

TGF- β conditions intestinal T cells to express increased levels of miR-155, associated with down-regulation of IL-2 and *itk* mRNA

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Transforming growth factor (TGF)- β , is an immunosuppressive cytokine that inhibits T-cell activation. We hypothesized that TGF- β mediates its immunoinhibitory effects by modulation of micro RNA (miRNA)-155 (miR-155). Interleukin (IL)-2 and interferon- γ are down-regulated by TGF- β in activated CD4 peripheral blood T cells and lamina propria T cells (LPT), but miR-155 is upregulated ninefold specifically in LPT. Consequently, this study focuses on the role of TGF- β -enhanced miR-155 on LPT immune responses. TGF- β induces miR-155 in both freshly isolated and LPT lymphoblasts, whereas other inducible miRNAs are not regulated by TGF- β . Using MAMI bioinformatics database, we determined that inducible T-cell kinase (*itk*) is a functional target of miR-155 that exhibits an inverse mRNA response to that of miR-155. To determine experimentally that miR-155 regulates *itk*, transfection experiments were performed that demonstrated miR-155 overexpression decreased *itk* and IL-2 mRNA, whereas antagonism of miR-155 restored both mRNAs in activated cells. These findings describe a TGF- β -dependent function for miR-155 in modulating cytokine and T-cell immune responses in the gut.

INTRODUCTION

Transforming growth factor (TGF)- β 1 is a ubiquitous and multifunctional cytokine, central to modulation of host defense. Secreted by regulatory T cells (Tregs) and non-immune cells, TGF- β has a key role in experimental models of oral tolerance and in the pathogenesis of experimental colitis.^{1–3} Many studies in murine models of inflammatory bowel disease have shown that the presence of functional TGF- β is associated with either complete protection from the development of colitis or reduced severity of colitis.^{4,5} Adoptive co-transfer of CD4+ CD25– colitogenic T cells and CD4+ CD25+ Tregs into RAG–/– recipient mice prevents colitis, which is reversed upon administration of anti-TGF- β 1 antibody, indicating that protection from colitis is mediated by TGF- β ⁶ acting on pathogenic T cells and that in the absence of this interaction, pathogenic T cells escape Treg control. Additional studies with Tregs from TGF- β R2 knockout mice suggest that TGF- β is not only a mediator of Treg function but is also required for Treg development.⁶ Functionally, TGF- β inhibits the entry of mouse CD4 and CD8 T cells into cell cycle progression, proliferation, and interleukin (IL)-2 production in Smad3-dependent and independent manner.⁷

These and other studies in murine models emphasize the role of TGF- β in regulating T-cell function in the intestinal mucosa. In humans, altered TGF- β levels are proposed as a reason for unrestricted immune reactivity, leading to acute and chronic inflammation with subsequent tissue damage.^{8,9} In addition, patients with inflammatory bowel disease have inappropriate T-cell responses to antigenic components of their own intestinal microflora that can be traced back to an abnormal profile of regulatory cytokines like TGF- β .^{10,11} However, the molecular mechanism by which TGF- β inhibits intestinal T cells is poorly understood. Previously, we reported that TGF- β treatment can directly inhibit human effector/memory peripheral blood T-cell (PBT) activation and proliferation, by blocking the expression of cell cycle progression regulators and IL-2 secretion¹² through modulation of T-cell receptor (TCR) signaling. In the current report, we show that human lamina propria T cells (LPT), known to be hypo-responsive to immune activation,¹³ are still inhibited by the suppressive effects of TGF- β via post transcriptional modification of TGF- β -targeted molecules. Inducible T-cell kinase (*itk*), a TCR signaling molecule belonging to the *tec* family of kinases and involved in T-cell activation,

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is modulated by TGF- β ¹⁴ and T cells from *itk*-null mice show reduced IL-2 production *in vitro* as well as *in vivo*.¹⁵ However, the mechanism of inhibition is unknown.

Short, endogenous, non-coding oligonucleotides called micro RNAs (miRNAs) regulate gene expression and have been implicated in various diseases¹⁶ and more recently in immune regulation.¹⁷ miRNAs inhibit gene expression by annealing with the 3' untranslated region of target mRNA that facilitate either degradation of transcripts or directly inhibit protein synthesis. The role of miRNAs in immune homeostasis and its loss or gain of function during inflammation is a rapidly expanding area of investigation. Evidence for involvement of miRNAs in immunity has emerged from studies showing selective expression of miRNAs in the context of inflammation.^{18–20} MiR-155, encoded by the non-coding transcript of the B-cell Integration Cluster (*BIC*) gene, is an important modulator of innate and adaptive immune responses.^{21,22} It is expressed in human B-cell lymphomas and in activated mature B and T cells, macrophages, and DCs. A potential role of miR-155 in regulating adaptive immune responses was associated with reduced TCR-induced interferon (IFN)- γ release from *bic*^{-/-} CD4⁺ T cells²³ and B-cell receptor-mediated tumor necrosis factor- α production from B cells,²⁴ indicating that miRNA-155 facilitates cytokine release in lymphoid cells and possibly has a critical role in the pathogenesis of autoimmune inflammatory disorders.^{25,26} Apart from the role of miR-155 in inflammation, the latter modulates immunoregulatory cytokines as well. MiR-155 targets Smad2 to modulate a macrophage response to TGF- β ²⁷ and Smad5 to interfere with the TGF- β pathway leading to lymphomagenesis.²⁸ The pleiotropic nature of miR-155 is emphasized in studies showing TGF- β -mediated upregulation of miR-155 in epithelial cells resulting in epithelial mesenchymal transition²⁹ and down-regulation of miR-155 in human fibroblasts.³⁰ The current study focuses on a TGF- β mediated role of miR-155 in intestinal T cells.

Our studies show that miR-155 displays an inverse temporal distribution with IL-2 and *itk* in activated CD4 LPT in the presence of TGF- β . Consistent with the bioinformatic prediction that miR-155 targets *itk*, we show that overexpression of miR-155 in CD4 LPT causes *itk* mRNA levels to decline along with a modest decrease in IL-2 mRNA. Conversely, silencing miR-155 caused an increase of both *itk* and IL-2 mRNA expression in TCR-activated LPT, suggesting that TGF- β mediated immunosuppressive effects may, in part, be mediated through miR-155 modulation.

RESULTS

TGF- β inhibits TCR-activated PBT and LPT cytokine production

We previously reported that TGF- β inhibits activated human PBT cells by modulating TCR-mediated signaling, inducing cell cycle arrest and inhibition of IL-2 production.¹² To extend this study, we tested whether human intestinal T cells, known to be tolerant to antigenic stimulation, were similarly responsive to the immunosuppressive effects of TGF- β . CD4 PBT and LPT were pretreated with TGF- β for 24 h and stimulated in a polyclonal manner through the TCR by crosslinking CD3 and

CD28 in the presence or absence of TGF- β . Forty-eight hours post-stimulation, protein expression levels of IL-2 and IFN- γ were compared between PBT and LPT. Not surprisingly, CD4 LPT produced ~12-fold less IL-2 following TCR-stimulation compared with PBT (**Figure 1a,b**; open bars), while TGF- β suppressed IL-2 by twofold in both CD4 LPT and PBT (**Figure 1a,b**; comparing open with filled bars, $P < 0.005$ and $P < 0.05$, respectively). TGF- β -mediated suppression of IFN- γ , although less pronounced, reflects a similar profile as IL-2 (**Figure 1c,d**; comparing open with filled bars, $P < 0.05$ and $P < 0.04$, respectively), indicating that although hypo-responsive, CD4 LPT are suppressed by TGF- β .

mRNA of TGF- β -suppressed targets relates inversely with TGF- β upregulation of miR-155

To investigate whether TGF- β -mediated inhibition of protein expression is a reflection of mRNA levels, IL-2 and IFN- γ mRNA expression levels were evaluated and compared between CD4 PBT and LPT following TCR activation (**Figure 2a,b**). Parallel with protein levels (**Figure 1**), IL-2 mRNA was inhibited threefold by TGF- β in activated PBT and LPT (**Figure 2a**; comparing open with closed bars). TGF- β regulation of IFN- γ is less pronounced but remains significant (**Figure 2b**; comparing open with closed bars). Furthermore, TCR-activated cells in the presence of TGF- β showed decreased cyclin D1 to p21 ratio, suggesting an inhibition of cell proliferation (**Figure 2c**). As TGF- β inhibition is initiated at the transcriptional level (**Figure 2a–c**), we sought to investigate a potential role for candidates of mRNA regulation. The expression of miR-155, an miRNA predominantly expressed in immune cells and induced upon T-cell activation,^{23,26,27} was assessed comparing PBT with LPT. TCR activation with costimulation induced a threefold expression in PBT and a robust sevenfold induction of miR-155 in LPT (**Figure 2d**; comparing open with closed bars). Relative expression of miR-155 was normalized against untreated PBT and LPT, both of which were assigned values of 1 (not included in **Figure 2d**). As miR-155 is inducible only upon T-cell activation, treatment with TGF- β in unstimulated PBT and LPT had no effect on miRNA expression (data not shown). Compared with PBT, miR-155 is induced almost ninefold in activated LPT (**Figure 2d**; open bars), suggesting that LPT may contain larger reserves of precursor miR-155. In addition, TGF- β pretreatment further upregulates miR-155 expression eightfold in LPT, compared with similarly treated PBT (**Figure 2d**; black bars, $P = 0.04$). Thus, TGF- β inhibits LPT production of cytokines and cell cycle proteins while inducing miR-155 and is thus chosen as a suitable model system to investigate miR-155 regulation.

Selective modulation of miR-155 by TGF- β

The observed upregulation of miR-155 in CD4 LPT lymphoblasts led us to test TGF- β upregulation of TCR-dependent induction of miR-155 in the more physiologically relevant but less abundant, freshly isolated CD4⁺ purified LPT. A significant 3.5-fold increase in miR-155 levels in TGF- β -treated activated LPT (**Figure 3a**; bar 4 compared with bar 3, $P < 0.05$) recapitulates miR-155 induction observed in TGF- β -treated activated

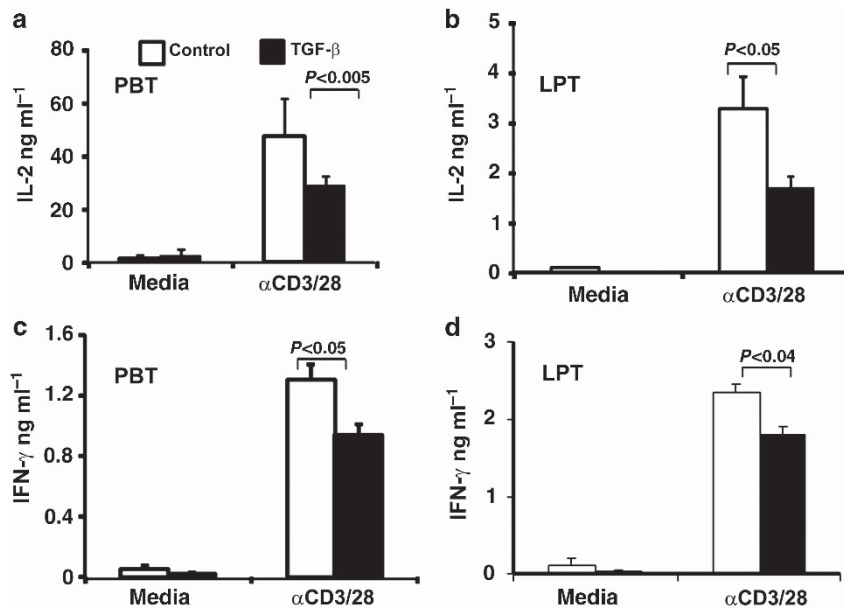


Figure 1 Transforming growth factor (TGF)- β inhibits T-cell-derived interleukin (IL)-2 and interferon (IFN)- γ production. Peripheral blood T cells (PBT) and lamina propria T cells (LPT) were activated with PHA and expanded in IL-2 for 8 days. CD4⁺ cells were purified by positive selection and pretreated with 3 ng ml⁻¹ TGF- β (filled bars) for 24 h or left untreated (open bars). Following pretreatment, cells were stimulated with plate-bound α CD3 (1 μ g ml⁻¹) and soluble α CD28 (1 μ g ml⁻¹) in the presence or absence of 3 ng ml⁻¹ TGF- β , respectively. Conditioned media was collected from triplicate wells 48 h after stimulation and evaluated for (a and b) IL-2 and (c and d) IFN- γ production by enzyme-linked immunosorbent assay. Data is presented as a mean of $n=4 \pm$ s.e.m.; significance between treated and untreated cells was compared using a Student's *t*-test.

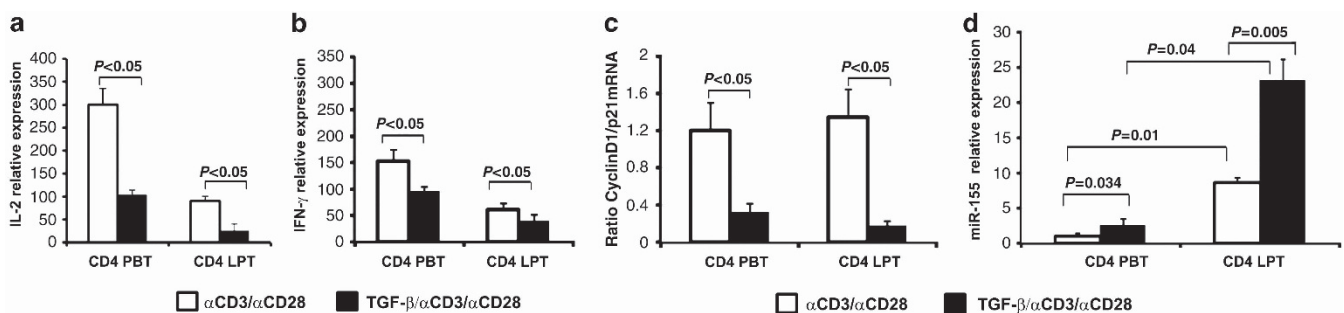


Figure 2 Transforming growth factor (TGF)- β inhibits cytokine mRNA accumulation and induces micro RNA-155 (miR-155) in TCR (T-cell receptor)-activated CD4⁺ T cells. CD4⁺ peripheral blood T cells (PBT) and lamina propria T cells (LPT) were purified, pretreated with 3 ng ml⁻¹ TGF- β (filled bars) for 24 h or left untreated (open bars), and activated as described in **Figure 1**. RNA was extracted 24 h after stimulation and evaluated for (a) interleukin (IL)-2, (b) interferon (IFN)- γ , (c) cyclin D1 and p21 mRNA by quantitative PCR. (d) miR-155 levels were determined 12 h after stimulation. For a–c, mRNA levels in unstimulated PBT and LPT were assigned a value of 1 (data not shown). Eukaryotic elongation factor 1A1 was used as a housekeeping gene to normalize cytokine expression. Fold change in expression of miR-155 was calculated using U6 levels to normalize expression. Data is presented as a mean of $n=4 \pm$ s.e.m.; significance between treated and untreated cells was compared using a Student's *t*-test.

lymphoblasts and subsequent experiments have been conducted with IL-2-expanded lymphoblast cells designated CD4⁺ LPT. To investigate whether TGF- β modulates other inducible immune-associated miRNAs,^{16,18,20} CD4⁺ LPT were activated for 12 h to assess alternative miRNA expression. MiR-9, miR-21, and let-7a were induced comparably with miR-155; however, unlike miR-155 (**Figure 3b**; comparing bars 1 and 2, $P=0.007$), TGF- β pretreatment and its subsequent presence in cell culture did not increase expression of any of these miRNAs (**Figure 3b**), portentous that TGF- β specifically upregulates miR-155.

Increased miR-155 expression is associated with decreased T-cell activation in TGF- β -treated LPT

Experimental evidence demonstrating c-Maf, a T-helper type 2 (Th2) transcription factor, and IFN- γ to be targets of miR-155 regulation^{23,31} led us to test the mRNA expression of these cytokines in TGF- β -treated activated CD4⁺ LPT that exhibit high miR-155. Both c-Maf and IFN- γ were inhibited by TGF- β 2.5-fold and threefold in TCR-activated CD4 LPT (**Figure 4a,b**; comparing bar 3 with bar 4, $P=0.01$ and $P=0.005$, respectively). To examine the temporal distribution of miR-155 in the

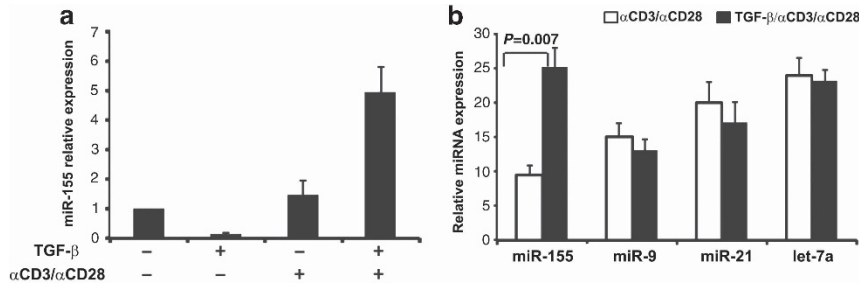


Figure 3 Transforming growth factor (TGF)-β induces micro RNA-155 (miR-155) in freshly isolated CD4+ lamina propria T cells (LPT) and selectively regulates miR-155 expression. (a) Freshly isolated CD4+LPT and (b) CD4+LPT lymphoblasts were pretreated with TGF-β (controls were untreated) followed by T-cell receptor activation in the presence or absence of TGF-β for 24 and 12 h, respectively. RNA was extracted to measure induction of (a) miR155 in fresh LPT and (b) miR-155, miR-9, miR-21, and let-7a in LPT lymphoblasts. Fold change in miRNA was normalized to U6. Data is presented as a mean of two donors for (a) and four donors±s.e.m. for (b); significance between treated and untreated cells was compared using a Student's *t*-test.

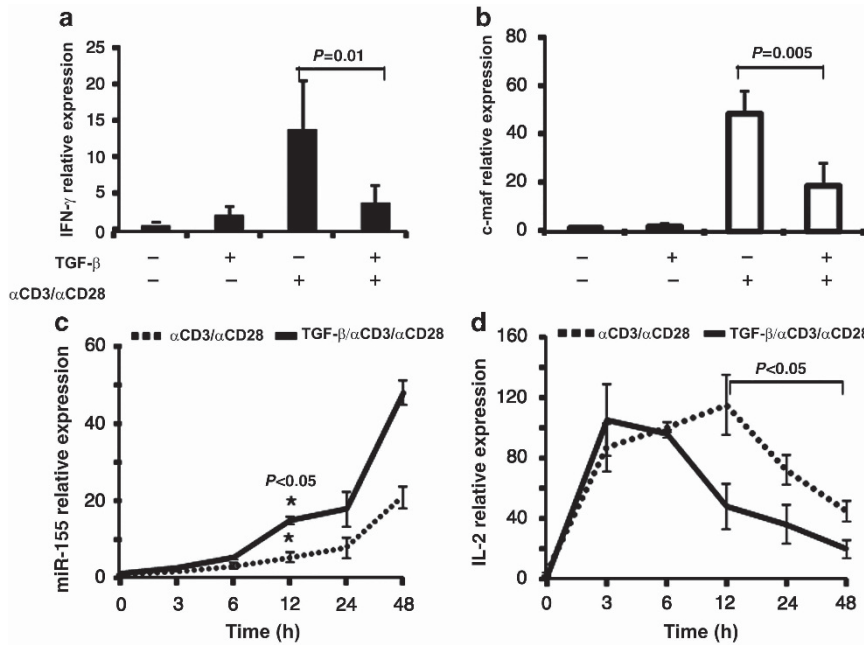


Figure 4 Prolonged upregulation of micro RNA-155 (miR-155) is inversely related with interleukin (IL)-2 production. CD4+LPT (lamina propria T cells) were pretreated (or not in controls) with 3ng ml⁻¹ transforming growth factor (TGF)-β and activated with αCD3/αCD28 for different time points for up to 48 h in the presence or absence of TGF-β. RNA was extracted to quantify (a) interferon (IFN)-γ and (b) c-Maf 24 h after T-cell receptor activation. To examine the temporal distribution of miR-155 expression profile with decrease in IL-2 mRNA, (c) miR-155 and (d) IL-2 were measured. Fold change in miRNA was normalized to U6, whereas fold change of mRNA levels were normalized to eukaryotic elongation factor 1A1. Data are an average of five independent experiments (*n*=5) and error bars represent s.e.m.

same cells that show decreased target levels, we monitored the miRNA expression of miR-155, which increased progressively up to 50-fold in 48 h compared with the level at the onset of culture (Figure 4c, solid line). This increase is 2.5-fold greater than CD4+LPT activated in the absence of TGF-β (Figure 4c, dashed line, *P*<0.005 at 12 h). Increasing miR-155 levels preceded a steady decrease in the expression of IL-2 mRNA over time when measured up to 48 h (Figure 4b). As IL-2 was neither a reported nor an algorithmic predicted target of miR-155, it was incumbent to query for an alternative target for T cell- and IL-2-associated miR-155.

MiR-155 is predicted to target itk

To explore the biological relevance of TGF-β-dependent upregulation of miR-155, we queried the miRNA-mRNA database (<http://mami.med.harvard.edu>) for molecular targets of miR-155 that are associated with IL-2 expression, as the latter was not predicted to be a candidate for miR-155. Itk, a cytoplasmic signaling molecule in T cells demonstrated to modulate IL-2 production,¹⁵ was predicted to be the target of miR-155. The 3' untranslated region of itk and a 7-mer seed region of miR-155 scored the highest probability of miRNA-mRNA annealing (Figure 5a). We next investigated the modulation of

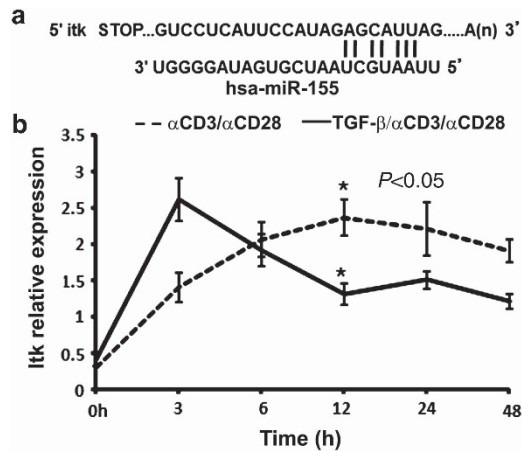


Figure 5 Inducible T-cell kinase (Itk) is a predicted target of micro RNA-155 (miR-155) and decreases in a transforming growth factor (TGF)- β -dependent manner. (a) Schematic representation of a 7-mer complementarity between the seed sequence of miR-155 and 3' untranslated region of itk mRNA. (b) CD4+LPT (lamina propria T cells) were pretreated with TGF- β and activated as described previously. RNA was extracted at indicated time points and itk expression quantified by quantitative PCR. Fold change in itk was normalized to eukaryotic elongation factor 1A1. Data are the mean of three independent experiments ($n=3$), * $P < 0.05$ where indicated.

itk mRNA in activated CD4+LPT exposed to TGF- β . An initial increase in itk at 3 h post T-cell stimulation is followed by a progressive decline for up to 48 h. A significantly decreased level of itk expression is demonstrated in TGF- β -treated CD4+LPT compared with untreated CD4+LPT after 12 h, a time point coincident with high levels of miR-155 under the same conditions (Figure 4b, $P=0.035$). A Pearson correlation study was conducted between miR-155 and itk mRNA expression over time to reveal a negative 0.21 value, confirming that they are inversely regulated throughout the time course.

Overexpression of miR-155 in CD4 LPT decreases itk and IL-2 mRNA

Given that there is an inverse association between miR-155 levels, IL-2, and itk mRNA in TCR-activated CD4+LPT treated with TGF- β , we used a gain of function approach to analyze the contribution of miR-155 in modulating the said target levels. Cells were transfected with 100 nM concentrations of precursor miR-155 followed by stimulation through the TCR as described previously to allow for maturation of miRNA. A transfection efficiency of about 60% was achieved as assessed by green fluorescent protein expression plasmid transfection (data not shown). Overexpressed miR-155 served to mimic TGF- β 's effect on activated LPT. As expected untransfected cells show an eightfold induction of miR-155 after 24 h of activation relative to unstimulated CD4+LPT (Figure 6a, bar 2 compared with bar 1). Activated CD4+LPT overexpressing miR-155 exhibited a further increase by 2.5-fold compared with non-transfected cells activated through the TCR (Figure 6a; bar 4 vs. bar 2, $P < 0.05$). To delineate a function for miR-155

in transfected cells, itk and IL-2 mRNA were assessed following polyclonal stimulation for 24 h. Whereas TCR-activated CD4+LPT express fourfold more itk mRNA compared with unstimulated cells (Figure 6b, bars 1 & 2), ectopic expression of miR-155 caused a fourfold decrease in itk mRNA compared with untransfected cells (Figure 6b bar 2 vs. bar 4, $P < 0.05$), suggesting that overexpressed miR-155 decreases itk mRNA expression that may subsequently influence T-cell activation. Consequently, we show a 2.5-fold decrease in IL-2 mRNA in miRNA overexpressed cells relative to non-transfected cells (Figure 6c, bar 2 vs. bar 4, $P < 0.05$). Given the role of cyclin D1 and p21 in the regulation of cell cycle progression, we also examined cyclin D1 and p21 mRNA levels to assess the effect of miR-155 on CD4+LPT proliferation. Quantification by quantitative PCR revealed a fourfold reduction in the ratio of cyclin D1 to p21 in miR-155 overexpressed cells compared with non-transfected CD4+LPT following TCR activation (Figure 6d, comparing bar 2 with bar 4, $P < 0.05$). To determine whether overexpression of miR-155 had any effect on other inducible miRNAs, the levels of miR-21 and let-7a were determined and revealed that both miRNAs were induced by TCR activation (Figure 6e, second and fourth open bar); however, overexpression of miR-155 had no effect on either miRNA (Figure 6e, second and fourth filled bar). Lastly, transfection with 100 nM scrambled miRNA produced no effect on miR-155 or target levels itk and IL-2 (Figure 6d). Overall, these data suggested that TGF- β -enhanced TCR induction of miR-155 impairs IL-2 production by affecting itk transcriptional activity.

MiR-155 silencing restores itk and IL-2 gene expression

To confirm the observation that overexpression of miR-155 affects itk, IL-2, and T-cell activation, we performed the reverse loss of function experiment and silenced miR-155 by transfecting with a small interfering RNA (siRNA) to miR-155 to evaluate its effect on CD4+LPT activation. As expected, TCR activation induced miR-155 and this upregulation was augmented in the presence of TGF- β (Figure 7a, bar 1 vs. bar 2 vs. bar 3). Transfection with 50 nM anti-miR-155 decreased miR-155 expression from 17-fold to fivefold (Figure 7a, comparing bar 3 with bar 6, $P < 0.05$), establishing efficiency of transfection with anti-miRNA. To analyze the effect of miR-155 silencing on T-cell function, both itk and IL-2 mRNA were evaluated in miR-155-silenced cells. Activated CD4+LPT show increased itk mRNA that is inhibited by TGF- β (Figure 7b, bars 2 and 3); however, this inhibition is reversed upon blocking miR-155 and itk mRNA is increased from 1.5-fold to 4.5-fold (Figure 7b, bar 3 vs. bar 6, $P=0.04$) and is comparable with its expression in non-transfected activated CD4+LPT (Figure 7b, bar 2). IL-2 mRNA profile in miR-155-silenced CD4+LPT is reflective of itk pattern of expression (Figure 7c). Transfection by anti-miR-155 increased IL-2 mRNA from 2.6-fold in TGF- β -treated untransfected cells to about fourfold (Figure 7c, bar 3 vs. bar 6, $P=0.04$). Although there is a slight decrease in IL-2 mRNA in transfected cells with and without TGF- β , this difference is not significant. As excess induction of miR-155 in CD4 LPT negatively affects cell proliferation, we asked whether silencing the miRNA can

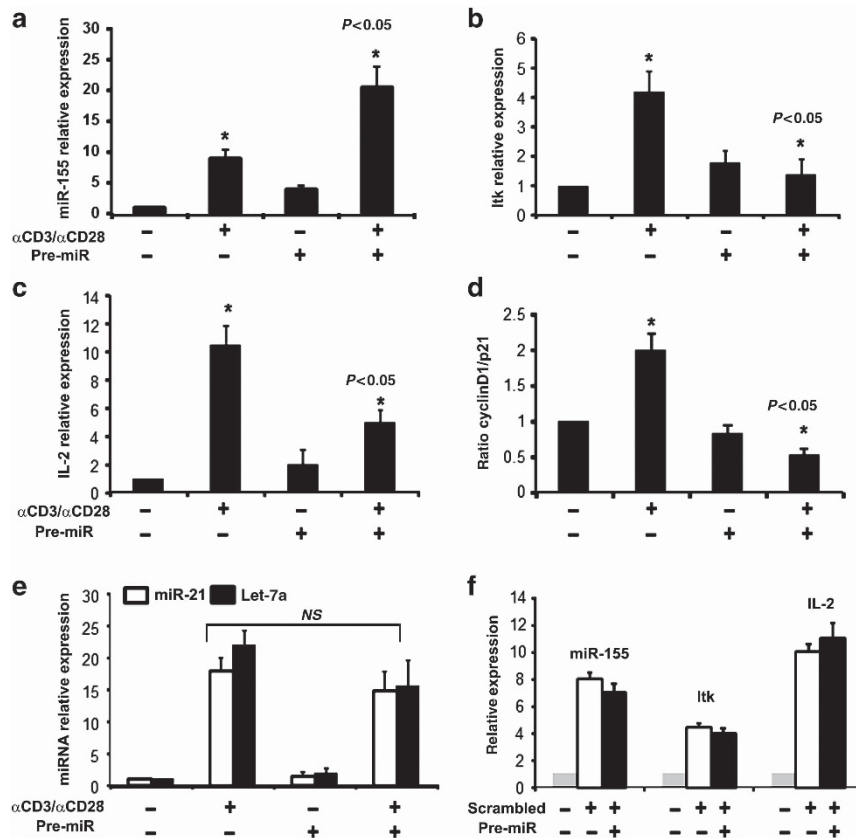


Figure 6 Overexpression of micro RNA-155 (miR-155) in CD4+LPT (lamina propria T cells) inhibits inducible T-cell kinase (itk) and interleukin (IL)-2 mRNA. CD4 LPT were transfected with precursor miR-155 at 100 nM and rested for 16 h. Following stimulation with α CD3/ α CD28 for 24 h, cells were subject to RNA extraction. (a) miR-155 levels were evaluated by quantitative PCR (qPCR) to assess efficiency of transfection and U6 levels used to normalize expression. Relative miR-155 expression from unstimulated mock-transfected cells was used for comparison and set to a value of 1. mRNA levels of (b) *itk*, (c) IL-2, (d) cyclin D1 and p21 mRNA expressed as a ratio, were measured by qPCR relative to eukaryotic elongation factor 1A1 to normalize expression. Control miRNAs (e) miR-21 and let-7a levels were also evaluated from the same cultured cells, whereas (f) miR-155, *itk*, and IL-2 miRNA and mRNA were quantified from T cells transfected with 100 nM scrambled miRNA cultured under similar conditions as described above. * $P < 0.05$. Data are the average of three separate donors in independent experiments and error bars represent s.e.m. NS, not significant.

reverse the observed inhibition. The observed increase in ratio of cyclin D1 to p21 upon T-cell activation is decreased in TGF- β -treated cells (Figure 7d bar 2 vs. 3), illustrating TGF- β -mediated suppression. Blocking miR-155 in TCR-activated cells increased the cyclin D1/p21 ratio, indicating miR-155-mediated inhibitory effect on T-cell proliferation (Figure 7d bar 3 vs. bar 6, $P < 0.05$). However, other inducible miRNAs, miR-21 and let-7a were unaltered by the transfection (Figure 7e) and transfection with a scrambled siRNA to miR-155 had no effect on either miR-155 or *itk* and IL-2 (Figure 7f). Taken together, our data reveals a role for TGF- β -dependent increase of miR-155 in affecting T-cell activation by modulation of *itk* and IL-2.

DISCUSSION

In this study, we demonstrate a TGF- β -dependent role for miR-155 in regulating intestinal T-cell activation. Based on our current findings that miR-155 is enhanced by TGF- β predominantly in mucosal T cells, and our earlier report¹² that TGF- β inhibits IL-2 by forcing T cells to enter cell cycle arrest, we propose that TGF- β exerts its anti-proliferative effects on LPT through

increased accumulation of miR-155. As the precise molecular mechanism by which miR-155 mediates immunosuppression of LPT is unknown, a bioinformatic query of predicted binding sites for miR-155 revealed *itk* as a potential target of miR-155. Subsequent gain and loss of expression experiments established a functional role for TGF- β -enhanced miR-155 in regulating *itk* mRNA. Thus, our results expand our current understanding of the immunoregulatory activity of miR-155 to include an immunoinhibitory function in the mucosa. TGF- β is a pleiotropic cytokine that has been shown to maintain immunological balance, as mice deficient in TGF- β develop multi-organ inflammatory immune infiltrate at an early (2–3 weeks) age.³² Interestingly, elevated levels of TGF- β have been detected in inflammatory bowel disease patients despite chronic inflammation, raising the possibility that cells may be resistant to the inhibitory effects of TGF- β . In addition, defects in the proteosomal degradation of Smad-7, an inhibitor of TGF- β signaling pathway are partially responsible for disruption of inhibitory effects of TGF- β on lamina propria mononuclear cells (LPMCs) of Crohn's disease patients.³³ Naive T-cell differentiation upon

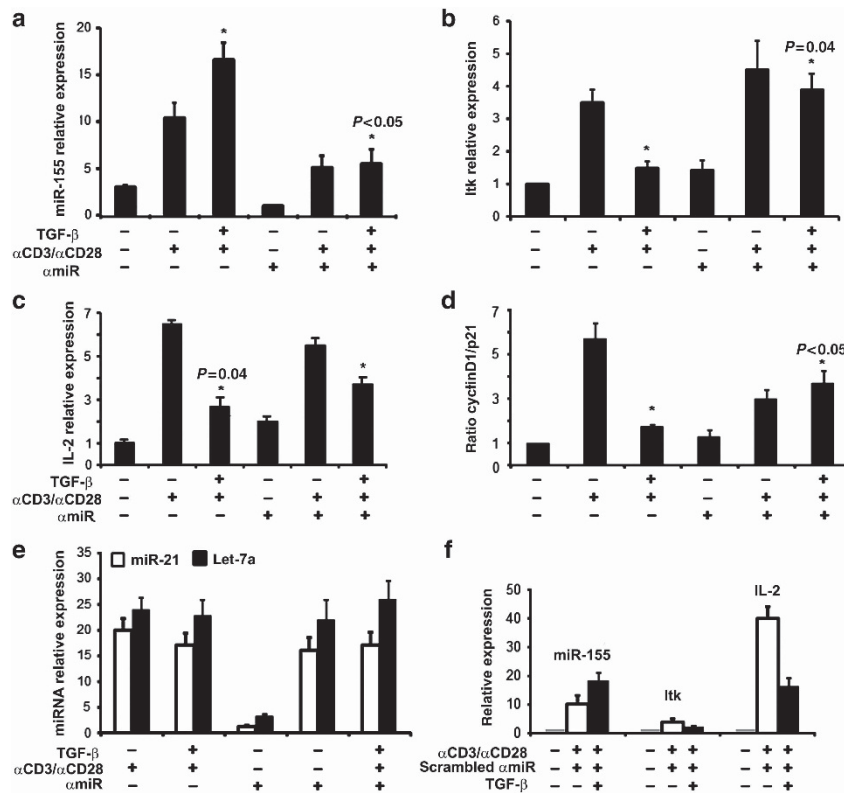


Figure 7 Micro RNA-155 (miR-155) silencing in transforming growth factor (TGF)- β -treated CD4 lamina propria T cells (LPT) increases interleukin (IL)-2 and inducible T-cell kinase (itk). TGF- β -treated and untreated CD4 LPT were transfected with 50 nM anti-miR-155 or scrambled small interfering RNA (siRNA), rested for 5 h following transfection and subjected to activation with α CD3/ α CD28 for 24 h. RNA was extracted to measure (a) miR-155 levels to assess transfection efficiency, (b) itk, (c) IL-2, and (d) cyclin D1 and p21 expressed as a ratio. Non-specific miRNAs (e) miR-21 and let-7a levels were evaluated to serve as controls and (f) miR-155, itk, and IL-2 levels were evaluated in cells transfected with 50 nM scrambled siRNA. Fold change in miRNA expression is normalized to U6 and fold changes in mRNA expression normalized to eukaryotic elongation factor 1A1. Relative expression of miR-155 was calculated using mock-transfected unstimulated cells set to 1. IL-2 and itk mRNA levels from unstimulated cells were used for comparison and set to 1. Data is an average of three independent experiments, error bars represent s.e.m.

antigen stimulation is inhibited by TGF- β by interfering with TCR-activated calcium flux and NFATc (nuclear factor of the activated T cells) translocation to the nucleus.¹⁴ In mature T cells, the major effect of TGF- β is thought to be at the level of inhibition of T-cell proliferation and IL-2 production,³⁴ yet the process by which TGF- β inhibits T-cell activation remain poorly understood.

Our data demonstrating the direct effect of TGF- β in modulating IL-2, IFN- γ , and cell cycle progression regulators in TCR-stimulated CD4+ LPT lymphoblasts, recapitulates TGF- β -mediated inhibition demonstrated by others in mouse and human T cells.^{14,35} We, therefore, hypothesized that TGF- β exerts its inhibitory effects post transcription through excess production of endogenous miR-155.

Induction of specific miRNAs has been associated with numerous immunological processes, including cell differentiation, polarization, and tolerance.³⁶ For instance, increased expression of miR-21 is associated with effector and memory T-cell differentiation, indicating that the miRNA profile changes dynamically during T-cell differentiation.³⁷ The hypo-responsive phenotype of naive CD4 T cells obtained from umbilical cord

blood, compared with adult naive CD4 T cells, is a result of elevated expression of miR-184 in cord blood CD4 T cells via down-regulation of NFAT.³⁸ Although miR-181 is an intrinsic modulator of T-cell sensitivity to antigen,³⁹ miR-146 influences many signaling pathways, including activation-induced cell death in TCR-activated T cells.⁴⁰ These and other reports implicate a substantial role for miRNAs in numerous T-cell-mediated immune responses.

Although the role of miRNAs in immunity is an area of intense research, fewer studies have been targeted toward the induction and regulation of miRNAs in the mucosa. MiR-143 and miR-145 have been demonstrated to function as colon cancer tumor suppressors and loss of these miRNAs increases susceptibility to chronic inflammation and neoplastic progression in inflammatory bowel disease.⁴¹ Evidence shows that an epithelial microRNA, miR-375, mediates the epithelium-lymphocyte crosstalk necessary for mounting protective Th2 responses against gut-associated pathogens.⁴² MiR-155 stands out as an immune-associated miRNA that is upregulated in response to multiple stimuli and in multiple immune cells, thus acquiring the designation of a multifunctional RNA.⁴³ MiR-155 is induced by

Toll-like receptor ligands, inflammatory cytokines, and specific antigens in monocytes, macrophages, B cells, and T cells,^{24,44,45} identifying it as a critical mediator of immune cell development, function, and disease.^{23,25} MiR-155 is rapidly induced in B cells and is involved in immunoglobulin class-switching through inhibition of activation-induced deaminase.⁴⁶ In T cells, miR-155, induced upon antigen stimulation through the TCR imparts Tregs with competitive fitness by direct binding to suppressor of cytokine signaling (SOCS1) mRNA.⁴⁷ In natural killer cells, miR-155 regulates IL-12 and IL-18 mediated IFN- γ production by binding to and down regulating SHIP1 mRNA.⁴⁸ Additionally, mice deficient in miR-155 display a range of immune pathology from impairment of T-cell lineage specificity, B-cell dysfunction, increased susceptibility to pathogenic infections and, autoimmunity, suggesting that miR-155 functions predominantly as a positive regulator of inflammation.^{49–51} MiR-155 is also induced by TGF- β in epithelial cells causing epithelial mesenchymal transition by direct interaction of the Smad4 signaling pathway with the promoter region of miR-155.²⁹ Despite these reported functions of TGF- β in innate and adaptive immune cells, the effect of TGF- β -enhanced miR-155 on human TCR-activated CD4 + LPT has not been explored. These reports together with our data suggest that the level of miR-155 expression and its function is dependent on the physiological environment.

The function of miRNA-155 in intestinal T cells is just beginning to be explored. One report demonstrates that miR-155-null mice have impaired Th1 and Th17 responses, and these mice are susceptible to *Helicobacter pylori* infection due to an intrinsic defect that causes T cells to be refractory to TCR signals.⁴⁹ This report supports the notion that in an infection model miR-155 expression is required for Th1/Th17 differentiation and is consistent with our findings that in the absence of miR-155, *itk* and IL-2 mRNA is increased (Figure 7), while IFN- γ and IL-17 remain unchanged (data not shown).

Our data demonstrates a significant increase in miR-155 following TCR activation of CD4 + LPT treated with TGF- β , consistent with reports that TGF- β can induce miR-155 expression in epithelial cells through the Smad4 pathway.²⁹ In comparison to its role in the mucosa, TGF- β induced only a modest increase in miR-155 in PBT, suggesting that CD4 + LPT may retain larger reserves of precursor miR-155, a speculation that calls for further investigation. Moreover, this induction is specific to miR-155 as other inducible miRNAs like miR-21, miR-9, and let-7a, although enhanced upon TCR activation, are not increased upon exposure to TGF- β (Figure 3b). This is consistent with reports showing miR-9 and miR-155 interfering with the Toll-like and IL-1 receptor signaling pathways in myeloid cells, implying that they may negatively regulate innate immune responses.^{52,53}

The MAMI miRNA-mRNA database (<http://mami.med.harvard.edu>) was queried to detect *itk* as a predicted T-cell-associated target for miR-155. *Itk* is positioned downstream of Src kinases in TCR-mediated signaling and studies in *itk*-null mice establish that *itk*⁵⁴ has a critical role in antigen-specific IL-2 production by T cells¹⁵ that is inhibited upon exposure to TGF- β .¹⁴ In keeping with the algorithmic prediction, *itk* mRNA

levels were inversely related to miR-155 levels in treated cells implying a probable cause and effect. Interestingly, we observed that miR-155-overexpressing cells display reduced *itk* and IL-2 mRNA in accordance with a decrease in the ratio of cyclin D1 to p21, suggesting that upregulated miR-155 acts as an inhibitor of T-cell activation as well as proliferation. In contrast to what we report, a recently published study reports that a human Jurkat cell line overexpressing miR-155 had no effect on the co-transfected 3' untranslated region of *itk*.⁵⁵ However, in our study, TGF- β does not upregulate miR-155 in Jurkat cells, although miR-155 is induced in non-transformed primary intestinal LPT and PBT. This discordance may, therefore, be strictly due to cell type specificity. In agreement with a previous study demonstrating miR-155 as a negative regulator of cellular activation through down-regulation of inflammatory mediators using gastric epithelial cells in an *H. pylori* infection model,⁵⁶ our evidence points to miR-155 as the immunosuppressive mediator in TGF- β -treated gut T cells. An examination of the function of miR-155 in knockout mice splenocytes reported decreased IFN- γ and increased IL-4 upon stimulation.²³ These authors speculated that the lack of miR-155 may lead to a preferential development of Th2 response. However, we conclude that TGF- β stimulation may induce miR-155 in excess of what is induced solely by T-cell activation, to curb the intensity of T-cell activation. Excess miR-155 in the presence of TGF- β may contribute to mucosal tolerance.

METHODS

Isolation of T lymphocytes from lamina propria and peripheral blood and CD4 T-cell purification. Written informed consent for blood was obtained from consecutive donors and donors of surgical samples of the large intestine who are blinded to the investigators considered and discarded tissue. Acquisition of blood and tissue samples conformed to and was approved by the University Hospitals Case Medical Center IRB.

Peripheral blood mononuclear cells were isolated using Ficoll Hypaque (Sigma-Aldrich, St Louis, MO) density separation. The cells were stimulated with 0.5% phytohemagglutinin for 48 h in the presence of 5 ng ml⁻¹ IL-2 in RPMI1640 containing 10% fetal bovine serum (Invitrogen Life Technologies, Grand Island, NY), 2.5% hydroxyethyl piperazineethanesulfonic acid and 2.5% PSF (penicillin, streptomycin, fungizone; Invitrogen Life Technologies). T cells were subsequently expanded with 5 ng ml⁻¹ of IL-2 (R&D Systems, Minneapolis, MN) for 8 days before CD4 purification. Intestinal LPMCs were isolated from histologically normal surgical resections of patients undergoing bowel resection for various medical conditions, including rectal prolapse, diverticulitis, and tumors. Sections considered for LPMC isolation are 10 cm away from the affected region. LPT was isolated and expanded as described.⁵⁷ Briefly, the mucosal layer was removed and cut into strips and subjected to sequential washes with dithiothreitol (Fisher Scientific, Fair Lawn, NJ) and EDTA (Sigma-Aldrich) to remove the mucus and the epithelial layers, respectively, followed by an 8 h digestion with collagenase and deoxyribonuclease (Worthington Biochemical, Lakewood, NJ). LPMCs were separated from the crude cell suspension by layering on Ficoll Hypaque density gradient. LPTs were expanded in LPMC media containing RPMI1640, 10% FCS, 2.5% PSF, and 1.5% hydroxyethyl piperazineethanesulfonic acid for 8–10 days by which time only T cells survive and attain their log phase of growth. CD4+ T cells from IL-2-expanded PBT and LPT were purified on days 8 and 12, respectively, by positive selection using CD4 microbeads from Miltenyi Biotec (Auburn, CA) and assessed for purity by flow cytometry to yield 98% pure population of CD4 + LPT or PBT.

TGF- β stimulation. CD4+ LPT and PBT were incubated in the presence or absence of 3 ng ml⁻¹ TGF- β for 24 h, followed by activation with plate-bound anti-CD3 (1 μ g ml⁻¹; Ortho Diagnostic Systems, Raritan, NJ) and soluble anti-CD28 (1 μ g ml⁻¹; Ancell Laboratories, Bayport, MN) antibody for 12, 24, or 48 h. TGF- β was maintained at the same concentration in the respective wells for the indicated time. To show that miR-155 levels are modulated by TGF- β , CD4+ LPT were exposed to varying concentrations of TGF- β for 24 h followed by activation with plate-bound anti-CD3 and soluble anti-CD28, where the cells continued to be exposed to the same concentrations of TGF- β for 12 and 48 h. Control cells were activated for the same duration in the absence of TGF- β . After the indicated time, cells were lysed and total RNA extracted.

Prediction of specific miRNAs involved in IL-2 regulation. The MAMI (Meta Mir:target Inference), a meta predictor of human micro RNA targets (<http://mami.med.harvard.edu/>) was queried to predict and score the extent of complementarity between 3' untranslated region of IL-2 and itk mRNA and its cognate human miRNA species. The database uses the miRanda algorithm to score mRNA-miRNA interaction based on thermodynamic stability rules set by the Vienna RNA folding routines.⁵⁸ Interactions that scored the highest were selected for further validation by real time PCR.

RNA extraction and miRNA detection. Total RNA was extracted using the Ambion (Austin, Tx) miRvana isolation kit and quantified with the Nanodrop ND1000 (Thermoscientific, Wilmington, DE). Using 0.5 μ g of RNA, cDNA was synthesized using the SA Biosciences (Frederick, MD) RT² first strand synthesis kit for 2 h at 37°C. MiRNA quantification was performed in a Biorad iCycler (Hercules, CA) using iQ-SYBR-Green Super Mix (BioRad). MiRNA detection and analysis were done by the comparative threshold cycle method, using U6 expression for normalization.⁵⁹ All miRNA primers were purchased from SA Biosciences. The reactions were performed in triplicate.

Real-time PCR with reverse transcriptase. cDNA was synthesized using Superscript II Reverse Transcriptase for 50 min at 42°C and used for subsequent quantitative real-time PCR as described above. Itk and IL-2 RNA were quantified by the comparative threshold method using eukaryotic elongation factor 1A1 for normalization. The following primers were used: itk, forward primer 5'-GGTCATTGGTGTGCTGATG-3' and reverse primer 5'-TCTGCAATTTTCAGCCAGTTG-3'; IL-2, forward primer 5'-TGCAACTCCTGTCTTGCAATT-3' and reverse primer 5'-GCCTTCTTGGGCATGTAATAA-3'; and eukaryotic elongation factor 1A1, forward primer 5'-CTTTGGGTGCGTTTGCTGTT-3' and reverse primer 5'-CCGTTCTCCACCACTGATT-3'. All reactions were performed in triplicate.

Antagomir treatment. Antagomirs to miR155 and scrambled siRNA were purchased from Ambion. CD4+ LPT were pretreated (or not) with 3 ng ml⁻¹ TGF- β for 24 h and transfected with 50 nM anti-miR-155 or scrambled siRNA, using an Amaxa Nucleofector (Gaithersburg, MD). Transfection was carried out according to the manufacturer's instructions for unstimulated T cells provided in the Human T-cell kit (VPA-1002, Amaxa, Gaithersburg, MD) using program U-14. Cells were rested in RPMI media at 37°C for 5 h post transfection and subsequently used in cell culture assays. Transfection efficiencies, as assessed by transfection with control green fluorescent protein vector, were approximately 60%.

Overexpression of miR155. CD4+ LPT were transfected using 100 nM pre-miR-155 by the same procedure as described above. Transfection with scrambled siRNA was used as a negative control. Transfected cells were rested at 37°C in RPMI media for 16 h and then plated on anti-CD3-coated plates in the presence of anti-CD28. After 24 h of activation, cells were harvested for RNA isolation, cDNA preparation, and real time PCR.

Cytokine analysis by ELISA. Conditioned medium was collected at 48 h from CD4+ LPT and PBT stimulated as described above. IFN- γ and IL-2

secreted from activated cells were detected in the medium by a sandwich ELISA (eBioscience, San Diego, CA) and the plates read with a multiwell plate reader (Molecular Devices, Sunnyvale, CA) and analyzed using Soft Max Pro 4.3 LS computer analysis software (Molecular Devices).

Statistical analysis. The Student's *t*-test was used to determine the statistical significance of experimental results. A *P*-value of ≤ 0.05 was considered significant. The results were represented as the mean plus or minus s.d. from three or more independent experiments. A Pearson correlation study was conducted between miR-155 and itk mRNA expression to determine whether they were inversely regulated throughout the time course.

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DISCLOSURE

The authors declare no conflict of interest.

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