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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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Abstract

Transforming Growth Factor (TGF)-β, is an immunosuppressive cytokine that inhibits T cell activation. We hypothesized that TGF-β mediates its immuno-inhibitory effects by modulation of micro (mi)RNA-155. IL-2 and IFN-γ are down-regulated by TGF-β in activated CD4 peripheral blood T cells (PBT) and lamina propria T cells (LPT), but miR-155 is up-regulated 9-fold 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 while 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.

Keywords

TGF-β; miR-155; LPT; itk; IL-2

Introduction

Transforming growth factor (TGF)-β1 is a ubiquitous and multifunctional cytokine, central to modulation of host defense. Secreted by regulatory T cells and non-immune cells, TGF-β plays a key role in experimental models of oral tolerance and in the pathogenesis of experimental colitis[1-3]. Many studies in murine models of IBD have shown that 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

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upon administration of anti-TGF-β1 antibody, indicating that protection from colitis is mediated by TGF-β1[6] acting on pathogenic T cells and that in the absence of this interaction, pathogenic T cells escape T reg control. Additional studies with Tregs from TGF-βRII KO 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 IL-2 production in Smad3 dependent and independent manners[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 (IBD) 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 PBT activation and proliferation, by blocking the expression of cell cycle progression regulators and IL-2 secretion[12] through modulation of T cell receptor (TCR) signaling. In the current report we show that human LPT, known to be hypo-responsive to immune activation[13], are inhibited by the suppressive effects of TGF-β via post transcriptional modification of TGF-β-targeted molecules. Itk, a TCR signaling molecule belonging to the tec family of kinases and involved in T cell activation, 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, noncoding oligonucleotides called micro RNAs (miRNAs) regulate gene expression and have been implicated in various diseases[16] and more recently in immune regulation[17]. miRNAs inhibit gene expression by annealing with the 3′untranslated region (UTR) 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] and 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 IFN-γ release from bic$^{-/-}$ CD4+ T cells[23] and B cell receptor-mediated TNF-α production from B cells[24], indicating that miRNA-155 facilitates cytokine release in lymphoid cells and possibly plays 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 up-regulation of miR-155 in epithelial cells resulting in epithelial mesenchymal transition (EMT)[29] 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 and compared 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. 48 h post-stimulation protein expression of IL-2 and IFN-γ were compared between PBT and LPT. Not surprisingly, CD4 LPT produce ~12-fold less IL-2 following TCR-stimulation compared to PBT (Fig. 1a, b open bars), yet TGF-β suppressed IL-2 by 2-fold in both CD4 LPT and PBT (Fig. 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 (Fig. 1c,d comparing open to 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 if TGF-β mediated inhibition of protein expression is a reflection of mRNA levels, IL-2 and IFN-γ mRNA expression was evaluated and compared between CD4 PBT and LPT following TCR activation (Fig 2a,b). Parallel with protein levels (Fig. 1), IL-2 mRNA was inhibited 3-fold by TGF-β in activated PBT and LPT (Fig 2a comparing open bars to closed bars). TGF-β regulation of IFN-γ is less pronounced but remains significant (Fig 2b comparing open to 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 (Fig 2c). Since TGF-β inhibition is initiated at the transcriptional level (Fig 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 to LPT. TCR activation with co-stimulation induced a 3-fold expression in PBT and a robust 7-fold induction of miR-155 in LPT (Fig. 2d comparing open bars 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 Fig. 2d). Since 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 to PBT, miR-155 is induced almost 9-fold in activated LPT (Fig. 2d open bars), suggesting that LPT may contain larger reserves of precursor miR-155. In addition, TGF-β pretreatment further up-regulates miR-155 expression 8-fold in LPT), compared to similarly

treated PBT (Fig. 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 up-regulation 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 (Fig. 3a bar 4 compared to bar 3, $p<0.05$) recapitulates miR-155 induction observed in TGF-β treated activated lymphoblasts and subsequent experiments have been conducted with IL-2 expanded lymphoblast cells designated CD4+LPT. To investigate if TGF-β modulates other inducible immune-associated miRNAs[16, 18, 20], CD4+LPT were activated for 12 hours to assess alternative miRNA expression. MiR-9, miR-21, and let-7a were induced comparably to miR-155, however unlike miR-155 (Fig. 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 (Fig. 3b), portentous that TGF-β specifically up-regulates miR-155.

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

Experimental evidence demonstrating c-Maf, a 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 3-fold in TCR activated CD4 LPT (Fig 4a,b comparing bars 3 with bar 4, p=0.01 and p=0.005 respectively). To examine the temporal distribution of miR-155 in the same cells that show decreased target levels we monitored the miRNA expression of miR-155, which increased progressively up to 50-fold in 48h compared to the level at the onset of culture (Fig. 4c, solid line). This increase is 2.5 fold greater than CD4+LPT activated in the absence of TGF-β (Fig. 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 48h (Fig. 4b). Since 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 biologic 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, since 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′UTR of itk and a 7-mer seed region of miR-155 scored the highest probability of miRNA-mRNA annealing (Fig. 5a). We next investigated the modulation of itk mRNA in activated CD4+LPT exposed to TGF-β. An initial increase in itk at 3h post T cell stimulation is followed by a progressive decline for up to 48h. A significantly decreased level of itk expression is demonstrated in TGF-β-treated CD4+LPT compared to untreated CD4+LPT

after 12h, a time point coincident with high levels of miR-155 under the same conditions (Fig. 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.

Over expression 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 stimulating through the TCR as described previously to allow for maturation of miRNA. A transfection efficiency of about 60% was achieved as assessed by GFP expression plasmid transfection (data not shown). Overexpressed miR-155 served to mimic TGF-β's effect on activated LPT. As expected un-transfected cells show an 8-fold induction of miR-155 after 24 h of activation relative to un-stimulated CD4+LPT (Fig. 6a, bar 2 compared to bar 1). Activated CD4+LPT overexpressing miR-155 exhibited a further increase by 2.5-fold compared to non-transfected cells activated through the TCR (Fig 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 4-fold more itk mRNA compared to un-stimulated cells (Fig 6b, bars 1& 2), ectopic expression of miR-155 caused a 4-fold decrease in itk mRNA compared to un-transfected cells (Fig 6b bar 2 vs bar 4, $p<0.05$), suggesting that overexpressed miR-155 decreases itk mRNA expression that may 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 (Fig. 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 qPCR revealed a 4-fold reduction in the ratio of cyclin D1 to p21in miR-155 over expressed cells compared to non-transfected CD4+LPT following TCR activation (Fig 6d, comparing bar 2 with bar 4, $p<0.05$). To determine if 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 (Fig 6e, 2^{nd} and 4^{th} open bar), however overexpression of miR-155 had no effect on either miRNA (Fig 6e, 2^{nd} and 4^{th} filled bar). Lastly, transfection with 100nM scrambled miRNA produced no effect on miR-155 or target levels itk and IL-2 (Fig 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 over expression 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 an siRNA to miR-155 to evaluate its effect on CD4+LPT activation. As expected, TCR activation induces miR-155 and this upregulation is augmented in the presence of TGF-β (Fig. 7a, bar 1 vs. bar 2 vs. bar 3). Transfection with 50 nM anti-miR-155 decreased miR-155 expression from 17-fold to 5-fold (Fig 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 (Fig. 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 (Fig. 7b, bar 3 vs. bar 6, p=0.04) and is comparable to its expression in non-transfected activated CD4+LPT (Fig 7b, bar 2). IL-2 mRNA profile in miR-155 silenced CD4+LPT is reflective of itk pattern of expression (Fig. 7c). Transfection by anti-miR-155 increased IL-2 mRNA from 2.6-fold in TGF-β treated untransfected cells to about 4-fold (Fig. 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. Since excess induction of miR-155 in CD4 LPT negatively affects cell proliferation, we asked whether silencing the miRNA can 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 (Fig 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 (Fig 7d bar 3 vs. bar 6, $p<0.05$). However other inducible miRNAs, miR-21 and let-7a were unaltered by the transfection (Fig 7e) and transfection with a scrambled siRNA to miR-155 had no effect on either miR-155 or itk and IL-2 (Fig 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 report[12] that TGF-β inhibits IL-2 by forcing cells to enter cell cycle arrest, we propose that TGF-β exerts its anti-proliferative effects on LPT through increased accumulation of miR-155. Since 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 wk) age[32]. Interestingly, elevated levels of TGF-β have been detected in IBD 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 (LPMC) of Crohn's disease (CD) patients[33]. Naïve T cell differentiation upon antigen stimulation is inhibited by TGF-β by interfering with TCR-activated calcium flux and NFATc 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 precise mechanism(s) by which it 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 at the translational level 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 naïve CD4 T cells obtained from umbilical cord blood, compared to adult naïve CD4 T cells, is a result of elevated expression of miR-184 in cord blood CD4 T cells via down-regulation of NFAT[38]. Whereas miR-181 is an intrinsic modulator of T cell sensitivity to antigen[39], miR-146 influences many signaling pathways, including activation induced cell death (AICD) 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 IBD[41]. Evidence shows that an epithelial microRNA, miR-375, mediates the epithelium-lymphocyte crosstalk necessary for mounting protective T helper type 2 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 (TLR) 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 (AID)[46]. In T cells miR-155, induced upon antigen stimulation through the TCR, renders regulatory T cells with competitive fitness by direct binding to suppressor of cytokine signaling (SOCS1) mRNA[47], while in NK cells, miR-155 regulates IFN-γ production in part due to its interaction with 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 EMT 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 had not been explored. These reports together with our data suggest that the level of miR-155 expression and its function is context dependent.

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 *H. 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 (Fig. 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 by TCR activation, are not increased by upon exposure to TGF-β (Fig. 3b). This is consistent with reports showing miR-9 and miR-155 interfering with the Toll like and IL-1 receptor (TIR) signaling pathways in myeloid cells, implying that they may negatively regulate innate immune responses[52, 53].

The 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] plays a critical role in antigen specific IL-2 production by T cells[15] 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′UTR of itk[55]. However, in our hands TGF-β does not up-regulate 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 immuno-suppressive 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 that 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.

PBMC (peripheral blood mononuclear cells) were isolated using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO) density separation. The cells were stimulated with 0.5% PHA for 48h in the presence of 5 ng/ml IL-2 in RPMI1640 containing 10% Fetal Bovine Serum (Invitrogen Life Technologies, Grand Island, NY), 2.5% HEPES and 2.5% PSF (Penicillin, streptomycin, fungizone) (Invitrogen Life Technologies). T cells were subsequently expanded with 5 ng/ml of IL-2 (R&D Systems, Minneapolis, MN) for 8 days prior to 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 DTT (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 Corporation, 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% HEPES 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 day 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 TGF-β for 24 h, followed by activation with plate-bound anti-CD3 (1 μg/ml) (Ortho Diagnostic Systems, Raritan, NJ) and soluble anti-CD28 (1 μg/ml, 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 24h 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 database was queried to predict and score the extent of complementarity between 3′UTR of IL-2 and IL-2 inducible T cell kinase (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 μg of RNA, cDNA was synthesized using the SA Biosciences (Frederick, MD) RT^2 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). MicroRNA 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 RT-PCR

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 (EEF) 1A1 for normalization. The following primers were used, itk forward primer 5′GGTCATTGGTGTGCTGATG3′ and reverse primer 5′TCTGCAATTTCAGCCAGTTG3′; IL-2, forward primer 5′TGCAACTCCTGTCTTGCATT3′ and reverse primer 5′GCCTTCTTGGGCATGTAAAA3′ and EEF1A1 forward primer 5′CTTTGGGTCGCTTTGCTGTT3′ and reverse primer 5′CCGTTCTTCCACCACTGATT3′. 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 TGF-β for 24h, transfected with 50 nM anti-miR-155 or scrambled siRNA, using an Amaxa Nucleofector (Gaithsburg, MD). Transfection was carried out according to the manufacturer's instructions for un-stimulated T cells provided in the Human T cell kit (VPA-1002) 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 GFP vector, were approximately 60%.

Overexpression of miR155

CD4+LPT were transfected using 100 nM premiR-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) and the plates read with a multiwell plate reader (Molecular Devices) and analyzed using Soft Max Pro 4.3 LS computer analysis software.

Statistical analysis

The Student *t* test was used to determine the statistical significance of experimental results. A *p*-value of 0.05 or less was considered significant. The results were represented as the mean plus or minus SD from 3 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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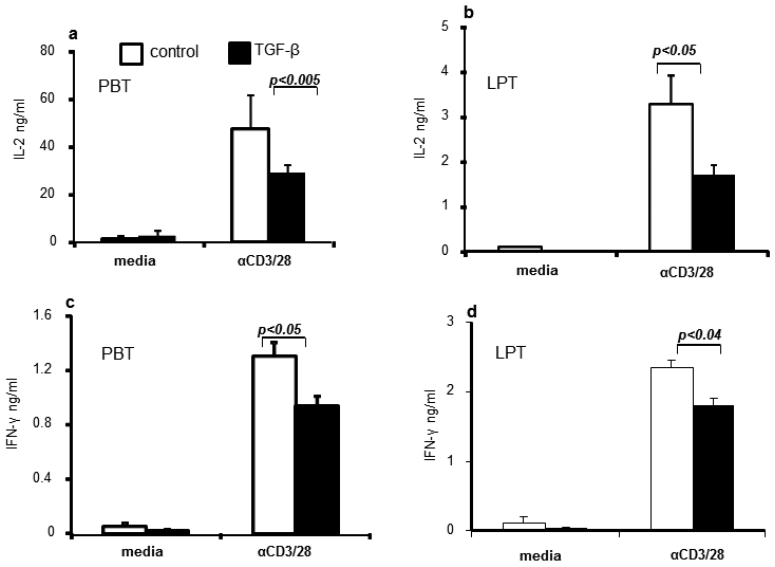

Figure 1. TGF-β inhibits T cell derived IL-2 and IFN-γ production

PBT and 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 TGF-β (filled bars) for 24 h or left untreated (open bars). Following pretreatment cells were stimulated with plate-bound αCD3 (1 μg/ml) and soluble αCD28 (1 μg/ml) in the presence or absence of 3 ng/ml TGF-β, respectively. Conditioned media was collected from triplicate wells 48 h after stimulation and evaluated for IL-2 (a,b) and IFN-γ (c,d) production by ELISA. Data is presented as a mean of n=4 ± SEM; significance between treated and untreated cells was compared using a Students t-test.

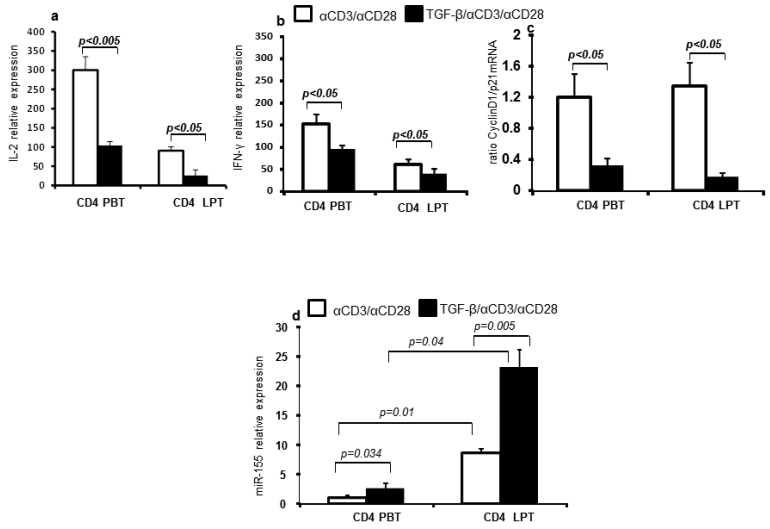

Figure 2. TGF-β inhibits cytokine mRNA accumulation and induces miR-155 in TCR-activated CD4+ T cells

CD4+ PBT and LPT were purified, pretreated with 3 ng/ml TGF-β (filled bars) for 24 h or left untreated (open bars), and activated as described in Fig.1. RNA was extracted 24 h after stimulation and evaluated for IL-2 (a), IFN-γ (b), cyclin D1 and p21 (c) mRNA by qPCR. miR-155 levels were determined 12 h after stimulation (d). For a-c, mRNA levels in un-stimulated PBT and LPT were assigned a value of 1 (data not shown). EEF1A1 was used as 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 ± SEM; significance between treated and untreated cells was compared using a Students t-test.

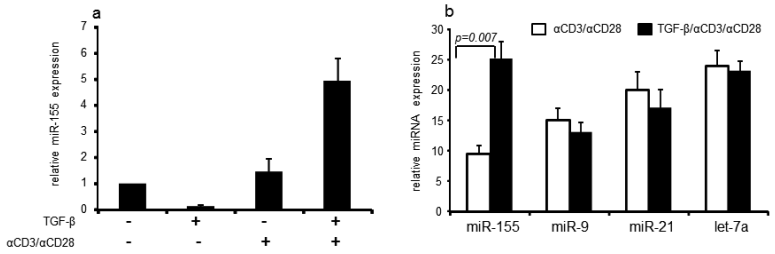

Figure 3. TGF-β induces miR-155 in freshly isolated CD4+ LPT and selectively regulates miR-155 expression

(a) Freshly isolated CD4+LPT and (b) CD4+LPT lymphoblasts were pretreated with TGF-β (controls were untreated) followed by TCR activation in the presence or absence of TGF-β for 24 h 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 2 donors for Fig. 3a and 4 donors ± SEM for Fig. 3b; significance between treated and untreated cells was compared using a Students t-test.

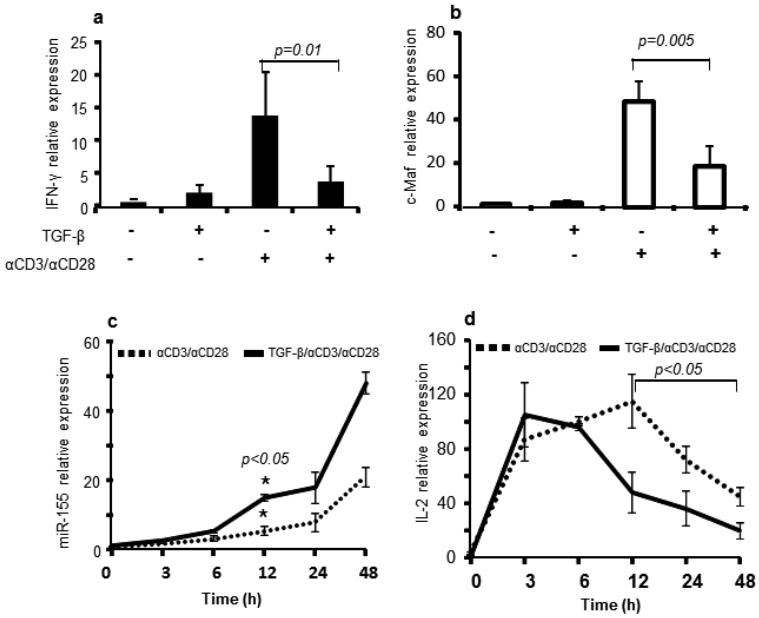

Figure 4. Prolonged up-regulation of miR-155 is inversely related with IL-2 production

CD4+LPT were pretreated (or not in controls) with 3 ng/ml 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) IFN-γ and (b) c-Maf 24 h after TCR 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 EEF1A1. Data are an average of 5 independent experiments (n=5) and error bars represent SEM.

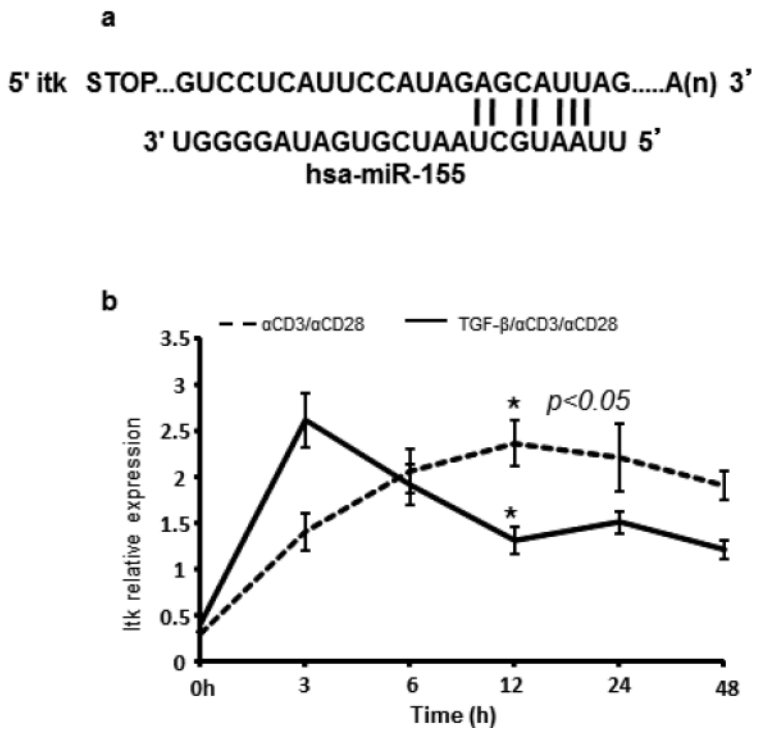

Figure 5. Itk is a predicted target of miR-155 and decreases in a TGF-β-dependent manner

(a) Schematic representation of a 7-mer complementarity between the seed sequence of miR-155 and 3′UTR of itk mRNA. (b) CD4+LPT were pretreated with TGF-β and activated as described previously. RNA was extracted at indicated time points and itk expression quantified by qPCR. Fold change in itk was normalized to EEF1A1. Data are the mean of 3 independent experiments (n=3), $p<0.05$ where indicated.

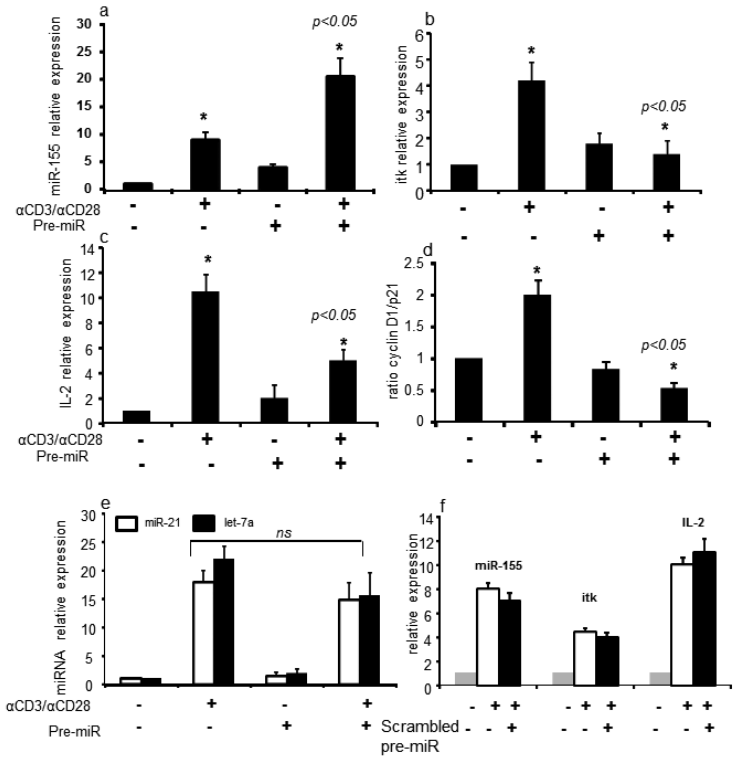

Figure 6. Over expression of miR-155 in CD4+LPT inhibits itk and IL-2 mRNA

CD4 LPT were transfected with precursor miR-155 at 100 nM and rested for 16 h. Following stimulation with αCD3/αCD28 for 24 h, cells were subject to RNA extraction. (a) miR-155 levels were evaluated by qPCR to assess efficiency of transfection and U6 levels used to normalize expression. Relative miR-155 expression from un-stimulated 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 EEF1A1 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 100nM scrambled miRNA cultured under similar conditions as described above. Data are the average of three separate donors in independent experiments and error bars represent SEM.

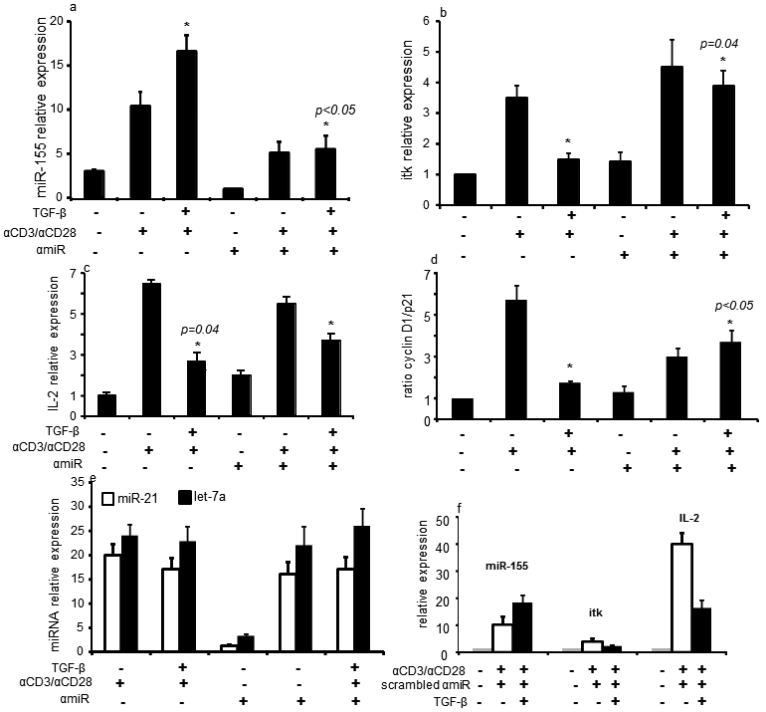

Figure 7. MiR-155 silencing in TGF-β-treated CD4 LPT increases IL-2 and itk

TGF-β-treated and untreated CD4 LPT were transfected with 50nM anti-miR-155 or scrambled siRNA, rested for 5h following transfection and subject to activation with αCD3/αCD28 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 50nM scrambled siRNA. Fold change in miRNA expression is normalized to U6 and fold changes in mRNA expression normalized to EEF1A1. Relative expression of miR-155 was calculated using mock-transfected un-stimulated cells set to 1. IL-2 and itk mRNA levels from un-stimulated cells were used for comparison and set to 1. Data is an average of 3 independent experiments, error bars represent SEM.