Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members

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Abstract

IL-35 is a cytokine of the IL-12 family, existing as a heterodimer of IL-12p35 and Ebi3. IL-35 has anti-inflammatory properties and is produced by regulatory T cells in humans and mice, where it is required for optimal suppression of immune responses. Distinct from other IL-12 cytokines, the expression of IL-35 has not been described in antigen presenting cells. In view of IL-35 immune-regulatory properties, we investigated the expression, regulation and function of IL-12p35 and Ebi3 in human monocyte derived dendritic cells (moDCs) and tolerogenic DCs (tolDCs). These tolDCs did not produce IL-12p70 or the homodimer IL-12p40. We demonstrate that tolDCs completely lack transcriptional expression of IL-12p40. However, tolDCs maintain mRNA expression of IL-12p35 and Ebi3. Using intracellular flow cytometry and western blot analysis we show that tolDCs produce Ebi3 and IL-12p35, and both can be enhanced upon stimulation with IFN-γ, LPS or CD40L. tolDCs supernatant has the capacity to suppress T-cell activation. Using *IL12A* silencing we demonstrate that IL-12p35 is required for tolDCs to reach their full suppressive potential. Taken together, our results indicate that tolDCs produce IL-35, providing an additional novel mechanism by which tolDCs elicit their tolerogenic potential.

Introduction

Dendritic cells (DCs) represent a heterogeneous family of professional APCs that have many functions in both the initiation and maintenance of immunity and immunological tolerance [1, 2]. Several factors, including IL-10, TGF-β, Vitamin D3 and corticosteroids have been shown to modulate DC function in vitro and in vivo, and lead to the generation of tolerogenic DCs (tolDC) [3, 4]. These tolDCs represent an altered status in DC differentiation and are currently considered in the design of therapeutic strategies to treat

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patients suffering from autoimmune disease [5] and those undergoing transplantation [6, 7]. Impaired expression of IL-12 in tolDCs is thought to be a crucial requirement for these cells to elicit their tolerogenic potential, and indeed many tolDC populations have been shown to lack IL-12 [4, 8]. However the IL-12 family is ever expanding and little is known about the regulation and expression of other IL-12 family members in tolDCs.

The IL-12 family is composed of four heterodimeric complexes each consisting of an alpha chain (IL-12p35, IL-23p19 and IL-27p28) and a beta chain (Ebi3 and IL-12p40) [9, 10]. The IL-12p40 subunit can pair with either IL-12p35 or IL-23p19 to yield IL-12 and IL-23 respectively [11]. The second beta chain subunit Ebi3, can pair with 2 other alpha chain subunits, IL-27p28 and IL-12p35, to yield IL-27 and IL-35 respectively. Although there is a large degree of chain sharing within this family, each cytokine possesses a unique and distinct function. IL-12 and IL-23 have primarily pro-inflammatory properties and play an important role in the development of Th1 and Th17 T helper cell subsets respectively. IL-27 possess dual roles playing a protective role in tumour immunity[12, 13], but as an immunoregulatory cytokine can induce Tr1 cells [14] and lead to the generation of a tolerogenic DC expressing elevated levels of CD39 [15]. The most recently characterised member of this family is IL-35, a potent anti-inflammatory cytokine, which has been shown to be required for the optimal suppressive activity of regulatory T-cell populations in both mice and man [16-18]. DCs are known producers of three family members, IL-12, IL-23 and IL-27. In tolDCs little is known about the expression of these family members aside from the characteristic loss of IL-12 production and limited transcriptional data on IL-23 and IL-27. For IL-35, production by myeloid cells in human or mouse has not yet been demonstrated.

In this study we addressed the expression profile and regulation of all IL-12 family members in human DC and tolDCs with a particular emphasis on the subunits which combined make up IL-35. We show that tolDCs did not produce detectable levels of bioactive IL-12p70, the homodimer IL-12p40 or express IL-12p40 transcripts. However tolDCs maintain mRNA and protein expression of IL-12p35 and Ebi3. We show that supernatants of tolDCs are sufficient to suppress T-cell proliferation, and that supernatants of cells silenced for IL-12p35 are hampered in their suppressive potential, thereby implying a role for IL-35 in tolDC function.

Results

Dexamethasone treated DCs display typical features of tolDCs with low CD86 but high B7H1 and B7DC

We have previously demonstrated that Dex treated DCs display reduced levels of costimulatory molecules [8]. To further investigate the phenotypical characteristics of DC and tolDC, freshly isolated monocytes were cultured in the presence of GM-CSF and IL-4, with the addition of Dexamethasone to generate tolDC. In an immature state both DCs expressed comparable levels of CD86 and immunoregulatory molecules B7H1 (CD274) and ILT3 (CD85k), while HLA-DR and B7DC (CD273) were more abundantly expressed on tolDCs (Fig.1). Stimulation using IFN-γ, LPS or PGN resulted in the generation of mature DCs as shown by increased expression of costimulatory molecules including CD86 and

HLA-DR (Fig.1A,B). tolDCs were significantly impeded in their ability to upregulate CD86 or HLA-DR upon stimulation with any agents used (Fig.1A,B). In contrast tolDCs upregulated B7H1 (Fig.1C) and to a lesser degree B7DC and ILT3 (Fig.1D,E).

To fully ascertain the allostimulatory capabilities of tolDCs, immature and mature DC/tolDCs were co-cultured with allogenic CD4^{+} T lymphocytes, after which the T cells were analysed for CFSE dilution (Fig.1F). DCs were potent inducers of T-cell proliferation which was further enhanced when the DCs were first matured for 24 hours with LPS or PGN. Conversely tolDCs were hampered in their ability to stimulate allogenic T cells regardless of maturation stimulus used (Fig.1G). In line with the proliferation data DCs induced a strong IFN-γ production by the T cells, whereas tolDCs induced almost negligible amounts of IFN-γ production (Fig.1H). In summary the tolDCs used in this study display typical characteristics of myeloid tolerogenic DCs.

TolDC maintain expression of IL-12p35 in the absence of IL-12p40

A key characteristic of tolDCs is their ability to preserve production of immunoregulatory cytokines while maintaining low to absent production of inflammatory cytokines. Upon activation, DCs showed strong production of IL-12p40 and IL-12p70, whereas these cytokines were completely absent in supernatants of activate tolDCs (Fig.2A,B).

In contrast, tolDCs produced significantly higher levels of IL-10 upon stimulation as compared to DC (Fig.2C). To further explore the expression of IL-12 in DCs and tolDCs we assessed by RT-PCR the expression of *IL12A* (IL-12p35) and *IL12B* (IL-12p40), together making IL-12p70. In line with our ELISA data DCs showed a strong *IL12B* expression upon stimulation while tolDCs were greatly inhibited in this ability (Fig.2D). However, tolDCs maintained their expression of *IL12A* and expressed significantly higher basal levels compared to DC (Fig.2E). Upon stimulation, where *IL12B* remains absent (Fig.2D), *IL12A* expression is significantly increased in tolDCs to comparable and even higher levels than DC (Fig.2E).

Differential kinetics of LPS induced IL-12 family members in DC and tolDC

The IL-12 family is composed of 4 related yet distinct heterodimeric cytokines and chain sharing has become a quintessential feature of this cytokine family [19, 20]. We demonstrated that tolDCs lack expression of *IL12B* (IL-12p40) yet maintain expression of *IL12A* (IL-12p35) (Fig.2). We investigated the expression of all family members in immature unstimulated DC and tolDCs and found that although tolDCs expressed lower levels of *IL12B* compared to DC, they maintained expression of all other family members and *IL10* (Fig.3A). Notably both *IL27B* (Ebi3) and *IL12A* expression was even higher in tolDC compared to DC.

We further investigated the regulation of the IL-12 family in DC and tolDC in LPS triggered cells. We found that DCs displayed a pronounced upregulation of *IL12A* and *IL27A* (IL-27p28) with the expression of each subunit peaking at 6 hours (Fig.3C,F). This was distinct from *IL10* and *IL23A* (IL-23p19) which showed the greatest expression at 2 hours

(Fig.3G,E), and *IL27B* which gradually increased over time and reached its highest level at 48 hours (Fig.3D).

While *IL12B* was rapidly induced in DCs, expression remained very low in tolDCs (Fig.3B). tolDCs demonstrated only a minor increase in *IL12A* and *IL27B* despite possessing up to 100 fold higher basal level of expression compared to DC (Fig.3C,D). *IL23A* expression was also higher in tolDC at time 0 but gradually decreased over time before returning to the initial expression level (Fig.3E). *IL27A* expression was comparable between the two cell types up until 6 hours, after which a drop off was noted for tolDC (Fig.3F).

Finally although *IL10* started off with a higher level of expression in tolDC the pattern of upregulation was comparable between the two cell types with a transient upregulation at 2 hours followed by a gradual decrease over time (Fig.3G). The relative abundance of both *IL27B* and *IL12A* transcripts in unstimulated tolDC compared to DC, in contrast to other IL-12 family subunits, is intriguing and may indicate some inherent regulation of these subunits within tolerogenic DCs.

Stimulation of tolDCs favours upregulation of the immunoregulatory chains IL-12p35, Ebi3 and IL-27p28

Although tolDCs specifically maintained the expression of subunits involved in immunoregulatory processes (Fig.3), they remained relatively refractory to LPS stimulation in terms of IL-12 family expression. We investigated if this profile was intrinsic for these cells or activation dependent.

We observed that although DCs expressed relatively abundant levels of *IL12B*, particularly upon stimulation with LPS, IFN-γ+LPS or Poly I:C, transcripts of this subunit were significantly reduced in tolDC (Fig.4A).

We demonstrate that *IL12A* is maintained in tolDCs and expressed comparably to DC across the different stimuli used. Notably treatment with a combination of IFN-γ+LPS yielded the highest *IL12A* expression in tolDCs (Fig.4B). IFN-γ, and a combination of IFN-γ+LPS also displayed the greatest increase in *IL27B* expression when comparing DC to tolDCs (Fig.4C), with CD40 ligation showing the next highest expression in tolDC.

IL27A levels were comparable between both DCs but IFN-γ yielded the strongest induction of *IL27A* transcripts in tolDCs (Fig.4D). In summary tolDCs do not express *IL12B* but do maintain expression of *IL12A, IL27A* and *IL27B* which can be further induced upon activation with IFN-γ, IFN-γ+LPS and CD40 ligation.

TolDC produce low levels of IL-27 but maintain production of IL-35 related chains

We investigated if our observations regarding transcriptional expression of IL-27p28, Ebi3 and IL-12p35 were translated into protein by assessing production in DC and tolDCs. Using an IL-27 ELISA we found that DCs, but not tolDCs, produce IL-27 upon IFN-γ+LPS stimulation, although we did find considerable variation across the 6 donors analysed (Fig. 5A). Notably a combination of IFN-γ+LPS or CD40L, with either IFN-γ or LPS, was the best inducers of IL-27 production in DCs while the individual stimuli alone were poor

inducers (Fig.5B). Under all conditions tolDCs were significantly hampered in their IL-27 production (Fig.5B).

Ebi3 and IL-12p35 can dimerise to form IL-35; however there are no reliable methods for the detection of IL-35 in human cellular supernatants. We established by intracellular fluorescent microscopy that tolDCs do express the individual chains, Ebi3 and IL-12p35, and that production of both can be increased upon stimulation with IFN-γ or IFN-γ+LPS (Fig.5C). To quantify this, we measured Ebi3 and IL-12p35 by intracellular flow cytometric staining in both DC and tolDC. We found that although tolDCs had lower basal levels of Ebi3 compared to DC, they showed a stronger induction upon stimulation (Fig.5D). The relative increase in Ebi3 expression upon stimulation with IFN-γ or IFN-γ+LPS in tolDCs was 6 fold or higher (Fig.5D). Although tolDC have a lower basal level of IL-12p35 compared to DC, they increase expression comparably to DC upon stimulation with IFN-γ or IFN-γ+LPS (Fig.5E). To further reinstate our findings we performed a western blot for IL-12p35 and Ebi3 on whole cell lysates. In line with our flow cytometry and microscopy data, tolDCs do express both IL-12p35 and Ebi3, which appears to increase upon stimulation. Importantly, in contrast to DC, tolDC do not express IL-12p40 using any stimuli assessed (Fig.5F). Together this suggests that tolDCs may be more likely to produce IL-35 due to the lack of competition between Ebi3 and IL-12p40 for available IL-12p35.

TolDCs require expression of IL-12p35 to elicit their full tolerogenic potential

To assess the possible secretion of IL-35 by tolDC, we investigated the suppressive activity of tolDC supernatants on αCD3/CD28 activated T cells. Supernatants from CD40 ligated tolDCs demonstrated a pronounced inhibition of T-cell proliferation which was not observed with mock activated tolDCs (Fig.6A). Using different tolDC and T-cell donors, we found that supernatant from CD40L activated tolDC consistently induced suppression ranging from 30-80% (median 60%) (Fig.6B).

The tolDCs used in this study do not express or produce IL-12p40 (Fig.2-5) therefore in our model IL-12p35 but not Ebi3 knockdown could be used as a method to specifically target IL-35 without affecting other IL-12 family members. To establish the role of IL-35 production by tolDC in T-cell suppression, we performed RNA interference of *IL12A*. By mRNA analysis we found at least a tenfold inhibition of *IL12A* expression in activated tolDCs (Fig.6C). Importantly *IL27B* and *IL10* remained unaffected by IL-12A silencing, and use of a non-specific siRNA did not affect *IL12A* expression (Fig.6C). Using supernatants from CD40L stimulated tolDCs we found that *IL12A* silenced supernatants were significantly impaired in their suppressive capacity (Fig.6D,6E), thereby indicating that IL-12p35 is required by tolDCs to elicit their full tolerogenic potential. However, it should be noted that *IL12A* silenced supernatants do retain some of their suppressive potential indicating that other mediators of suppression e.g. IL-10 are also likely to play an important role in this setting.

To confirm that the *IL12A* siRNA directly targeted IL-12p35 protein we demonstrated, in activated control DCs, that IL-12p70 was up to 60% inhibited while IL-12p40 was unaffected (Fig.6F). As a consequence, the capacity of DC supernatant to induce IFN-γ production in activated T cells was significantly reduced (Fig.6G).

Discussion

The IL-12 family is gaining increasing attention as being a key regulator of immunity and immunological tolerance [20, 21]. A common feature of tolerogenic DCs is the lack of IL-12, however little is known about the expression, regulation or function of the other family members in human tolDCs. In this study we focused on the IL-12/IL-35 axis and found that tolDCs maintain, and can increase expression of Ebi3 and IL-12p35, and that IL-12p35 is required for their full tolerogenic potential.

Despite the remarkable degree of chain sharing between the four members of the IL-12 family, each one has its own unique biological function. IL-12 and IL-23 are pro-inflammatory cytokines crucial for the development of Th1 and memory Th17 cells respectively [22, 23]. On the other hand IL-27 and IL-35 are inhibitory and immunoregulatory cytokines [16, 24, 25]. IL-35, although a relatively new player within this family, has already been shown to induce proliferation of iTr35, a regulatory T-cell subset which possesses a profound anti-inflammatory phenotype with the ability to reduce inflammation in animal models of IBD and EAE [16]. With the striking immunoregulatory functions of IL-35 it became evident to us that the possible secretion of IL-35 from tolerogenic DCs could be a new previously unexplored mechanism into how these cells can suppress T-cell responses.

To study this we used a well characterised human tolDC and first established that they displayed typical features associated with regulatory DC populations-low CD86, high B7H1 [26] and poor ability to stimulate allogenic T cells [27]. In addition we observed that these cells express high levels of the endocytic receptor CD206 as well as the immunoregulatory molecules B7DC [28, 29] and ILT3 [30].

Our focus was on the cytokine repertoire of these cells and one of the more intriguing qualities of tolDCs is the profound lack of IL-12p40/70 production regardless of stimulation used. We have shown that tolDCs completely lack IL-12p40 expression yet preserve their transcriptional expression of the other IL-12 family members, in particular those who, upon dimerisation are associated with anti-inflammatory properties, namely Ebi3, IL-12p35 and IL-27p28.

Analysing LPS triggered DCs, we observed that IL-12p35/p40 were transient and superimposable with both subunits reaching their highest level at 6 hours in DCs. A similar peak in expression was noted for IL-27p28, though the increase in expression appears to occur more rapidly than for p40/p35. Conversely Ebi3 does not peak until 24-48 hours post activation in DCs. This differential pattern of expression has been likewise noted in previous studies [31, 32] and has fuelled speculation that Ebi3 may have a unique biological function independent of its typical pairing subunits.

Recent evidence showed that type 2 anti-inflammatory macrophages express high level of Ebi3 [33, 34]. We have also observed elevated Ebi3 levels in M-CSF generated human M2 compared to GM-CSF generated M1 macrophages (unpublished data). Intriguingly, the authors show that intracellular Ebi3 expression can block LPS induced M1 to M2 transition and IL-12p70 production [33], suggesting that Ebi3 may play a role in maintaining the

regulatory functions of M2 macrophages. Further work is needed to fully extrapolate this data to human macrophage subsets.

In our study with dendritic cells we not only observed consistently higher levels of Ebi3 in tolDCs, but also IL-12p35. We observed minimal upregulation when the tolDCs were stimulated with LPS so we addressed whether this was an intrinsic characteristic of these cells or was activation dependent. Notably IFN-γ seemed to have the greatest effect on tolDC in terms of Ebi3/p35/p28 expression with just a moderate increase noted in DCs. This may be explained by the finding that tolDCs express higher levels of IFN-γR1/2 and STAT1 compared to DC (data not shown).

Although IFN-γ is well acknowledged as an inflammatory mediator, in recent years it has also been appreciated as a key coordinator of immunity with many anti-inflammatory molecules also under its control, including B7H1 [35] and IDO [36]. More recently IFN-γ has even been demonstrated to lead to the generation of a tolerogenic DC population in vitro [37]. In fact, Ebi3 and IFN-γ have been demonstrated to be important to limit allograft rejection in mouse, whereby IFN-γ upregulation of Ebi3 in autologous tolerogenic DCs was found to be a crucial mechanism in graft maintainance [38]. It is intriguing that in our current study, using human cells, Ebi3 was also strongly upregulated by IFN-γ in tolDCs.

As described earlier Ebi3 can dimerise with either IL-27p28 or IL-12p35 to yield IL-27 or IL-35 respectively. In view of the expression of IL-27p28 in tolDCs we investigated IL-27 production in both DC and tolDC. IL-27 has been shown to be produced by DCs stimulated with Poly I:C [39] or macrophages stimulated with IFN-γ+LPS [40]. We did not find any striking effect with Poly I:C in our study but did find that IFN-γ+LPS induced a strong IL-27 production in DCs. In addition we show that CD40 ligation alone or in particular when combined with IFN-γ or LPS can also induce IL-27 production. In contrast tolDCs had low levels of IL-27 in their supernatants with only a combination of IFN-γ with either LPS or CD40L showing any detectable levels. This may be explained by the rapid loss of IL-27p28 transcripts in tolDC after 6 hours which we observed using LPS induced kinetics. Further work is required to evaluate whether IL-27p28 transcripts are less stable in tolDC compared to DC or are possibly targeted for degradation. Unfortunately there are no reliable methods to determine IL-35 in cellular supernatants and to date all co-IPs in the literature regarding IL-35 have been conducted using murine cells (both T and B cells) [41, 42]. We have tried extensively using various antibody combinations but were unable to detect any significant signal via western blot. This may be due to the sensitivity of the assay, the combination of antibodies used or the documented difficulty in detecting human IL-35 which others have already commented on [20, 43]. However we did show that tolDCs do express the individual chains required to form IL-35 and hypothesised that tolDCs may be more likely to produce IL-35 due to the lack of competition between Ebi3 and IL-12p40 for available IL-12p35. It is clear that expression alone is not sufficient to conclude that tolDCs will produce bioactive heterodimer therefore we established the functional significance of our findings by performing a T-cell proliferation assay in the presence of supernatants from activated tolDC and *IL12A* silenced tolDC. Using the supernatants from control silenced tolDCs we demonstrated a robust inhibition of T-cell proliferation while supernatants from *IL12A* silenced tolDCs were significantly impaired in this ability, indicating that tolDCs

require IL-12p35 to elicit their full tolerogenic potential. It should be noted that silencing *IL12A* alone is not sufficient to fully reverse the suppressive capabilities of tolDC supernatants indicating that additional soluble mediators may work independently or in concert with IL-35. Considering the abundance of IL-10 in tolDC stimulated supernatants and its stable presence in *IL12A* silenced cells it would seem likely that IL-10 is responsible for some of the remaining suppressive potential of *IL12A* silenced tolDCs.

There has been much speculation as to whether DCs, particularly tolerogenic DCs could produce IL-35 [21]. To date there is little information about the stimuli that drive IL-35 production aside from IL-35 itself, which has been show to generate iTr35 regulatory T cells characterised by IL-35 production. It would be extremely interesting to test whether the generation of DCs in the presence of IL-35 leads to the development of a tolerogenic population which can additionally produce IL-35.

It has been reported that IL-12p35 needs to be part of a heterodimer in order to be secreted [44]. IL-12p35 can heterodimerise with IL-12p40 or Ebi3 and the tolDCs used in this study do not produce IL-12p40. Although we cannot exclude that there may be a yet unidentified additional chain which IL-12p35 may pair with it, it seems likely considering the abundance of IL-12p35 and Ebi3 in tolDCs, that the partial loss of suppressive capacity in IL-12A silenced tolDCs may be a reflection of the loss of IL-35.

Taken together, our results suggest that human tolDCs produce IL-35, an exciting new immunoregulatory cytokine, providing an additional novel mechanism by which these cells can elicit their tolerogenic potential.

Materials and Methods

Generation of human monocyte derived DCs

Human monocytes were isolated from buffy coats and DCs were generated as previously described [45]. TolDCs were generated by addition of Dexamethasone (10^{-6}M Dex) (Pharmacy, LUMC, Leiden, The Netherlands), at the start of culture. On day 6 cells were harvested, washed and stimulated with 200 ng/mL LPS (*E.Coli 0127:B8* Sigma-Aldrich, Zwijndrecht), 20μg/ml Poly I:C, 10 μg/ml PGN (InvivoGen, France) or 100 ng/ml IFN-γ (Peprotech, Germany). CD40L-activation was performed with CD40L transfected L cells (L-CD40L) in a DC: L cell ratio of 5:1. Non-transfected L cells served as control cells.

DC-T cell co culture

Total CD4$^+$ lymphocytes were negatively selected from PBMCs using CD4$^+$ T-cell isolation kit II (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany), and subsequently labelled with carboxyfluorescein diacetate succinamidyl ester (CFSE) (Molecular Probes, Europe BV Leiden, The Netherlands) as described before[10]. Labelled CD4$^+$ T cells were co-cultured with allogeneic DCs at a 10:1 T cell/DC ratio for 5 days. The T cells were then harvested and proliferation was assessed by CFSE dilution.

Surface and Intracellular flow cytometry

For cell surface flow cytometric analysis, cells were stimulated overnight, harvested, washed, and stained for 30 minutes at 4°C in FACS buffer (PBS, 0.5% heat inactivated NHS, 1% BSA, 0.02% NaN_3) with anti-CD86, anti-CD14 MΦ P9 (BD biosciences, San Diego, CA, USA), anti-B7H1, anti B7DC, anti-ILT3 (Biolegend, ITK Diagnostics, the Netherlands), anti- HLADR (B8.11.2, ATCC), anti-Mannose Receptor (clone D547, msIgG1 in house) or anti-DC-SIGN (R&D Systems, Wiesbaden, Germany). Nonconjugated antibodies were detected with PE-conjugated goat-anti-mouse Ig (Dako, Glostrup, Denmark). Isotype matched control antibodies were used to determine the level of background staining. For intracellular staining cells were stimulated overnight, followed by incubation in the presence of Brefeldin-A (Sigma-Aldrich, Zwijndrecht) for a further 5 hours. The cells were then harvested, washed and fixed in PBS containing 4% formaldehyde and 1% heat inactivated FCS, washed with PBS containing 10% heat inactivated FCS and permeabilised with perm buffer (PBS, 0.5% saponin, 1% heat inactivated FCS) for 10 minutes. Cells were stained with anti-Ebi3-allophycocyanin or anti-IL-12p35-allophycocyanin (both R&D Systems, Wiesbaden, Germany) for 30 minutes. allophycocyanin -conjugated isotype matched control antibodies were used to determine the level of background staining. The fluorescence was measured on a FACS Calibur flow cytometer, and data were analysed with FlowJo Software (Tree star, USA).

mRNA isolation, cDNA synthesis, and real-time PCR

mRNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Digestion of genomic DNA was performed by using the on-column RNase-free DNase set. Reverse transcriptase system kit (Promega, Leiden, The Netherlands) was used to synthesise cDNA conforming to the manufacture's instruction and was stored at −20°C for further analysis. Specific primers (Table I) for human *IL12A, IL12B, IL23A, IL27A, IL27B, IL10* and *GADPH* were designed using the computer software Oligo explorer and synthesised at Biolegio. Quantitative PCR was performed using SYBR Green qPCR master mix (Bio-Rad, Veenendaal, The Netherlands). GAPDH was used as the endogenous reference gene. Data analysis was performed using Bio-Rad CFX Manager Software (Bio-Rad). For each sample, the relative abundance of target mRNA was calculated from the obtained C_t values for the target gene and expressed relative to the endogenous reference gene *GAPDH*.

Cytokine production

Cell culture supernatants were harvested after 48 hours and frozen at −80°C until analysis. Subsequently they were tested for the presence of IL-12p70, IL-12p40, (Biolegend) IL-10 (Sanquin, Amsterdam, The Netherlands), IL-27 and IFN-γ (eBioscience) by ELISA according to manufacturer's instructions.

Western Blotting

DCs were stimulated for 16 hours, followed by the addition of Brefeldin A for a further 5 hours. Cells were harvested, washed in ice-cold phosphate-buffered saline and lysed in medium stringency lysis buffer for 30 minutes. The lysates were centrifuged at 13,000 rpm,

the supernatants harvested and protein concentration was determined by a Pierce assay. The samples were diluted in $1\times$ SDS loading buffer, boiled for 5 minutes in reducing conditions, followed by separation on 10% SDS-PAGE gel. Gels were transferred to nitrocellulose membranes, blocked with TBS plus 0.1% Tween-20 (TBS-T) and 3% BSA or milk and probed with indicated primary antibodies; purified anti-IL-12p40, anti-IL-12p35 (Santa Cruz Heidelberg, Germany) or purified anti-Ebi3 (Abnova, Huissen The Netherlands). After incubation with HRP-conjugated secondary antibody, proteins were detected with super signal ECL system. Blots were stripped and β-actin was used as a loading control using HRP conjugated anti-β actin (Abcam, Cambridge, UK).

Immunofluorescence microscopy

Cells were cultured on 8 well chamber slides (NUNC) in the presence of stimuli and Brefeldin-A. The cells were subsequently washed and fixed in PBS containing 4% formaldehyde, 1% heat inactivated FCS followed by washing with PBS containing 10% heat inactivated FCS and permeabilised with perm buffer (PBS, 0.5% saponin, 1% heat inactivated FCS) for 10 minutes. The DCs were then stained using IL-12p35-allophycocyanin or anti-Ebi3 (Abnova), followed by goat anti-mouse-Alexa[488] (Molecular Probes). Hoechst was used for nuclear staining.

RNA interference

DCs were transfected with 50 nM siRNA through the use of the transfection reagent Lipofectamine 2000 (Life Technologies, Bleiswijk, the Netherlands) and were used for experiments 24 hours after transfection. The following SMARTpool siRNAs were used (Dharmacon, Fisher Scientific, Netherlands): IL-12A and nontargeting siRNA as a control. Silencing of expression was verified by real-time PCR in tolDC and IL-12p40/p70 ELISAs in DC.

T cell proliferation assays

Naïve CD4$^+$ T cells were isolated by negative selection using naïve CD4$^+$ T-cell isolation kit II, from cord blood derived PBMCs. Cells were stimulated as described previously [16]. Briefly cells were plated at a density of 25,000/well in 96 well round bottom plates in the presence of αCD3/28 coated beads. Supernatants from stimulated tolDCs were harvested and incubated with the T cells at a dilution of 1:2. T-cell proliferation was assessed by the addition of ^{3}[H]-thymidine (0,5 μCi/well) for the last 8 hours. Percentage suppression was calculated by 1-(cpm T cells + tolDC sup/cpm T cells alone) times 100.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism (Graph Pad Software, San Diego, CA) using a one-tailed t-test. P-values   0.05 were considered statistically significant.

Acknowledgements

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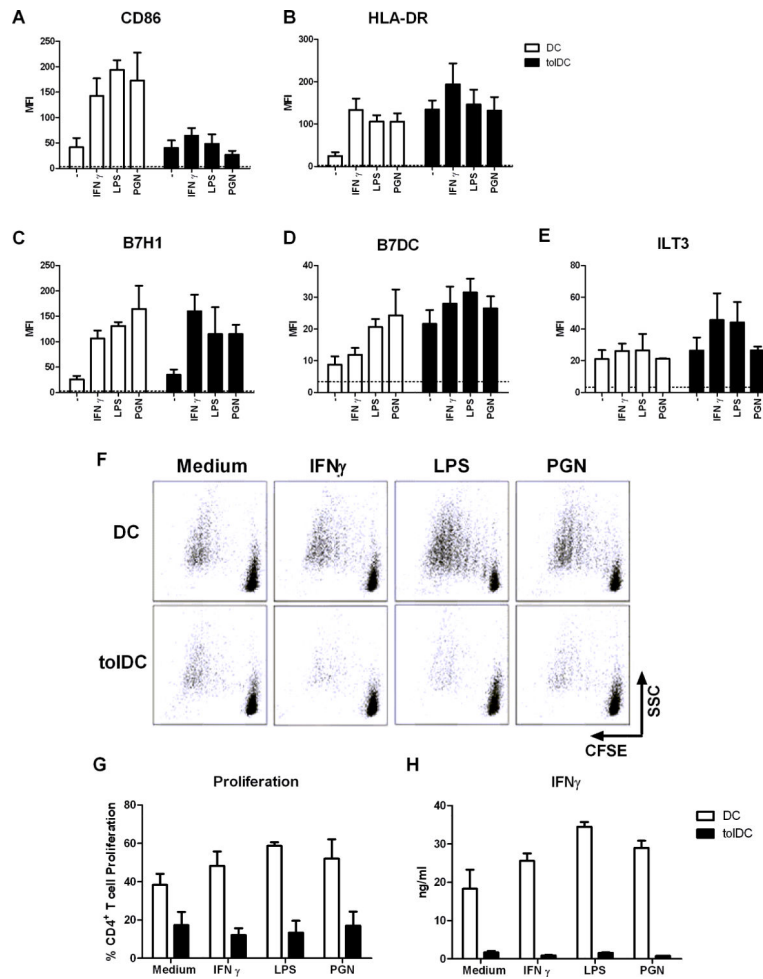
Figure 1. DCs generated in the presence of dexamethasone display a tolerogenic phenotype and have a reduced allostimulatory capacity

Monocytes were isolated from PBMCs and cultured for 6 days in the presence of IL-4 and GM-CSF to obtain immature DC. tolDC were generated by the addition of 10^{-8} Dexamethasone to the moDC cultures at day 0. Cells were activated overnight with IFN-γ, LPS or PGN. (A-E) The expression of (A) CD86, (B) HLA-DR, (C) B7H1, (D) B7DC and (E) ILT3 and on DCs and tolDCs was analysed using flow cytometry. Dashed line indicates the mean MFI of isotype controls used. (F) DC and tolDC were harvested on day 6 and either unstimulated or stimulated with IFN-γ, LPS or PGN for 24 hours. The DCs were then harvested and co cultured with CFSE labelled allogenic CD4$^+$ T cells at a ratio of 1:10. On day 5 the T cells were harvested and the CFSE staining was analysed using flow cytometry. (G) Percentage proliferation as determined in (F) of 3 independent experiments (H) T-cell culture supernatants were harvested on day 5 and analysed for IFN-γ production. (A-E, G and H) Data are shown as mean ± SD from 3 independent experiments.
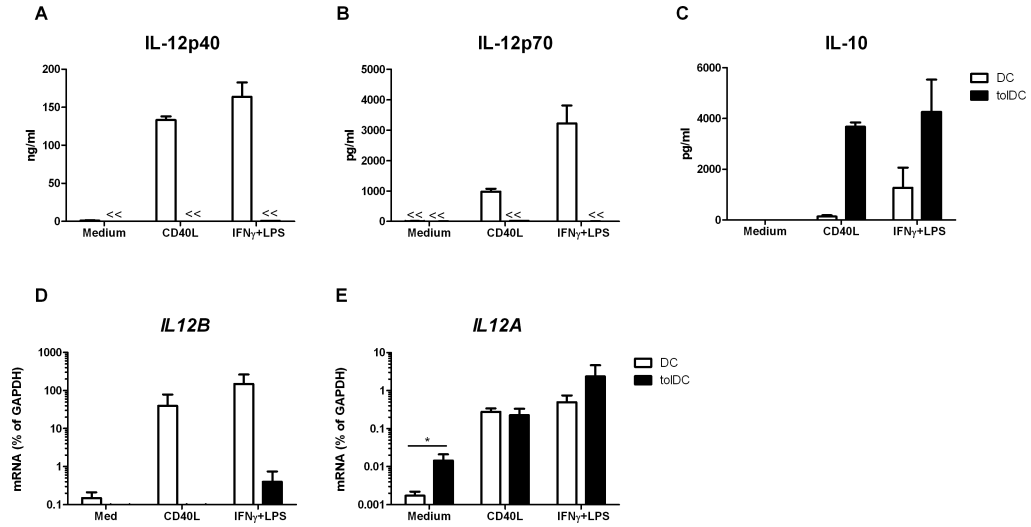
Figure 2. TolDC maintain expression of IL-12p35 in the absence of IL-12p40

Dendritic cells were harvested after 6 days of culture and stimulated with irradiated CD40L cells at a ratio of 1:5 DCs, or a combination of IFN-γ+LPS. After 48 hours the supernatants were harvested and, (A) IL-12p40, (B) IL-12p70 and (C) IL-10 levels were measured by ELISA. (A-C) Data are shown as mean ±SD (n=2 replicates) and are pooled from 6 independent experiments. (D and E) Cells were stimulated for 6 hours after which mRNA was isolated followed by cDNA synthesis. The transcript levels of (D) *IL12B* (E) *IL12A* were determined by RT-PCR. GAPDH mRNA expression from the same samples was used as an endogenous reference gene (relative mRNA expression). Data are shown as mean ±SD from 4 independent experiments. Untransfected L cells were used as a control for CD40L cells and yielded results in line with medium conditions (data not shown), *p 0.0143 using a one-tailed T-test.
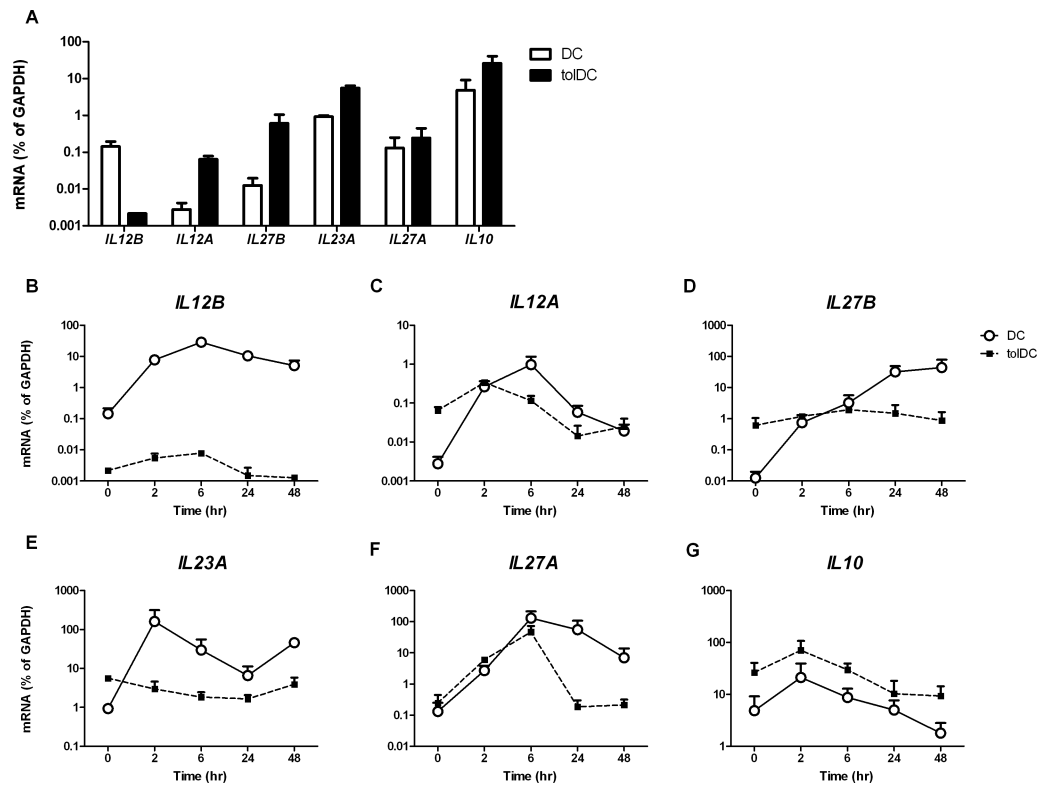
Figure 3. Kinetic expression of IL-12 family members and IL-10 mRNA in LPS-treated DCs and tolDCs

Cells were harvested on day 6 mRNA was isolated followed by cDNA synthesis. The transcript levels of (A) *IL12B, IL12A, IL27B, IL23A, IL27A* and *IL10* mRNA expression was determined by RT-PCR in unstimulated cells. Cells were stimulated with LPS for indicated time points; mRNA was isolated followed by cDNA synthesis. The transcript levels of (B) *IL12B*, (C) *IL12A*, (D) *IL27B* (E) *IL23A*, (F) *IL27A* and (G) *IL10* were determined by RT-PCR. GAPDH mRNA expression from the same samples was used as an endogenous reference gene (relative mRNA expression). (A-G) Data are shown as mean ± SD of 2 independent experiments.
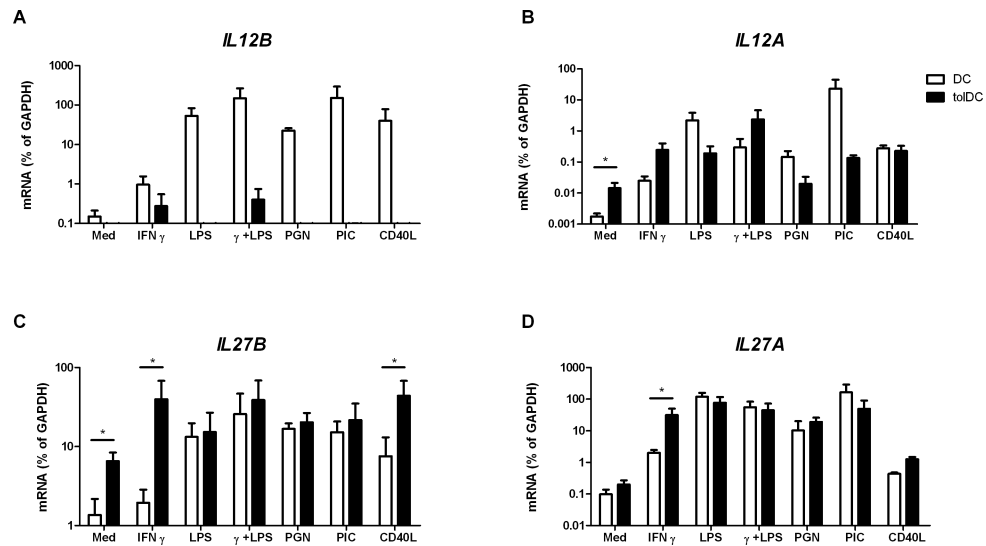
Figure 4. Stimulation of tolDCs favours upregulation of the immunoregulatory chains IL-12p35, Ebi3 and IL-27p28

Dendritic cells were stimulated for 6 hours with IFN-γ,LPS,PGN,Poly (I:C) or a combination of both IFN-γ+LPS. Irradiated CD40L cells were added at a ratio of 1:5. The transcript levels of (A) *IL12B*, (B) *IL12A,* (C) *IL27B*, (D) *IL27A* were determined by RT-PCR. GAPDH expression from the same samples was used as an endogenous reference gene (relative mRNA expression). Data are shown as mean ± SD of 3-5 independent experiments. Untransfected L cells were used as a control for CD40L cells and yielded results in line with medium conditions (data not shown), *p 0.0286, using one tailed T-test.
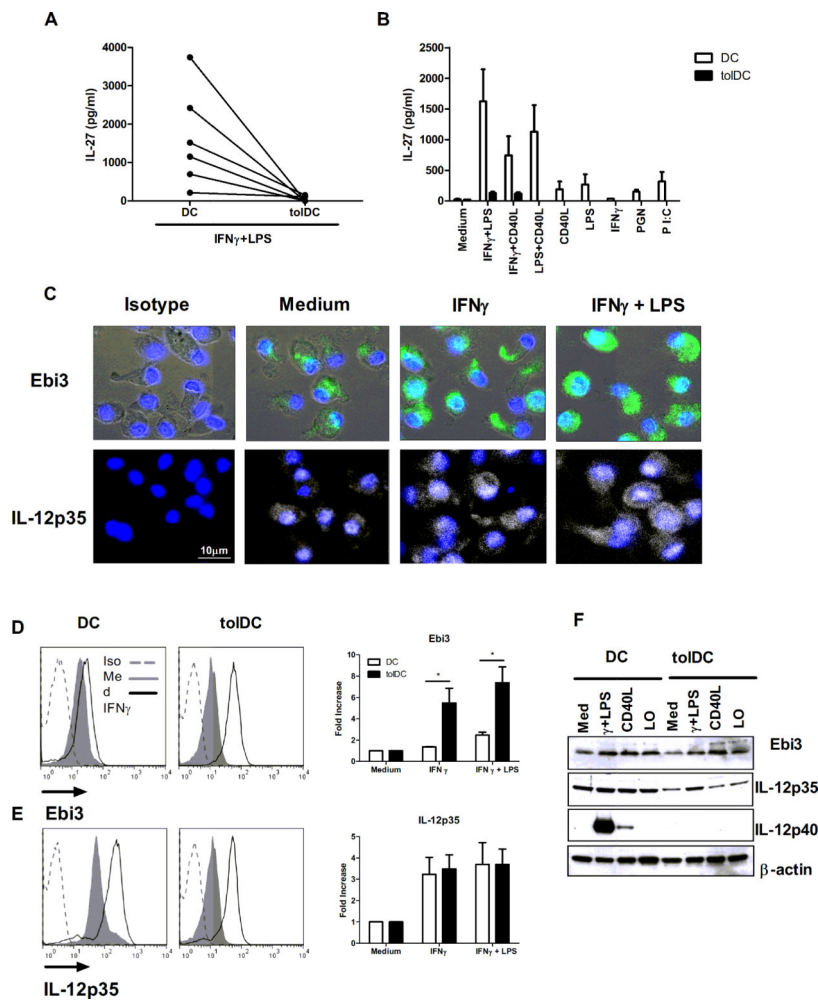
Figure 5. TolDC maintain and can enhance their protein expression of IL-12p35 and Ebi3 in the absence of IL-12p40

(A) DCs and tolDCs were harvested on day 6, replated and stimulated with IFN-γ+LPS;. (B) Cells were stimulated with IFN-γ+LPS, IFN-γ+CD40L, LPS+CD40L or CD40L for 48 hours. (A and B) IL-27 levels were assessed by ELISA. Data are shown as mean ± SD (n=2 replicates) and are pooled from 3-6 independent experiments. (C) tolDCs were cultured on 8 well chamber slides and either untreated, with IFN-γ or IFN-γ+LPS followed by overnight incubation with Brefeldin A. The cells were then fixed and permeabilised, followed by incubation with purified anti-huEbi3 or IL-12p35-allophycocyanin. Ebi3 was detected using GaM-Alexa[488].

(D and E) DCs and tolDCs were either untreated or stimulated for 16 hours with IFN-γ alone or IFN-γ+LPS, followed by incubation with Brefeldin A. The cells were then harvested, fixed and permeabilised, followed by incubation with (D) Ebi3-allophycocyanin or (E) IL-12p35. Data shown is representative of 3 independent experiments, with graphed data showing the mean ± SD of those 3 experiments. (F) DCs were treated as in (D, E) for the generation of cellular lysates. Western blot was performed with the indicated primary antibodies. Blots are representative of 3 independent experiments, *p 0.05 using one-tailed T-test.
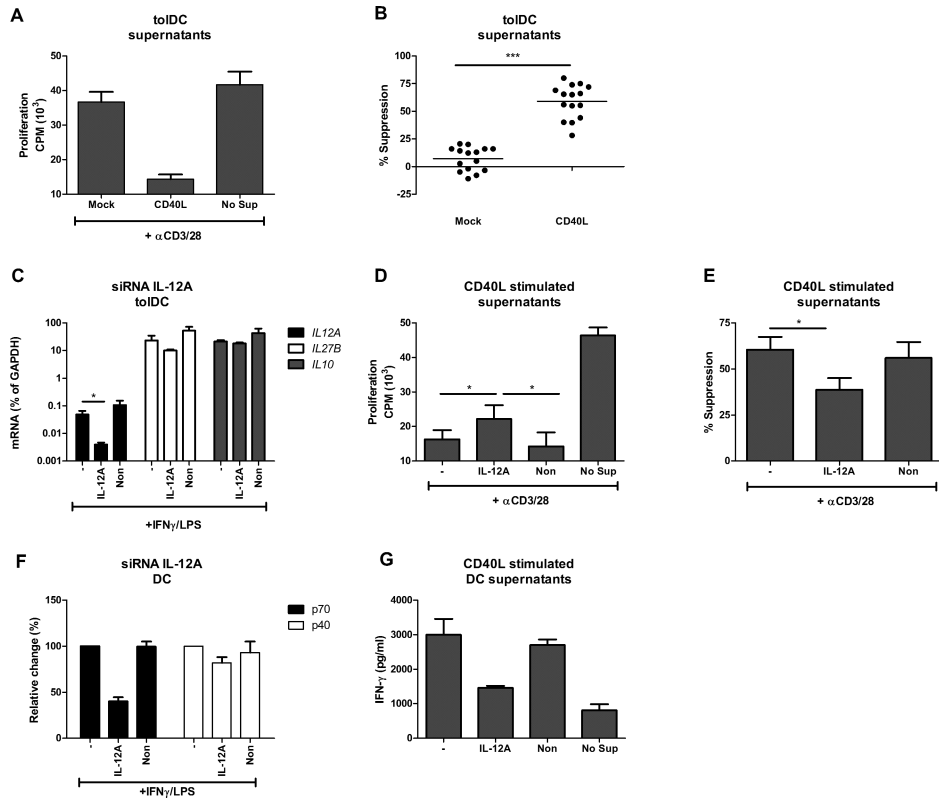
Figure 6. TolDCs require IL-12p35 in order to elicit their full tolerogenic potential
(A and B) tolDCs were stimulated with irradiated CD40L or mock transfected cells.
Supernatants were incubated with αCD3/28 stimulated naïve CD4$^+$ T cells. ^{3}H incorporation
was determined during the last 8-12 hours of culture by liquid scintillation. (A) Data are
shown as mean ± SD of CPM from triplicate cultures. B) Each data point represents a single
experiment and the bar indicates the mean ± SEM. Data are representative from 15
independent experiments. (C-E) *IL12A* silenced or control treated tolDCs were stimulated as
described above. (C) mRNA expression was analyzed by RT-PCR. (D) CPM and (E)
percentage of inhibition are shown as mean ± SD of 3 independent experiments.
(F and G) DCs were treated with mock (−), IL-12A, or Non-target siRNA, and then
stimulated with IFN-γ+LPS. (F) IL-12p70, IL-12p40 production was assessed by ELISA.
Data are shown as mean +SD from 4 independent experiments. (G) siRNA treated DCs were
stimulated with CD40L, supernatants were harvested and then incubated with αCD3/28
activated total CD4$^+$ T cells for 3 days. IFN-γ levels were determined in the T-cell
supernatants by ELISA. Data are shown as mean +SD (triplicate culture) and are
representative of 3 independent experiments. ***p 0.0001, *p 0.0114, using one tailed T-
test.

Table I

Real Time PCR Oligonucleotide sequences

Gene	NCBI ID	Protein ID	Forward Sequence	Reverse Sequence
IL12B	3593	IL-12p40	CAGCAGCTTCTTCATCAGGG	GAGTACTCCAGGTGTCAGG
IL12A	3592	IL-12p35	CCAGAGTCCCGGGAAAGTC	ACCAGGGTAGCCACAAGG
IL27B	10148	Ebi3	TGGATCCGTTACAAGCGTC	AGTTCCCCGTAGTCTGTG
IL27A	246778	IL-27p28	GAGGGAGTTCACAGTCAGC	GCAGGAGGTACAGGTTCAC
IL23A	51561	IL-23p19	CCAAGGACTCAGGGACAAC	CTGAGGCTTGGAATCTGCTG
GAPDH	2597	GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG