal inflammation. We noticed that up to 12–13 wk of age, the weight of wild-type and bic/miR-155-deficient mice was similar. Although older bic/miR-155-deficient mice were underweight (data not shown), histological analysis revealed no evidence of significant gastrointestinal inflammation. The lack of pathological lesions in bic/miR-155-deficient mice suggested that Treg suppressor capacity was not significantly impaired. However, as bic/miR-155 regulates the function of T cells and myeloid cells (14, 15, 19), concurrent defective T cell effector function might have protected the mice from spontaneous IBD. Moreover, although we found that bic/miR155$^{-/-}$ Tregs efficiently suppressed wild-type T cell proliferation in vitro, the mechanisms for in vivo suppression may be different. To assess the intrinsic suppressive function of bic/miR-155-deficient Tregs in vivo, we evaluated their ability to prevent colitis when cotransferred with wild-type $CD4^+CD45RB^{high}$ T cells into immune-deficient recipients (17). In common with the wild-type Tregs, bic/miR155$^{-/-}$$CD4^+CD25^+$ T cells protected the $Rag2^{-/-}$ recipients from the colitogenic effect of cotransferred $CD4^+CD45RB^{high}$ T cells (Fig. 4). Taken together, our results suggest that bic/miR-155 regulates the development of $Foxp3^+$ cells, but not their suppressor function. As miR-155 has been reported as a Foxp3 target, miR-155 may contribute to the stability of the transcriptional program induced by Foxp3 by repressing unwanted genes. In addition, the reduced expression of CD103 on bic/miR-155$^{-/-}$ Tregs may affect their recruitment to sites of infection (20). Additional experiments are required to identify the molecular targets of miR-155 in developing Tregs. As miRNAs play a key role in the control of Treg homeostasis and function (10–12), other miRNAs, alone or in combination with miR-155, are likely to prove important for Treg biology.

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Disclosures

The authors have no financial conflict of interest.

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