IL-27: An endogenous constitutive repressor of human monocytes

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Abstract

Interleukin (IL)-27 is a pleiotropic cytokine that initially was described as being pro-inflammatory and an inducer of T helper (Th)1 cells. In contrast, it has also been described as an anti-inflammatory cytokine in that it suppresses pro-inflammatory Th17 cells and induces anti-inflammatory IL-10 producing T regulatory (Tr)1 cells. While the majority of studies have been focused on the effects of IL-27 on T cells, human antigen-presenting cells express high levels of the IL-27 receptor \textit{ex vivo}, in addition to being the major producer of IL-27. We report here that human monocytes are repressed by endogenous IL-27, in that the addition of an anti-IL-27 neutralizing antibody increases the production of pro-inflammatory cytokines \textit{ex vivo}. We observed that neutralizing monocyte-derived IL-27 leads to increased IL-17A production by CD4+ T cells and a down-regulation of the IL-17 modulating ectonucleotidase CD39 on monocytes. The locus that contains the \textit{IL27} gene has been linked to susceptibility for type 1...
diabetes (T1D). Interestingly, *ex vivo* monocytes from subjects with T1D produce more IL-27 suggesting this upregulation of IL-27 acts as a negative feedback loop to attempt to counterbalance the proinflammatory immune response in the disease state. In summary, we provide evidence that IL-27 is an endogenous regulator of human monocytes and has consequences on CD4+ T cell phenotype, particularly Th17 cells.

**Keywords**

IL-27; Monocytes; T1D; Th17 cells; Autoimmunity

1. **Introduction**

Antigen presenting cells (APCs), and specifically monocytes appear to be the major producers of IL-27 [1]. IL-27 is a heterodimeric cytokine composed of two subunits, the IL-27p28 and Epstein-Barr virus-induced gene 3 (EBI3) chains [1]. Consistent with IL-27 being a pleiotropic cytokine, many different cell types express the receptor [2], including the majority of human circulating monocytes [3]. The IL-27 receptor (IL-27R) is a heterodimer composed of the IL-27Ra (WSX-1/TCCR) subunit and the gp130 subunit [2]. IL-27Ra is unique for the binding of IL-27, while gp130 is shared with a number of other cytokines including IL-6 [4].

Genome Wide Association Studies (GWAS) have shown linkage of the locus that contains the *IL27* gene to type 1 diabetes (T1D) [5], however, the mechanism by which IL-27 may influence disease course in humans has not been elucidated. Examining the role of IL-27 in mouse models of autoimmunity has shown IL-27 to function as both a pro-inflammatory [6,7] and immunomodulatory cytokine [8-10], in agreement with its known pleiotropic functions and widely distributed receptor expression [2]. Extensive analyses in mouse models of autoimmunity have shown that IL-27 can be a potent immunosuppressive cytokine that inhibits development of pathogenic T helper (Th)17 cells and induces IL-21 and IL-10 producing Tr1 cells [8,11,12]. In diabetic mouse studies, the results have been conflicting with two reports suggesting IL-27 has a pathogenic effect [13,14], and one study suggesting it is beneficial [15].

IL-27 upregulates the expression of CD39, an immunoregulatory ectonucleotidase that hydrolyzes ATP and ADP to AMP, in mouse dendritic cells [16]. It has also been shown that human monocytes and monocyte-derived cells respond to exogenous IL-27, by inducing pro-inflammatory cytokines [2,17]. In other contradictory studies, it was shown that while pre-treatment with exogenous IL-27 augments toll-like receptor (TLR) induced cytokine production [18], it also suppressed TNF-α and IL-1β induced cytokines [19]. Here, rather than examining the effect of exogenous IL-27, we examined the effect of endogenously-produced IL-27 on human monocytes.
2. Materials and methods

2.1. Study subjects

Peripheral venous blood was obtained in compliance with protocols approved by the Institutional Review Boards of Partners Healthcare or the Joslin Diabetes Center. The PhenoGenetic Project recruited healthy subjects 18 to 50 years old who are free of known inflammatory and chronic infectious diseases. This living tissue bank consists of healthy subjects from the general population of Boston, MA. The median age was 24, with 62.7% of subjects being women. For our experiments comparing these control subjects with those with T1D, peripheral venous blood was obtained from 20 recent-onset (< 1 year from disease onset) T1D subjects (mean age +/− SD, 26.3 ± 12.0 years; 35% female), 22 long-term (> 1 year from disease onset) T1D subjects (mean age +/− SD, 26.5 ± 5.8 years; 50% female), and 32 healthy subjects (mean age +/− SD, 26.7 ± 7.1 years; 55% female) from the Joslin Diabetes Center and PhenoGenetic Project. Peripheral blood mononuclear cells (PBMCs) were separated using density centrifugation on Ficoll-Hypaque (GE Healthcare). PBMCs were frozen at a concentration of 1–3 × 10^6/mL in 10% DMSO (Sigma-Aldrich)/90% FCS (Atlanta Biologicals). After thawing the PBMCs were washed in PBS.

2.2. Neutralization of endogenous IL-27

Monocytes were isolated from PBMCs by negative selection (Miltenyi Biotec). 5 × 10^4 monocytes/well were incubated with or without 10 μg/mL neutralizing anti-IL-27 antibody (R&D Systems or Shenandoah Biotechnology) for 40 h in 96-well polypropylene plates (Corning, 3879) in HL-1 medium supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin, 0.1 mM each nonessential amino acids, 1 mM sodium pyruvate (all from Lonza), and 1% heat-inactivated human male AB serum (Omega Scientific) at 37 °C/5% CO₂. Supernatants were examined for secreted cytokines using the Luminex bead-based assay (Millipore). The mean fluorescence intensity (MFI) of CD39 surface expression was measured at 24 h by flow cytometry using an anti-CD39 antibody (Biolegend). For gene expression analysis, 1 × 10^5 monocytes were incubated with or without 10 μg/mL neutralizing anti-IL-27, 10 μM BMS-345541 (Sigma Aldrich) or isotype antibody (R&D Systems). For the time-course experiments, the monocytes were also incubated with or without the addition of 0.5 μg/mL IL-1Ra (R&D Systems) for 4, 8 or 24 h in 96-well polypropylene plates. RNA was isolated using the RNAeasy kit (Agilent Technologies, Palo Alto, CA) and converted to cDNA via reverse transcriptase by random hexamers and Multiscribe reverse transcriptase (Life Technologies, Foster City, CA). The primers used for this study were purchased from Life Technologies. The values are represented as the difference in Ct values normalized to β2-microglobulin for each sample as per the following formula: relative RNA expression = (2^{−ΔΔCt}) × 10^{3}. Five samples were analyzed using a custom nanostring codeset [20] (NanoString Technologies).

2.3. Pre-treatment with anti-IL-27/rIL-27

1 × 10^5 monocytes/well were incubated with either 10 μg/mL neutralizing anti-IL-27 antibody, or 25/100 ng/mL recombinant IL-27 (R&D Systems) for 16 h in supplemented
HL-1 media at 37 °C/5% CO₂ and then activated with 10 ng/mL LPS (Sigma) for 4 h in 96-well polypropylene plates. Gene expression was analyzed using real-time PCR.

2.4. T cell/monocyte co-culture assays

Monocytes and CD4+ T cells were isolated from PBMCs by negative selection (Miltenyi Biotec). T cells were cultured with monocytes (1:1) in the presence of 1 μg/mL plate-bound anti-CD3 Ab (OKT3, BD Bioscience) in complete HL-1 medium and 5% human serum for 5 days with or without neutralizing anti-IL-27 antibody in polystyrene plates (Corning, 3799). Alternatively, the monocytes were pre-incubated with anti-IL-27 or anti-CD39 for 2 h, washed and then incubated with the CD4+ T cells. After 5 days, the T cells were transferred to a new 96-well plate and incubated with rIL-2 (20 U/mL) for an additional 7 days. The T cells were then stimulated with PMA (50 ng/mL) and ionomycin (250 ng/mL) (Sigma Aldrich) in the presence of GolgiStop (BD Biosciences) for 3 h. Cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 1% FBS and 0.1% saponin. The cells were then stained with allophycocyanin-IL-17A (eBioscience). The data were acquired on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

2.5. IL-27 protein and RNA measurements

5 × 10⁴ monocytes/well were incubated in the presence of Golgistop (BD Bioscience) for 16 h in 96-well polypropylene plates at 37 °C/5% CO₂. Cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 1% FBS and 0.1% saponin. The monocytes were then stained with PE-anti-IL-27 antibody (Abcam). The data were acquired on a FACSCalibur machine and analyzed with FlowJo software. To measure IL-27p28 and EBI3 mRNA levels, 1 × 10⁵ monocytes were re-suspended in RNA lysis buffer immediately after monocyte isolation.

2.6. Statistics

Significant differences were calculated with Prism 5.0 software (GraphPad) using standard paired two-tailed t-tests or one-way ANOVA. The Nanostring data was analyzed using a t-test as well. To correct for potential batch effects we have also applied an Empirical Bayes priors distribution estimation framework (Combat v. 2.0) [21], as implemented in Gene Pattern [22] using a parametric method.

3. Results

3.1. IL-27 acts as an endogenous repressor of monocytes

As monocytes are known to be a major producer of IL-27 [1], we examined the effect of endogenously produced IL-27 on human monocytes. We found that blocking IL-27 using a neutralizing anti-IL-27 antibody (R&D Systems) resulted in increased production of a panel of cytokines from negatively-purified, non-stimulated monocytes (Fig. 1A-H), while antibodies blocking GM-CSF and IL-1 receptor antagonist (IL-1Ra) as well as the addition of recombinant IL-1Ra itself had little effect on cytokine production from the resting monocytes (Supplemental Fig. 1A-G) at 40 h. Indeed, blocking IL-27 significantly increased monocyte protein production of GM-CSF, IL-1α, IL-1β, IL-6, MIP-1β, and TNF-α, while marginally reducing IL-1Ra, the naturally produced inhibitor of IL-1, as
measured by a bead-based Luminex assay. Kinetic studies demonstrated that by four hours of treatment, *IL6* and *IL1β* mRNA levels have peaked in response to neutralization of IL-27 (Fig. II, J). We confirmed the increase of pro-inflammatory cytokine mRNA production in response to IL-27 neutralization with an additional IL-27 blocking antibody (Shenandoah Biotechnology) (Supplemental Fig. 2A-C). We conclude from this data that IL-27 functions as an endogenous constitutive repressor in the steady-state.

Surprisingly, IL-10 protein production was also significantly increased with neutralization of IL-27 at 40 h (Fig. 1H). We hypothesize that the increase in IL-10 production is a secondary effect occurring in response to the increase of the pro-inflammatory cytokines, not in response to the blockade of IL-27 signaling directly, and indeed we found that the kinetics of *IL10* mRNA up-regulation was delayed in comparison to *IL6* and *IL1β*, and was not significantly increased at 4 h with neutralization of IL-27 (Fig. 1K). The addition of IL-1Ra was able to reverse some of the effect of blocking IL-27, reducing the mRNA expression of *IL1β* and *IL6*, and reverting the expression level of *IL10* to the untreated levels (Fig. II-K), suggesting that the upregulation of IL-10 is secondary to induction of pro-inflammatory cytokines.

To investigate the mechanisms that mediate the effects of endogenous, monocyte-derived IL-27 on monocytes, we utilized a nanostring codeset of 386 genes, designed for examining activation pathways of innate immune cells [20]. We identified 45 genes that are significantly up-regulated with neutralization of IL-27 and two genes that are downregulated (Supplemental Fig. 3A). We confirmed our findings of increased inflammatory cytokines like *IL1β* and *IL6* mRNA expression with neutralization of IL-27. We found that inhibition of endogenous IL-27 led to the increase of a number of IκB/NFκB pathway members, such as NFKB1. Therefore, we inhibited the pathway using BMS-345541, an IκB kinase inhibitor, and found that the addition of the inhibitor blocked the anti-IL-27 increase in *IL1β* and *IL6* expression (Supplemental Fig. 3B,C).

### 3.2. IL-27 decreases IL-17A production

To further explore the influence of IL-27 on innate immune responses, monocytes were pre-treated with either neutralizing antibody or recombinant IL-27 protein followed by LPS stimulation. We found that pre-treatment with neutralizing antibody enhanced mRNA production of cytokines that support Th17 differentiation, *IL6* and *IL23p19*, while reducing the anti-Th17 cytokine, *IL27*, measured by quantitative PCR. Pre-treatment with recombinant IL-27 protein enhanced the Th1 and IL-21 inducing cytokines, *IL12p35* and *IL27*mRNA. The enhancement of IL-27 expression following pre-treatment with recombinant IL-27 protein suggests a positive feedback loop (Fig. 2A-D).

It has been shown that recombinant IL-27 directly reduces IL-17A production from purified T cells as well as expression of the Th17 associated transcription factor RORc/gt [23,24]. Our data indicate that monocytes deprived of endogenous IL-27 produce more Th17-polarizing cytokines upon LPS stimulation. To examine the influence of endogenously-produced IL-27 on monocyte-induced T cell polarization, CD4+ T cells were co-cultured with monocytes from allogenic subjects in the presence of anti-CD3, with or without neutralizing antibody to IL-27. Consistent with the published data showing the influence
of exogenous IL-27 on purified T cells [23,24], neutralization of monocyte-derived IL-27 results in an increased frequency of IL-17A-producing cells. We found the frequency of both IL-17A+ single-positive and IL-17A + IFNγ+ double positive T cells increased with neutralization of IL-27, while the frequency of IFNγ+ single-positive cells was unchanged (Fig. 3A-C). The increase of IL-17A+ T cells was confirmed with an additional neutralizing antibody (Supplemental Fig. 4). Both the T cells and the monocytes express IL-27R, therefore the neutralization of IL-27 prevents signaling in both the T cell and the monocyte, suggesting two different and complimentary mechanisms of IL-17A modulation; directly on the T cells and through modulation of monocyte cytokine production. To address this, monocytes were pre-treated with the IL-27 neutralizing antibody, washed and then cultured with CD4+ T cells. Monocytes deprived of IL-27 were able to increase the frequency of IL-17A producing T cells independent of the effect of IL-27 directly on the T cells (Fig. 3D), consistent with our finding that pre-treatment of monocytes with anti-IL-27 increased pro-Th17 cytokines upon activation (Fig. 2A, B).

IL-27, in addition to polarizing mouse dendritic cells away from a pro-Th17 phenotype, was also shown to upregulate the expression of CD39, and pretreatment of CD39-deficient mouse dendritic cells with IL-27 is unable to decrease IL-17A production [16]. Therefore, we examined the effect of neutralizing IL-27 on monocyte expression of CD39. Blocking the effect of IL-27 on monocytes led to a modest, but significant decrease in CD39 surface expression at 24 h and mRNA expression at 4 h after treatment (Fig. 4A, B). Since CD39 expression is critical for the phenotype of IL-27 conditioned mouse dendritic cells, we hypothesized that reduced human monocyte CD39 expression may be part of the mechanism for increased IL-17A T cell production upon blockade of monocyte-produced IL-27. Co-culture of T cells with monocytes that had been pre-incubated with neutralizing anti-CD39 antibody led to an increased frequency of IL-17A+ T cells, similar to the increase found with neutralization of IL-27 (Fig. 4C).

**Monocytes from Type 1 Diabetic Subjects Produce More IL-27.**

Monocytes from T1D subjects have an activated phenotype [25-27], and activation of monocytes induces IL-27 expression [28], therefore it is also possible that monocytes from T1D subjects are making more IL-27, as they do pro-inflammatory cytokines such as IL-1β [26,27]. We found that a higher frequency of unstimulated monocytes from recent-onset T1D subjects are producing IL-27 than monocytes from long-term T1D subjects or healthy control subjects (Fig. 5A), consistent with previous reports of increased serum levels of IL-27 in patients with T1D [29,30]. We also compared the gene expression of both chains of IL-27 from ex vivo monocytes from T1D subjects and healthy control subjects. The expression levels of *IL27p28* was increased in the recent-onset T1D subjects compared to the long-term T1D subjects and the healthy control subjects (Fig. 5B), while *EBI3* was unchanged (Fig. 5C). Finally, we also examined the response of the monocytes from T1D subjects to inhibition of endogenously-produced IL-27. The monocytes from T1D subjects had a trend of a reduced response to inhibition of endogenous IL-27 compared to monocytes from healthy control subjects, with the exception of IL-1α and MIP-1β, which were both significantly reduced (below 0.0007 with Bonferroni correction) in response to inhibition of IL-27 (Supplemental Fig. 5). This is consistent with the concept that monocytes from
individuals with T1D are in a slightly activated state ex vivo, as it is known that stimulation such as LPS induces IL-27 production [1] and we found that LPS stimulated monocytes did not respond to the inhibition of IL-27 as ex vivo monocytes did. (data not shown).

4. Discussion

The importance of IL-27’s role in attenuating autoimmunity, specifically by promoting regulatory Tr1 and suppressing Th17 cells has been identified [9,16], however IL-27 has also been shown to promote autoimmunity through modulation of Th1 and CD4+ Foxp3+ regulatory T cells populations [7,14,31] in murine models. This evidence led to the classification of IL-27 as having both pro-inflammatory and immune-regulatory functions. With the exception of a few studies [16,32] the focus of these murine reports has been on the response of T cells to IL-27, not innate immune cells, such as monocytes. However, human monocytes are not only major producers of IL-27, but also act as IL-27 responder cells as they express both chains of the receptor [2,3] [33].

While a number of studies have examined the response of primary human monocytes and the THP-1 monocytic cell line to recombinant IL-27, finding both a pro-inflammatory role increasing cytokines such as IL-1β, IL-6 and TNF-α 2,17,33 as well as an immune-regulatory role inducing IL-1Ra, there is little known about the response of monocytes to endogenous monocyte-produced IL-27. Here the ex vivo monocyte response to endogenous IL-27 was examined using a neutralizing antibody. Of note, polypropylene plates were used to minimize plastic adherence-induced activation of the monocytes while in culture. Our findings suggest a role for monocyte-derived IL-27 as a constitutive suppressor of pro-inflammatory responses from ex vivo monocytes, keeping the monocytes in a quiescent state.

Since IL-27 is a known inducer of IL-10,23 our finding of increased IL-10 protein production at 40 h upon neutralization of IL-27 was perhaps surprising. However, our kinetic studies demonstrate that the upregulation of IL10 mRNA is not observed at four hours as is the case with IL1β and IL6. The expression of IL10 is increased at eight hours, suggesting that the induction of its expression could be secondary to the induction of the pro-inflammatory cytokines. IL-10 induction is normally delayed compared to the pro-inflammatory cytokines after monocyte activation with stimuli such as LPS, as it works as a negative feedback loop, reducing the production of the pro-inflammatory cytokines.34 When we add IL-1Ra, an inhibitor of IL-1 signaling, we find that neutralization of IL-27 is unable to increase IL10 mRNA at any of the time points measured, consistent with it being induced secondarily to the pro-inflammatory cytokines.

The priming effects of exogenous IL-27 on monocytes have been previously measured. It has been shown that pre-treatment of monocyte-derived macrophages with IL-27 before TLR stimulation lead to enhanced production of pro-inflammatory cytokines such as TNF-α and IL-6.19,35 We also examined the effect of pre-treatment of monocytes with neutralizing antibody or recombinant IL-27, focusing on cytokines which are important for Th17 and Th1 differentiation. Contrary to the published findings, we found that production of IL6 was not increased with recombinant IL-27 pre-treatment, but was in fact increased with priming.
by IL-27 neutralizing antibody. It is unclear what experimental differences account for the variation, possibly our use of polypropylene plates, which reduces background activation, or that we are examining monocytes while the published papers used monocyte-derived macrophages.

We also examined the influence of monocyte-produced IL-27 on the production of IL-17A and IFNγ from CD4+ T cells. As predicted, we found that neutralization of monocyte-derived IL-27 led to an increased frequency of IL-17 producing CD4+ T cells, both IL-17A + IFNγ+ and IL-17A + IFNγ−. However, we saw no difference in the frequency of IL-17A-INFγ+ T cells with neutralization of IL-27. This finding in our co-culture system examining naturally produced IL-27 differs from a published finding using recombinant IL-27 on purified human T cells, which found increased IFNγ production, upon stimulation with IL-27.23 Our finding of CD39 expression as part of IL-27’s mechanism to reduce IL-17A is in agreement with a previous report demonstrating that CD39 was induced by IL-27, and these IL-27 pre-treated dendritic cells induced fewer IL-17A producing T cells after LPS stimulation.16

GWAS studies have implicated the IL27 locus as a susceptibility risk factor for T1D,5 suggesting that it may be an important cytokine for T1D. Therefore, we examined the frequency of IL-27 + monocytes in recent-onset T1D patients and long-term T1D patients compared to age- and sex-matched healthy control subjects at both the protein and RNA levels and found an increase in IL-27 production in the recent-onset T1D subjects. This is consistent with previous reports that found increased levels of IL-27 in serum from individuals with T1D.29,30 We had previously found that there is an increase in the frequency of IL-6 and IL-1β positive monocytes in individuals with T1D.26 This shows the complexity of monocyte cytokine production in terms of Th17 cell differentiation in T1D, an increase of two IL-17 inducing cytokines and one IL-17 inhibitory cytokine.

Our previous study demonstrated an increase of IL-17 positive T cells in the periphery of individuals with long-standing T1D26 and another correlation study showed an increase of IL-17+ β-cell–specific autoreactive CD4+ T cells in individuals with T1D.36 The role of IL-17 has also been investigated in mouse models of T1D. Th17 cells appear to only induce T1D once they convert to INF-γ producing Th1 cell in murine models.37,38 We found that pre-treatment of human monocytes with recombinant IL-27 led to an increase of the Th1 inducing cytokine IL-12 upon activation of the monocytes. This could be a potential function of IL-27 producing monocytes in T1D.

In conclusion, our report examined the endogenous function of monocyte-derived IL-27 in human monocytes and found that IL-27 behaves as a constitutive suppressor, limiting pro-inflammatory cytokine production. It also modifies the ability of monocytes to induce differentiation of CD4+ T cells to the Th17 phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
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Fig. 1.
Neutralization of endogenous IL-27 leads to increased cytokine production. (A-H)
Negatively isolated monocytes from healthy subjects were incubated with an IL-27 neutralizing antibody for 40 h. Supernatants were collected and cytokine production was measured using the Luminex platform. (I–K) IL1β, IL6 and IL10 mRNA was analyzed at 4, 8 and 24 h after incubation with the neutralization antibody with or without the addition of IL-1Ra. Each individual is represented as a symbol. *p < .05; **p < .01; ***p < .001.
Fig. 2.
Neutralization of endogenous IL-27 leads to increased pro-Th17 cytokines. (A-D) Ex vivo monocytes were pre-treated with either IL-27 neutralizing antibody or recombinant IL-27 protein for 16 h and then activated with LPS for 4 h. Relative expression of IL6, IL23p19, IL27p28 and IL12p35 were measured by Taqman PCR. Each dot represents an individual. **p < .01; ***p < .001.
Fig. 3.
Neutralization of endogenous IL-27 leads to increased CD4+ IL-17+ T cells. (A-C) The frequency of (A) IL-17+ IFNγ− and (B) IL-17+ IFNγ+, but not (C) IL-17-IFNγ+ T cells is increased after incubation with an anti-IL-27 antibody in the presence of monocytes. (D) The frequency of IL-17 positive T cells is increased when incubated with monocytes that were pre-treated with the anti-IL-27 antibody compared to untreated monocytes. *p < .05; **p < .01; ***p < .001.
Fig. 4. Neutralization of IL-27 leads to a reduction of CD39 (ENTPD1) expression. (A) The geometric mean of CD39 expression is reduced on monocytes after incubation with anti-IL-27 for 24 h, as determined by flow cytometry. (B) Monocyte ENTPD1 gene expression is reduced after 4 h of incubation with anti-IL-27. (C) The frequency of IL-17 positive T cells is increased when incubated with monocytes that were pre-treated with anti-CD39 compared to untreated monocytes. Each dot represents an individual.
Fig. 5.
Monocytes from subjects with recent-onset type 1 diabetes produce more IL-27. (A) The frequency of IL-27 positive monocytes was measured ex vivo using flow cytometry. The frequency of IL-27 positive monocytes is increased in subjects with recent-onset T1D compared to subjects with long-term T1D and healthy controls. (B, C) Gene expression of both chains of IL27 were measured by taqman PCR, only IL27p28 was increased in monocytes from recent-onset T1D subjects compared to healthy subjects.