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Downregulation of Inflammatory MicroRNAs by Ig-like Transcript 3 Is Essential for the Differentiation of Human CD8+ T Suppressor Cells

Chih-Chao Chang, Qing-Yin Zhang, Zhuoru Liu, Raphael A. Clynes, Nicole Suciu-Foca, and George Vlad

We have investigated the mechanism underlying the immunoregulatory function of membrane Ig-like transcript 3 (ILT3) and soluble ILT3Fc. microRNA (miRNA) expression profile identified genes that were downregulated in ILT3-induced human CD8+ T suppressor cells (Ts) while upregulated in T cells primed in the absence of ILT3. We found that miR-21, miR-30b, and miR-155 target the 3′-untranslated region of genes whose expression was strongly increased in ILT3Fc-induced Ts, such as dual specificity protein phosphatase 10, B cell CLL/lymphoma 6, and suppressor of cytokine signaling 1, respectively. Transfection of miRNA mimics or inhibitors and site-specific mutagenesis of their 3′-untranslated region binding sites indicated that B cell CLL/lymphoma 6, dual specificity protein phosphatase 10, and suppressor of cytokine signaling 1 are direct targets of miR-30b, miR-21, and miR-155. Primed CD8+ T cells transfected with miR-21&30b, miR-21&155, or miR-21&30b&155 inhibitors displayed suppressor activity when added to autologous CD3-triggered CD4 T cells. Luciferase reporter assays of miR-21 and miR-155 indicated that their transcription is highly dependent on AP-1. Analysis of activated T cells showed that ILT3Fc inhibited the translocation to the nucleus of the AP-1 subunits, FOSB and c-FOS, and the phosphorylation of ZAP70 and phospholipase C-γ1. In conclusion, ILT3Fc inhibits T cell activation and induces the generation of Ts targeting multiple inflammatory miRNA pathways. The Journal of Immunology, 2012, 188: 3042–3052.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BCL6, B cell CLL/lymphoma 6; DC, dendritic cell; DUSP, dual specificity phosphatase; FOS, FBJ murine osteosarcoma; hRuc, luciferase (Renilla); ILT3, Ig-like transcript 3; KD, knockdown; luc, luciferase (Firefly); miRNA, microRNA; PLC, phospholipase C; SOCS1, suppressor of cytokine signaling 1; TGFB1R2, TGFBR2; Treg, T regulatory cell; TOB1, transducer of ERBB2; Ts, T suppressor cell; 3′-UTR, 3′-untranslated region.

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Gene profile analysis of CD8+ T cells primed for 7 d in MLC in the presence or absence of ILT3Fc showed that several hundred genes belonging to >28 gene ontology categories were modulated by ILT3Fc (12). There was a striking increase in the expression of transcription factors belonging to a class of zinc finger transcriptional repressors including B cell CLL/Lymphoma 6 (BCL6), a known inhibitor of IFN-γ (13), IL-17 (14, 15), IL-5 (16, 17), and granzyme B (18) expression. BCL6 was found to be crucial to CD8 T cells prevented their differentiation into Ts, whereas transfection of BCL6 in primed CD8+ T cells resulted in the acquisition of Ts function (11). High expression of BCL6 was also found in humanized mice tolerating islet allografts (11). Other genes strongly upregulated by ILT3Fc included the dual specificity phosphatase (DUSP)8, DUSP10, TGF-βR 2 (TGFBR2), transducer of ERBB2 (TOB1), CXCR4, adrenergic β-2R, and suppressor of cytokine signaling (SOCS)1 (11, 12). All of these genes are involved in functional differentiation of T cells, and are targeted by micro-RNAs (miRNA), some of which are discussed in the current study.

miRNA, a newly discovered class of small noncoding RNAs, are key negative regulators of gene expression. As with conventional protein-encoding RNA, miRNA are transcribed by RNA polymerase II, and their expression is controlled by transcriptional factors. The mature miRNAs inhibit target mRNA translation or promote their degradation by directly binding to the 3'-untranslated region (3'-UTR) of target genes (19). Increasing evidence suggests that the differentiation of T cells with diverse effector or regulatory function is largely controlled by miRNAs, which integrate cues from the microenvironment to either turn on or silence lineage-specific genes (20, 21).

The aim of our present study was to identify the specific miRNAs that are involved in the upregulation of BCL6 and other genes related to the differentiation of CD8 T cells primed in the presence of ILT3Fc. To our knowledge, our current findings show for the first time how an immunomodulatory molecule, such as ILT3Fc, inhibits T cell immune/inflammatory responses by downregulating the expression of inflammatory miRNA.

Materials and Methods

Cell cultures

Preparation of PBMC and negative isolation of CD3+ T cells have been described previously (9). To generate allospecific CD8+ Ts, CD3+CD25+ T cells (50×10^6/ml anti-CD3 mAb (clone UCHT1; BD Biosciences). Magnetically sorted (BD Biosciences) in the presence of 2×10^6 magnetic beads) followed by incubation in anti-CD3–coated T cell activation plates (Promega) for 24 h. T cells were treated with ILT3Fc, which inhibited the MLC response by 40% to 90% (compared with control). Preparation of PBMC and negative isolation of CD3+ T cells have been described previously (9, 11). On day 7, CD8+ T cells were negatively selected from these cultures and tested for suppressor activity prior to use in miRNA or microarray assays. Allostimulated CD8+ T cells treated with ILT3Fc, which inhibited the MLC response by >80%, were used as Ts. Non–ILT3treated CD8+ T cells had no inhibitory activity and were used as controls.

Polyclinically activated CD8+ T cells were prepared from fresh PBMC by negative selection (using anti-CD4, -CD14, -CD20, -CD56–coated magnetic beads) followed by incubation in anti-CD3–coated T cell activation plates (BD Biosciences) in the presence of 50 μg/ml anti-CD28 Abs. After 2 d of incubation, cells were collected, washed twice, and transfected with either reporter constructs or RNA oligomers (miRNA mimics or hairpin inhibitors) using the nuclease method. Putative CD8+ suppressor cells were generated by transfection of polyclonally activated CD8+ T cells with individual hairpin inhibitors of miRNA or mixtures of two or three inhibitors. To test the ability of these putative Ts to inhibit autologous CD4 Th responses, transfected CD8 T cells (50×10^6/well) were co-cultured with CFSE-labeled CD4+CD25+ Th responders, and CD4+ monocytes at a 1:1:1 ratio, in the presence of 1 μg/ml anti-CD3 mAb (clone UCHT1; BD Biosciences). Magnetic cells were sorted CD4+CD25+ responder T cells were labeled with CFSE (1 μM CFSE (“CellTrace”; Invitrogen) for 10 min in PBS/0.1% BSA at room temperature. The reaction was stopped by addition of ice-cold culture medium containing 10% FCS for 5 min, followed by extensive washing. Proliferation of the CFSE-labeled CD4 Th responders was assessed at day 3 analyzing dye dilution by flow cytometry using a FACSCalibur instrument and CellQuest software (BD Biosciences). As controls, conditions were set up, in which no CD8 T cells, or no anti-CD3 mAb were added. CD8 T cells transfected with control RNA (no miRNA inhibitors) were also assayed.

Gene promoter and 3'-UTR constructs

Sequences corresponding to −1296 to +40 bp from the miRNA start of BCL6 to −748 to +52 from the RNA start of SOCS1 genes were cloned from genomic DNA by PCR using high-fidelity Taq and gene-specific primers (Supplemental Table I). These gene promoters, previously shown to be functional in response to various stimuli in reporter assays (22, 23), were subsequently cloned into HindIII or BglIII and Nhel sites of pGL3 basic Firefly-lucerase construct (Promega). Sequences corresponding to −410 to +40 bp from the miRNA start of mir-21 promoter were similarly constructed. This region includes two AP-1 sites, AP-1 Ψ (proximal) and AP-1 Ψ (distal), shown to be fully functional, PMA responsive, and critical for expression of mir-21 in 293T cells (24), mir-155 promoter construct and its AP-1 and NFκB binding sites mutants were provided by Dr. E. Flemmington (Tulane Health Sciences Center, New Orleans, LA) (25). The NF-AT/AP-1 basic promoter construct pGL4.30, which contains an enhancer element from the human IL-2 gene promoter, was obtained from Promega; the AP-1(miR21B) basic promoter construct was prepared by replacing a 140-bp NF-AT/AP-1 fragment of pGL4.30 with a triplet of the AP-1 Ψ site of the mir-21 gene promoter.

Full-length 3'-UTR of various genes (1.1 kb BCL6, 0.4 kb SOCS1, and 1.0 kb DUSP10) were obtained from CD8+ T cell cDNA libraries by PCRs using a high-fidelity Taq DNA polymerase (Invitrogen) and gene specific primers (Supplemental Table I), PCR products were first cloned into pgEM-T Easy Vector (Promega), excised from recombinant plasmids by Notl digestion, and subcloned into psiCHECK-2 luciferase reporter (Promega). The psiCHECK-2 vector contains an SV40 early enhancer/promoter, a synthetic Renilla luciferase (hRluc) gene flanked at the 3'-end by a Notl restriction site, and a synthetic poly(A) tail. All reporter constructs were completely sequenced from both ends.

miRNA mimics and hairpin inhibitors

Stability-enhanced miR-30b RNA oligonucleotide (miRDIAN 30b) and miR-155 oligonucleotide (miRDIAN Mimic-155), hairpin miRNA inhibitors (meridian hairpin inhibitor mir-21, mir-30b, and mir-155), and the control nontargeting RNA oligonucleotide (miRDIAN Mimic Negative Control number 1) were purchased from Dharmaco. miRNA mimics (0–10 nmol) were transfected into fresh, nonactivated CD8 T cells (3×10^6), and hairpin inhibitors (0–10 nmol) were transfected into polyclonally activated CD8+ T cells (3×10^6) using Amazaxa’s Human T cell Nucleofector Kit (Lonza).

Mutagenesis

Site-directed mutagenesis of AP-1 binding sites in mir-30b promoter constructs and mutagenesis of mir-30b and mir-155 binding sites in 3’-UTR of BCL6 and SOCS1 was performed using QuickChangeII (Stratagene) with mutated gene-specific primers and corresponding DNA templates (Supplemental Table I).

Transforms of reporter constructs and miRNA oligonucleotides

Polyclinically activated CD8+ T cells were generated by stimulation with anti-CD3/CD28 mAb in the presence or absence of ILT3Fc (50 μg/ml) as described above. After 48 h, 3–5×10^6 cells were collected and cotransfected with 3 μg pGL3-based (Promega) promoter constructs (containing either BCL6 or SOCS1 promoters and the Firefly lucerase, luc, gene) and 2 μg pGL4.70[hRluc] (Promega) (containing the constitutively expressed Renilla lucerase, hRluc, gene), using Amazaxa Human T cell Nucleofector Kit (Lonza). Sixteen hours after transfection, cells were lysed and assayed for both Firefly and Renilla lucerase activities in a single tube luminoimeter (Turner BioSystems 20/20). Normalized promoter activity was measured as units of Firefly lucerase activity divided by units of Renilla lucerase activity.

The 3’-UTR reporter gene activities in polyclonically activated CD8 T cells were similarly assayed, using 2 μg psCHECK-2 vectors. These vectors contained an SV40 promoter, the Renilla hRluc reporter gene, and the 3’-UTR of BCL6, SOCS1, or DUSP10 genes, in addition to a constitutive hRluc reporter gene. For normalization, hRluc activity was divided by internal Firefly luc activity. ILT3Fc treatment did not affect the normalized hRluc/luc activity in activated Jurkat or CD8 T cells transfected with the unmodified psCHECK-2 plasmid.

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For miRNA promoter reporter assays, Jurkat T cells (American Type Culture Collection) were pretreated with either human IgG or ILT3Fc for 16 h. Cells were transfected using Lipofectamine 2000 (Invitrogen) with one of the following Firefly luciferase plasmids: pGL3 containing mir-21 or mir-155 promoters, pGL4.30 [luc2/NFAT-RE/Hygro] containing the NFAT/AP-1 binding sites of the human IL-2 promoter, or pGL4.30 from which the NFAT/AP-1 sites were removed and replaced with three copies of the AP-1 site of the miR-21 promoter. The cells were cotransfected with pGL4.70 [Rluc] plasmid constitutively expressing Renilla luciferase (Promega) for assessing transfection efficiency and normalization. The cells were then stimulated for 48 h with PMA (10 nM) or mAb anti-CD3/CD28, and reporter gene readouts were performed and normalized as above.

**miRNA array and real-time PCR**

ILT3Fc-induced allospecific CD8+ Ts cells and primed (non-ILT3 treated) CD8+ T cell controls were generated in MLC as described above. Five micrograms of total RNA prepared from these cells using TRIzol (Invitrogen) was annealed to oligonucleotide primer mix and hybridized to 132 miRNA oligonucleotide probes. Streptavidin–HRP chemiluminescence was used for detection of miRNA expression (Signos). Real-time PCR detection of miRNA was performed using TaqMan Small RNA assays (Applied Biosystems). Relative expression of miRNA was normalized by U6 miRNA. Relative expression or protein encoding genes was normalized by GAPDH.

**Nuclear protein extraction and immunoblotting**

For signaling studies, Jurkat or primary CD8 T cells were stimulated with CD3/CD28 mAbs (2 μg/ml) and a cross-linking anti-mouse IgG Ab for the indicated times in the presence or absence of ILT3Fc (15 μg/ml). Total cell lysates (20 μg) prepared from CD8+ T lymphocytes or Jurkat T cells were loaded onto either 10 or 4–12% SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane, and probed with various Abs as described previously (8). In some experiments, a commercial protein extract kit (NucBuster; Novagen) was used to extract nuclear proteins. Polyclonal rabbit Abs recognizing various TCR and AP-1 signaling proteins (Cell Signaling Technology), SOCS1, BCL6 (Santa Cruz Biotechnology), and DUSP10 (Abcam) were used to probe the membranes.

**miRNA target prediction**

Several Web-accessible miRNA database searching programs were used for prediction of miRNA target sites. These include http://www.microrna.org, http://www.miRBase.org, and http://www.targetscan.org.

**Statistical analysis**

Differences between control and experimental groups (each consisting of a minimum of three individual experiments) were estimated using the t test for paired two-tailed distribution. Mean and SE were calculated for each group. Differences associated with p < 0.05 were considered significant.

**Results**

**ILT3 inhibits proinflammatory miRNAs expression in alloantigen-stimulated T cells**

We first explored the miRNA transcriptional profiles of CD8+ Ts generated in 7-d MLC containing ILT3Fc and in control CD8+ T cells allostimulated without ILT3Fc. A group of at least six miRNAs (miR-21, miR-146a, miR-30b, miR-30c, miR-29, and miR-155), which are expressed at negligible levels in unstimulated CD8+ T cells, were consistently upregulated by allosimulation in three independent experiments (Fig. 1A). When ILT3Fc was added to the cultures, the level of expression of these genes was strongly downregulated, as confirmed by real-time PCR analysis (Fig. 1B). Similarly, membrane ILT3 also inhibits the expression of miR-21, -30b, -146a, and -155 as demonstrated in experiments in which T cells were allostimulated with DC transfected with ILT3 small interfering RNA (ILT3KD-DC) or empty vector (control-DC). Upon priming with ILT3KD DC, CD8+ T cells, sorted after 16 h from these cultures, showed much higher expression of miR-21, miR-30b, miR-146a, and miR-155, as determined by RT-PCR, compared with CD8+ T cells stimulated with ILT3+ control DC (Fig. 1C). Hence, both membrane and soluble ILT3 inhibit miRNAs expressed by MLC-stimulated CD8+ T cells.

**Induction of BCL6, SOCS1, and DUSP10 by ILT3Fc is 3′-UTR dependent**

Computer aid searches for putative targets of these miRNAs showed that many of them were genes whose expression was upregulated (>3-fold) in CD8+ T cells allostimulated in the presence of ILT3Fc compared with CD8+ T cells primed in cultures without ILT3Fc (11, 12). Table I shows a partial list of these ILT3Fc-upregulated genes whose 3′-UTRs contain target sites for these ILT3Fc-modulated miRNAs. Genes induced by ILT3Fc treatment, which are predicted to be targeted by miR-21, include dual-specific phosphatases DUSP8 and DUSP10, known to be inhibitors of the MAPK pathway and cytokine production (26). Also included in this group are TGFBR2 and TOB1, involved in regulation of T cell responses (27, 28). Genes upregulated by ILT3Fc, which are predicted to be targeted by miR-30b and miR-146a, are BCL6 and CXCR4, respectively. There is already evidence that miR-146a controls expression of CXCR4 (29), miR-21 controls TGFBR2 (30), and miR-155 acts on SOCS1, a negative regulator of cytokine signaling through STAT1 (31–33). SOCS1 also contains a putative target site for miR-30b.

The upregulation of these target genes by ILT3Fc was further confirmed by real-time PCR analysis using three independent samples sets. ILT3Fc-induced inhibition of IFN-γ expression was found in all of our previous studies (9–12) and is shown as a control for the ILT3Fc effect (Fig. 2A). The findings that ILT3Fc induces upregulation of these genes and downregulation of the miRNA (which target them) prompted us to examine the underlying molecular mechanism.

The upregulated expression of BCL6, SOCS1, and DUSP10 is integral to the signature of ILT3Fc-induced CD8+ Ts. These genes are known inhibitors of cytokine production and TCR signaling and are targeted by miRNAs, which are suppressed by ILT3Fc. We thus analyzed the relationship between miRNAs and gene expression in the presence and absence of ILT3Fc.

Because target sites of miRNAs, with very few exceptions, are located in the 3′-UTR of messenger RNAs, we performed 3′-UTR reporter assays. PsiCHECK-2 luciferase reporter constructs made up of an SV40 promoter, the Renilla luciferase gene, and the 3′-UTRs of BCL6, SOCS1, or DUSP10 were transfected into CD8+ T cells, which had been stimulated for 2 d with anti-CD3/CD28 mAbs in the presence of absence of ILT3Fc. ILT3Fc induced the increase of luciferase reporter activity in primed CD8+ T cells transfected with BCL6, SOCS1, or DUSP10 3′-UTR reporters (Fig. 2B), confirming the importance of the 3′-UTR for the ILT3-induced upregulation of these genes. To investigate the role of the respective promoters on the induction of these genes by ILT3Fc, we generated pGL3-based promoter reporter constructs consisting of the Firefly luciferase gene under the transcriptional control of the BCL6 or SOCS1 promoters. These constructs were transfected into activated CD8+ T cells which had been stimulated for 2 d with anti-CD3/CD28 mAbs in the presence of absence of ILT3Fc. These experiments showed that ILT3Fc had no significant effect on luciferase activity and therefore the upregulation of Ts signature genes by ILT3Fc is not mediated through induction of transcriptional activity at the BCL6 and SOCS1 promoters (Fig. 2C). miR-30b, miR-155, and miR-21 target the 3′-UTR of ILT3Fc-inducible genes.

The dependence of ILT3Fc-induced upregulation of DUSP10, SOCS1, and BCL6 on their 3′-UTR suggested that the mRNA which target them may play a role in regulation of gene expression. We analyzed BCL6, SOCS1, and DUSP10 expression in CD8+ T cells by transient transfection of either gene-specific, chemically stabilized, dsRNA oligomers, that mimic the func-
tion of endogenous mature miRNA (miRNA mimic), or chemically modified, single-stranded antisense oligomers that inhibit miRNA function (miRNA hairpin inhibitor). Transfection efficiency of either unstimulated or polyclonally stimulated CD8+ T cells, monitored by FACS analysis of transfected GFP RNA oligomer, was ∼75%. The predicted miRNA target sites in the 3’-UTRs of BCL6, SOCS1, and DUSP10 are shown in Fig. 3A.

We transfected unprimed CD8+ T cells with miRNA mimics specific for miR-30b and miR-155. As a control, the cells were transfected with nontargeting RNA oligomers. Western blot analyses of BCL6 and SOCS1 proteins, using β-actin as an internal control, showed that overexpression of miR-30b downregulated the expression of BCL6 and SOCS1, whereas overexpression of miR-155 lead to downregulation SOCS1 (Fig. 3B) but not BCL6 (which has no target site for it). Inhibition of BCL6 and SOCS1 gene expression by these miRNA mimics increased with the amount added to the culture.

As a corollary, transfection of the polyclonally stimulated CD8+ T cells with gene-specific hairpin inhibitors to knock down the respective miRNAs induced the upregulation of the target genes (Fig. 3C). Hairpin inhibitors of miR-30b induced upregulation of BCL6 and SOCS1, whereas inhibitors of miR-155 upregulated only SOCS1. KD of miR-21 resulted in upregulation of DUSP-10 yet had no effect on BCL6, which lacks the target sequence for miR-21 (Fig. 3C).

To determine whether physical binding of miRNA to their target sites in 3’-UTR of the corresponding genes is required for their downregulation, we generated a series of reporters in which the consensus regions of miR-21, miR-30b, or miR-155 sites were mutated. Experiments in which these mutant reporters were transfected in stimulated CD8+ T cells from three different donors showed that mutations of miR-30b sites in 3’-UTR of BCL6 and SOCS1, and mutation of miR-21 site in 3’-UTR of DUSP10 abrogated their responsiveness to miRNA suppression, inducing

Table I. ILT3Fc-inducible genes contain potential binding sites for miRNAs, which are inhibited by ILT3Fc

<table>
<thead>
<tr>
<th>Gene Induced by ILT3Fc</th>
<th>Induction (Fold)</th>
<th>miRNA Sites in 3’-UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUSP8</td>
<td>11.23</td>
<td>miR-21</td>
</tr>
<tr>
<td>DUSP10</td>
<td>3.2</td>
<td>miR-21</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>3.3</td>
<td>miR-21</td>
</tr>
<tr>
<td>TOB1</td>
<td>4.9</td>
<td>miR-21</td>
</tr>
<tr>
<td>ZNF331</td>
<td>8.2</td>
<td>miR-146a</td>
</tr>
<tr>
<td>BCL-6</td>
<td>3.1</td>
<td>miR-30b</td>
</tr>
<tr>
<td>CXCR4</td>
<td>6.6</td>
<td>miR-146a</td>
</tr>
<tr>
<td>ADRB2</td>
<td>4.5</td>
<td>miR-21 and miR-30b</td>
</tr>
<tr>
<td>SOCS1</td>
<td>3.7</td>
<td>miR-30b and miR-155</td>
</tr>
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</table>

ADRB2, Adrenergic β-2R.
higher reporter activities than wild-type miRNA binding sequences (Fig. 3D). Mutation of miR-155 in 3′-UTR of SOCS1, as expected, also expressed higher reporter gene activities. To further determine whether downregulation of these miRNAs is essential for the ILT3-induced upregulation of genes involved in the differentiation of Ts, we analyzed the response of the endogenous target genes in CD8+ T cells that have been transfected with vectors containing constitutively expressed miR-30b, miR-21, and miR-155 mimics and then stimulated for 2 d with anti-CD3/CD28 mAbs in the presence or absence of ILT3Fc. ILT3Fc treatment no longer affected the endogenous expression of BCL6, DUSP10, and SOCS1 in cells transfected with these miRNA mimics (Fig. 4).

Taken together, these data indicate that BCL6, SOCS1, and DUSP10 are direct targets of posttranscriptional regulation mediated by miR-30b, miR-155, and miR-21, respectively. Hence, downregulation of these miRNAs by ILT3Fc in primed CD8+ T cells leads to upregulation of ILT3Fc-inducible gene expression.

**FIGURE 2.** Activation of BCL6, SOCS1, and DUSP10 genes by ILT3Fc is 3′-UTR dependent. (A) Real-time PCR analysis of selected genes expressed by CD8+ T cells allostimulated in the presence or absence of ILT3Fc in three independent sample sets. (B) Effect of ILT3Fc on BCL6, SOCS1, and DUSP10 3′-UTR reporter assays. Polyclonally activated CD8+ T cells stimulated in the presence of absence of ILT3Fc were transfected with psiCHECK-2 vectors containing the Renilla hRLuc gene linked to the 3′-UTRs of BCL6, SOCS1, or DUSP10 and a constitutively expressed Firefly luc gene. The luciferase activity was assayed 16 h later, and the normalized hRluc/luc values are represented. (C) Effect of ILT3Fc on the BCL6 and SOCS1 promoter activities. Polyclonally activated CD8+ T cells stimulated in the presence of absence of ILT3Fc were cotransfected with pGL3 plasmids containing the Firefly luc gene under the control of BCL6 or SOCS1 promoters and pGL4 plasmids containing a constitutively expressed Renilla hRluc gene. The luciferase activity was assayed 16 h later, and the normalized luc/hRluc values are represented. Mean and SEM of three experiments and statistical significance (*p < 0.05) are indicated.
CD8 T cells transfected with miRNA inhibitors suppress CD4 T cell responses

We sought to determine whether ILT3Fc-induced differentiation of Ts is due to inhibition of these inflammatory miRNA. For this, CD8 T cells from three different blood donors were activated by 2-d incubation with anti-CD3 and CD28 mAb and then transfected with individual or combinations of miRNA inhibitors. Sixteen hours after transfection, these CD8 T cells were added to autologous CFSE-labeled CD4+CD25 T cells in cultures containing 1 μg/ml anti-CD3 Abs and autologous APC. Cell proliferation assay showed that when CD8 T cells, which had been transfected with individual hairpin inhibitors of miR-21, -30b, or -155, were added to CD4+ T cells, there was no inhibition in T cell proliferation compared with control cultures containing nontargeted oligonucleotides (Fig. 5).

Although individually none of these miRNA inhibitors were sufficient to induce Ts, activated CD8 T cells transfected with mixtures of miR-21/30b, miR-21/155, or miR-21/30b/155 inhibitors suppressed CD3-activated proliferation of CD4 T cells by >50% in five independent experiments. ILT3Fc added to CD3-activated CD4 T cells inhibited proliferation by >80%. This suggests that miRNAs targeting DUSP10, BCL6, and SOCS1 act in concert, playing a role in the differentiation of CD8 T cells (Fig. 5).
ILT3Fc regulates microRNA gene expression by inhibiting their promoter activities

Having learned that ILT3Fc induces the upregulation of BCL6, DUSP10, and SOCS1 via downregulation of miRs, which target their 3' UTR, we tried to understand the mechanism of miRNA inhibition. For some of these experiments, we used the Jurkat T cell line whose miRNA expression profile is similar to that of activated human T lymphocytes and shows high binding of fluo-

rescinated ILT3Fc (>90%), lending itself as an ideal tool to study signaling effects downstream of the ILT3 ligand.

To determine whether miRNA gene promoters can be activated by anti-CD3/CD28 mAbs in Jurkat cells, Firefly luciferase reporter gene plasmids (pGL3 based), in which the reporter was under the transcriptional control of either miR-21 or miR-155 gene promoters, were cotransfected with a Renilla luciferase control reporter construct. After 48 h of incubation with CD3/CD28 mAbs,
normalized promoter activities (Firefly luciferase/Renilla luciferase) showed a 2- to 3.5-fold increase above the level seen in nonstimulated Jurkat cells (Fig. 6A). Mutations at the AP-1 binding sites in the miR-21 or miR-155 gene promoters completely abolished reporter gene activity in three independent experiments. This indicates that the transcriptional activities of miR-21 and miR-155 depend on AP-1.

We next tested the promoter activities of miR-21 and miR-155 genes in CD3/CD28-triggered Jurkat cells pretreated with various doses of ILT3Fc. The results indicated that ILT3Fc at concentrations as low as 5 μg/ml strongly inhibited miR-21 and miR-155 miRNA gene promoter activities (Fig. 6B). Hence, the AP-1 binding sites within the promoter regions of miR-21 or miR-155 were crucial to the inhibitory effect of ILT3Fc.

ILT3Fc inhibits basal TCR signaling and TCR-mediated nuclear localization of AP-1

Because AP-1 binding to the promoters of miR-21 and miR-155 is essential for their activation (24, 25), we investigated the possibility that the inhibitory effect of ILT3Fc on these miRNAs is mediated by modulation of AP-1 activity. The proximal AP-1 site of the miR-21 promoter and the distal NFAT/AP-1 site of the human IL-2 promoter were engineered as three tandem copies and inserted into the pGL4.30 Firefly luciferase reporter construct. These constructs were transfected in Jurkat cells, which were activated with PMA (10 nM) in the presence or absence of different concentrations of ILT3Fc (1.5–15 μg/ml). Reporter genes containing only AP-1 binding sites were strongly induced by PMA. ILT3Fc at 1.5–5 μg/ml consistently inhibited PMA-induced, AP-1–mediated reporter activities in a dose-dependent manner in three repeat experiments (Fig. 7A).

Because transcription factors binding to AP-1 site play an important role in TCR signaling, we investigated the possibility that TCR/AP-1 signaling pathways are affected by ILT3Fc. Jurkat or primary CD8 T cells were preincubated with 15 μg/ml ILT3Fc for 16 h and then stimulated by adding anti-CD3/CD28 Abs to the cultures over 3 h. We isolated nuclear fractions of cell extracts obtained from these cultures at various time points. Nuclear proteins from these cells were probed with Abs recognizing various subunits of AP-1 protein complexes. Western blot analyses showed that the FBJ murine osteosarcoma (FOS) proteins—c-FOS and two forms of FOSB—were rapidly synthesized and translocated into the nucleus of non–ILT3Fc-treated (control) Jurkat cells. After 3 h of stimulation, the level of c-FOS was already downregulated, whereas the level of FOSB was still upregulated. In ILT3Fc-treated Jurkat cells, c-FOS and FOS-B proteins were detected in the nucleus in lower amounts and at later times, indicating that ILT3Fc inhibited and delayed TCR signaling events (Fig. 7B).

These results suggest that the inhibitory activity of ILT3Fc is mediated through its effect on TCR signaling during very early stages of T cell activation. To identify TCR signaling proteins, which may be affected by ILT3Fc, we treated Jurkat cells and primary CD8 T cells with increasing concentrations of ILT3Fc for 16 h and then probed cell lysates with Abs recognizing different AP-1 pathway components. Normalization of the phosphorylated fractions (p) of ZAP70 and phospholipase C (PLC)-γ 1 by the

**FIGURE 6.** ILT3Fc inhibits miRNA promoter activities. (A) miRNA gene promoters were cotransfected with Firefly luciferase reporter plasmids containing miR-21 or miR-155 gene promoters and a control Renilla luciferase reporter construct into Jurkat cells. Mutation sites are indicated by “X.” Sites that are distal or proximal to the RNA start are indicated by “D” and “P,” respectively. Normalized luciferase activities in transfected Jurkat cells stimulated with CD3/CD28 mAbs or left untreated are shown. (B) ILT3Fc inhibits miR-21 and miR-155 promoter reporter activity. The promoter activity was tested in CD3/CD28-triggered Jurkat cells that had been pretreated with 5 μg/ml ILT3Fc. Error bars represent SE of three independent experiments. Differences between ILT3Fc-treated and nontreated cells are statistically significant (p < 0.05).
respective total amounts (T) of these proteins shows that ILT3Fc
inhibited the activation of these signaling molecules in a dose-
dependent manner (Fig. 7C). We found that the dual-specific
phosphatase DUSP10 was 2-fold higher in ILT3Fc-treated Jurkat
T cells, similar to the results obtained in ILT3Fc-induced Ts. The
finding that dephosphorylation of these TCR signaling proteins is
associated with the increased expression of DUSP10 suggests that
this dual-specific phosphatase modulates cell signaling pathways
in ILT3Fc-treated T cells.

Discussion
It is well established that inhibitory molecules expressed by APCs
have important roles in the induction of Treg/Ts (1). The ex-
pression of ILT3, a prototype of such inhibitory molecules, is
characteristically increased on the membrane of human tolero-
genic DC and induces the differentiation of human CD8 and CD4
Treg. Adaptive Ts/Treg induce the upregulation of ILT3 on DC
which in turn trigger the differentiation of new waves of Ag-
specific Ts/Treg (3, 4).

We have demonstrated that similar to membrane, soluble ILT3 is
a strong inducer of Ag specific CD8+ Ts (9–11). BCL6 is one of the
genes, which play a crucial role in the differentiation of ILT3-
induced Ts, because KD of this gene prevents Ts differentiation,
whereas overexpression of BCL6 in primed CD8+ T cells endows
them with suppressor activity (11).

Recently, the role of various miRNA in the differentiation of
effector and Treg has been described previously (33–35). The aim
of our study was to identify miRNAs, which regulate the expres-
sion of signature genes induced by ILT3 in human CD8+ Ts cells.
Our studies have demonstrated that miR-21, -30b, -146a, and -155
were downregulated in both membrane and soluble ILT3-induced
CD8+ Ts in conjunction with the upregulation of the genes whose
3'-UTR they target (DUSP10, BCL6, CXCR4, and SOCS1).

Consistent with our study showing that the downregulation of
miR-21, and miR-155 results in the upregulation of genes essen-
tial to Ts differentiation, such as DUSP10, and SOCS1, are recent
reports on the role of these two miRNAs in promoting autoimmune
inflammation and enhancing inflammatory T cell development (36,
poor concordance between humans and mice for lymphocyte analysis have unraveled numerous differences including the expression in human, but not mice, of KIR and ILT genes and of FOXP3 not only in Treg but also in Th cells (39, 40). There is also poor concordance between humans and mice for lymphocyte miRNA signature (41).

The miRNAs inhibited by ILT3 in primed CD8+ T cells target BCL6, which is crucial to the differentiation of Ts and some other genes with known immunoinhibitory functions such as SOCS1, DUSP1, DUSP8, DUSP10, and CXCR4 (11, 12). Although SOCS1 is a negative regulator of cytokine signaling (31, 33), DUSP10 acts on the MAPK pathways inhibiting TCR signaling (42). CXCR4, a receptor for the trafficking ligand CXCL12, was shown to contribute to the induction of T cell immunotolerance in mice (43).

CD8 T cells transfected with mixtures of miR inhibitors suppressed CD4 Th proliferation in response to CD3 triggering, suggesting that miRNAs targeting BCL6, SOCS1, and DUSP10 must act in concert to induce the differentiation of Ts.

ILT3Fc inhibits miR-21 and miR-155 at the transcriptional level in activated human T cells by downregulating AP-1 (24). ILT3Fc impaired the phosphorylation of Zap70 and PLC-γ and increased the expression of DUSP10. This dual-specific phosphatase is likely to modulate signaling pathways in primed T cells, which acquire suppressor activity upon exposure to ILT3. This finding is consistent with other studies, which demonstrated that DUSP10 dephosphorylates MAPK and JNK, attenuating the effect of TCR ligation and reducing the production of proinflammatory cytokines (44). Knockout of DUSP10 in mice leads to the overproduction of inflammatory cytokines and accelerated immune-mediated death after rechallenge with lymphocytic choriomeningitis virus (42). The finding that ILT3Fc regulates miRNA transcriptionally by targeting TCR/AP-1 signaling pathways suggests that this agent raises the threshold of TCR triggering.

A potential advantage of using ILT3Fc as an immunosuppressive agent emerges from its capacity to target multiple inflammatory miRNA pathways. Although each single miRNA acts simultaneously on hundreds of target genes, we found that inhibition of several inflammatory miRNAs is required to suppress T cell proliferation. Via simultaneously targeting the expression of several inflammatory miRNAs, ILT3Fc seems ideally suited to induce a cascade of inhibitory signals resulting in T cell anergy.

The strong immunosuppressive activity of ILT3Fc has been documented by its ability to suppress allograft rejection (10, 11), a phenomenon in which myriads of MHC-bound peptides are recognized via the direct and indirect pathways, triggering rejection (45). Therefore, it is most likely that recognition of a limited number of autoantigenic, tissue-specific peptides can be inhibited by ILT3Fc.

Information gained from studying the effect of ILT3Fc in the differentiation of Ts may readily be translated into clinical benefits for treatment of autoimmune diseases. Because exosomes can modify gene expression in a recipient cell (46, 47), it may become possible to deliver T cells signature genes or inhibitors of miR-21, -155, and -30b alone or in combination to generate CD8+ Ts. Alternatively, immune deficiencies may be treated by interfering with the generation and function of Ts, inhibiting their signature genes by targeted delivery of miRNA or by blockade of the ILT3 interaction with its T cell ligand.

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We thank Dr. Riccardo Dalla-Fava for excellent suggestions and Dr. Erik Flemmington for providing miR-155 promoter plasmids.

Disclosures

The authors have no financial conflicts of interest.

References

miRNA SIGNATURE OF ILT3-Fc–INDUCED CD8+ Ts


## Supplementary Table I.

### A. Primers used for generation of reporter constructs

#### 3'UTR reporter constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL6</td>
<td>5'-TGAAGCATGAGGTGATGAG-3'</td>
<td>5'-GCCGTAATGCAGTTTAGACACA-3'</td>
</tr>
<tr>
<td>SOCS1</td>
<td>5'-GACGCTTTCCTCCAGATT-3'</td>
<td>5'-AAAATATAAAATAGGATTCTGCACAC-3'</td>
</tr>
<tr>
<td>DUSP10</td>
<td>5'-ATGCTCGAGTGACAATGCTGGATGGA-3'</td>
<td>5'-GAGCTCTTCCCCTCCAGATT-3'</td>
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</tbody>
</table>

#### Promoter constructs: (underlines denote restriction site added for cloning into pGL3)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>5'-GCAGCTAGCTTTCTAAGTTGCCC-3'</td>
<td>5'-GCAAAGCTTTCCTCAGAGTAAGGC-3'</td>
</tr>
<tr>
<td>BCL6</td>
<td>5'-TGCTAACGTGCTTGAAGGACTCT-3'</td>
<td>5'-TCCAGATCTCTCTCTACAAATGC-3'</td>
</tr>
<tr>
<td>SOCS1</td>
<td>5'-CGATGCTAGCGTTTTCGGGCT-3'</td>
<td>5'-ACGTAAGCTTCCGAGTGGGGCGAAG-3'</td>
</tr>
</tbody>
</table>

### B. Mutagenesis (underlines denote positions of mutant sequences)

#### AP-1 sites in the miR-21 promoter

<table>
<thead>
<tr>
<th>Site</th>
<th>Primer 1</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>5'-CACCTTTCTTTGGATAAACACCCAGATTGTC-3'</td>
<td>5'-GTCTTTTTAGGACGACATAATACTCGGG-3'</td>
</tr>
<tr>
<td>Site 2</td>
<td>5'-TAGGGATGACACAAGCATAAACTTTCTCTTATCGT-3'</td>
<td>5'-GGTTTGAAACCAATTTAAGGAAGGTTCTGTTG-3'</td>
</tr>
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</table>

#### miR-30b recognition site in BCL6's 3'UTR

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</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-GTATTTCTTTTGCAAGTGACCGCCCAACTTTAAATG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TTTAAATACACCTTTGGAATTATTGTCGCTTAC-3'</td>
</tr>
</tbody>
</table>

#### miR-30b recognition site in SOCS1's 3'UTR

<table>
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</tr>
</thead>
<tbody>
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<td>Forward</td>
<td>5'-TGCAAGATACCTGGGATTATGTCGCTTCCAC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GTGCAAAGGATATCGGATTATGTCGCTTCCAC-3'</td>
</tr>
</tbody>
</table>

#### miR-155 recognition site in SOCS1's 3'UTR

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-AGTACGCGCCCGCCGCGGATGAGTGAGGGAC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CAGACTTCCACGCGCTTCCGCTTACGAGCC-3'</td>
</tr>
</tbody>
</table>