

# A proinflammatory cytokine interleukin-32 $\beta$ promotes the production of an anti-inflammatory cytokine interleukin-10

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## Introduction

Interleukin-32 (IL-32), originally reported as natural killer (NK) transcript 4, which was the IL-32 $\gamma$  isoform, is produced by activated T cells and NK cells and was thought to be secreted because it contains an internal signal sequence and lacks a transmembrane region.<sup>1</sup> However, the function of IL-32 has been unclear. Recently, IL-32 was defined as a proinflammatory cytokine that is induced in epithelial cells and monocytes by interferon- $\gamma$

## Summary

A new proinflammatory cytokine interleukin-32 (IL-32) has six isoforms. Although IL-32 can be detected in sera from patients suffering from Crohn's disease and rheumatoid arthritis, it is unclear which isoforms are involved. To this end, we investigated the functions of the most abundant IL-32 $\beta$  by generating K562-IL-32 $\beta$  stable cell lines. This report confirms, using IL-32 small interfering RNA, that IL-32 $\beta$  induces an anti-inflammatory cytokine IL-10 in K562-IL-32 $\beta$  cells and U937 promonocytic cells, which express endogenous IL-32 $\beta$  upon phorbol 12-myristate 13-acetate (PMA) treatment, and monocyte-derived dendritic cells (DC) upon lipopolysaccharide (LPS) treatment. Interleukin-32 $\beta$  was induced in monocyte-derived macrophages by LPS and in monocyte-derived DC by LPS, poly(I:C), or anti-CD40 antibody, but was not induced by PMA. We showed that IL-32 $\beta$  expression was increased in a time-dependent manner in monocyte-derived DC upon LPS treatment and peaked at 24 hr. Production of IL-10 was exactly coincident with IL-32 $\beta$  expression, but IL-1 $\beta$  and tumour necrosis factor- $\alpha$  production peaked at 6 hr after LPS treatment, then steeply declined. Interleukin-12 p40 was induced at 9 hr and gradually increased until 48 hr, at which time IL-32 $\beta$  and IL-10 were no longer increased. Knock-down of IL-32 $\beta$  by IL-32 small interfering RNA led to the decrease of IL-10, but the increase of IL-12 in monocyte-derived DC, which means that IL-32 $\beta$  promotes IL-10 production, but limits IL-12 production. We also showed that IL-10 neutralization increases IL-12, IL-1 $\beta$  and tumour necrosis factor- $\alpha$  production, which implies that IL-10 suppresses such proinflammatory cytokines. Taken together, our results suggest that IL-32 $\beta$  upregulates the production of an anti-inflammatory cytokine IL-10, and then IL-10 suppresses proinflammatory cytokines.

**Keywords:** cytokine; dendritic cell; inflammation; interleukin-10; interleukin-32

(IFN- $\gamma$ ) stimulation in a time-dependent manner.<sup>2-4</sup> Moreover, proteinase 3 has been identified as an IL-32 $\alpha$  binding protein that produces the active form of IL-32 $\alpha$ . Cleavage of IL-32 $\alpha$  by proteinase 3 induces macrophage inflammatory protein-2 in mouse Raw264.7 cells and IL-8 in human peripheral blood mononuclear cells (PBMC).<sup>5</sup> These observations suggest that IL-32 belongs to a proinflammatory cytokine group. Six alternatively spliced isoforms of IL-32 have been reported,<sup>3,6,7</sup> but the functional differences among the isoforms have not been elucidated.

Interleukin-10 is a well-known anti-inflammatory cytokine that suppresses immune responses by inhibiting the production of inflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 and the cell surface expression of major histocompatibility complex class II.<sup>8</sup> Interleukin-10 was originally known as a cytokine synthesis inhibitory factor (i.e. CSIF) that is produced by T helper type 2 cells upon stimulation with concanavalin A or antigen.<sup>9</sup> Myeloid cells are major sources of IL-10.<sup>10</sup> Myeloid cells, including antigen-presenting cells, are stimulated by pathogen-associated molecular patterns [e.g. lipopolysaccharide (LPS)] to produce proinflammatory cytokines such as IL-12, TNF- $\alpha$ , chemokines, prostaglandin and nitric oxide, whereas IL-10 prevents the induction of these inflammatory mediators.<sup>8,11</sup>

When infection with a pathogen occurs, proinflammatory responses must be triggered to regulate the proper immune responses. However, the inflammatory process must be resolved to prevent damage to the host.<sup>12</sup> The proinflammatory cytokine IL-12 has been reported to induce IL-10 and IFN- $\gamma$ , but suppress IL-4 in BALB/c mice infected with the intramacrophage parasite *Leishmania major* and in infected severe combined immunodeficient (SCID) mice.<sup>13,14</sup> Another proinflammatory cytokine, TNF- $\alpha$ , is also known to induce IL-10 in THP-1 cells.<sup>15</sup> Through these regulatory loops, the host maintains a homeostatic immune balance. In this study, we demonstrate that IL-32 $\beta$  induces the anti-inflammatory cytokine IL-10, which could be another regulatory loop of the innate immune response.

## Materials and methods

### Reagents and cell culture

The human erythroleukaemia K562 cell line and the promonocytic U937 cell line were grown in RPMI-1640 culture medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% fetal bovine serum (Hyclone, Logan, UT). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and rhIL-4 (Endogen, Woburn, MA) were used for the primary cultures. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St Louis, MO). Small interfering (si) RNA for IL-32 and non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO).

### Cloning and construction of stable cell lines

Human IL-32 $\alpha$  and IL-32 $\beta$  complementary DNA (cDNA) were amplified by polymerase chain reaction (PCR) and cloned into pcDNA3.1+ using *EcoRI* and *XhoI*. Stable clones were constructed by transfecting K562 cells with pcDNA3.1+, pcDNA3.1+IL-32 $\alpha$  or pcDNA3.1+IL-32 $\beta$

plasmids using a nucleofection kit V (Amaxa, Cologne, Germany) and then G-418 (1 mg/ml) resistant clones were selected for 3 weeks.

### Preparations of dendritic cells and macrophages from monocytes isolated with immunomagnetic beads

Dendritic cells (DC) and macrophages were obtained as previously described.<sup>16</sup> Briefly, human PBMC from healthy donors (Red Cross Blood Centre, Daejeon, Korea) were isolated by density centrifugation on Histopaque 1077 (Sigma). The Red Cross Blood Centre Committee (Seoul, South Korea) approved the use of these cells. Monocytes were purified by negatively depleting T, B and NK cells, erythrocytes, and granulocytes using mouse antibody-reactive immunomagnetic beads (Dyna, Oslo, Norway). Briefly, anti-CD2, -CD7, -CD16, -CD19, -CD56 and -CD235a-labelled PBMC were incubated with immunomagnetic beads for 30 min at 4 $^{\circ}$  with gentle rotation, and positive cells were removed using a Dynal magnet. Purified monocytes were found to be > 90% positive for the CD14 marker. Immature DC were generated by culturing monocytes in human DC medium containing 400 ng/ml rhGM-CSF and 20 ng/ml rhIL-4 or macrophage medium containing 10 ng/ml rhM-CSF (R&D Systems, Minneapolis, MN) in a 12-well plate at a concentration of  $5 \times 10^5$  cells per well.

### Transfections

K562-IL-32 $\beta$  and U937 cells were transfected with 1  $\mu$ g IL-32 siRNA and non-targeting control siRNA using a nucleofection kit V (Amaxa) as described. The siRNA for IL-32 or control siRNA were introduced into monocytes using a monocyte nucleofection kit (Amaxa) according to the manufacturer's instructions and then differentiated into DC by culturing in DC medium containing rhIL-4 and rhGM-CSF for 6 days. pcDNA3.1+IL-32 $\beta$  and pcDNA3.1+ empty vector were introduced into U937 cells by electroporation for ectopic overexpression.

### Reverse transcription-PCR and Western blot analysis

Total RNA from K562-vector, K562-IL-32 $\beta$ , U937 or DC was extracted using an easy-BLUE kit (iNtRON, Seongnam, South Korea). First-strand synthesis was performed using AMV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions then PCR-amplified using *iTag* polymerase (iNtRON). The following primers were used: IL-32 sense primer: 5'-ATGTGC-TTCCCGAAGGTCCTC-3'; IL-32 antisense primer: 5'-TC-ATTTTGAAGGATTGGGGTTC-3'; IL-10 sense primer: 5'-AACCTGCCTAACATGCTTCGA-3'; and IL-10 antisense primer: 5'-CTCATGGCTTTGTAGATGCCT. Cells were harvested and lysed in RIPA buffer (20 mM Tris-HCl,

pH 7.4, 150 mM NaCl, 0.5% nonidet P-40, 0.1% sodium deoxycholic acid, 0.05% sodium dodecyl sulphate, 10 mM  $\beta$ -phosphoglycerate, 10 mM sodium pyrophosphate, 1 mM sodium vanadate and protease inhibitor cocktail). Interleukin-32 was detected using a monoclonal antibody (mAb), KU32-52, as previously described.<sup>7</sup>

*Enzyme-linked immunosorbent assays*

The IL-32 enzyme-linked immunosorbent assay (ELISA) system was previously described.<sup>7</sup> Briefly, cell culture supernatants were incubated with mAb KU32-56-precoated on 96-well plates for 1 hr at room temperature. After washing three times with 0.05% Tween-20 in phosphate-buffered saline (PBST), wells were incubated with the biotinylated KU32-52 in 3% skim milk for 1 hr at room temperature, washed three times with PBST, then incubated with streptavidin peroxidase in 3% skim milk, followed by three washes with PBST. SureBlue TMB peroxidase substrate (KPL, Gaithersburg, MD) was added and the reaction was stopped by adding 50  $\mu$ l 1.25 M sulphuric acid. Enzyme activity was measured at 450 nm using an ELISA reader (Photoread, Berthold, Germany). Interleukin-10, IL-1 $\beta$ , TNF- $\alpha$ , IL-12 p70 and IL-12 p40 were measured in culture supernatants using the appropriate sandwich ELISA kits.

*IL-10 neutralization*

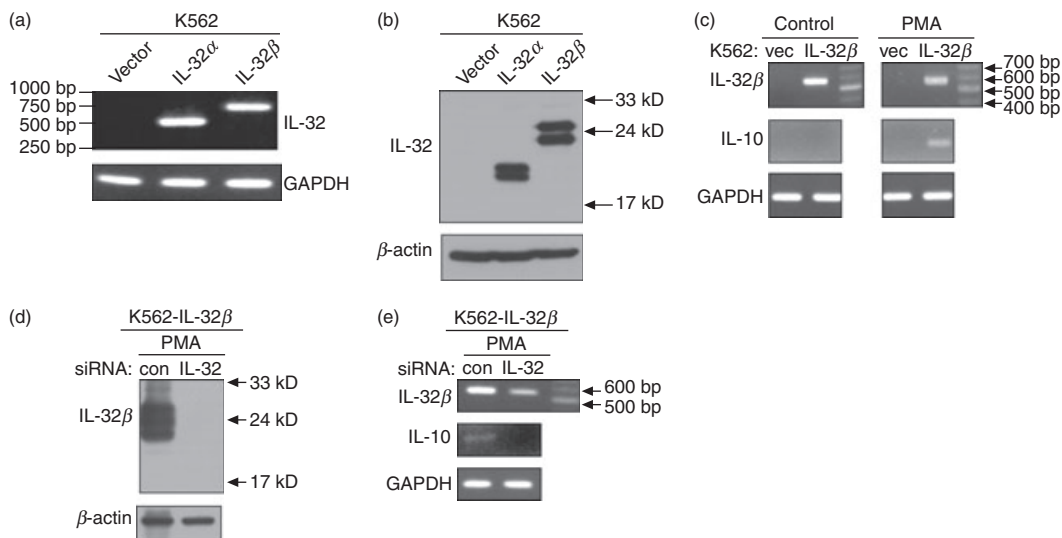
Monocyte-derived DC of  $1.5 \times 10^6$  cells/ml were treated with 10  $\mu$ g/ml IL-10 neutralizing mAb (R&D Systems) in

the presence or absence of 1  $\mu$ g/ml LPS for 24 hr. Cells were harvested for Western blotting, and culture media were collected and assayed for IL-10, IL-12 p70, IL-12 p40, IL-1 $\beta$  and TNF- $\alpha$  using the appropriate ELISA kits (R&D Systems).

**Results**

**IL-10 transcripts are induced in IL-32 $\beta$ -expressing K562 cells upon PMA stimulation**

We subcloned the full-length cDNA for human IL-32 $\alpha$  or human IL-32 $\beta$  into the expression vector pcDNA3.1+, then transfected these into the human erythroleukaemia cell line K562 to generate a constitutive expression system for IL-32 $\alpha$  or IL-32 $\beta$ . Strong expression of IL-32 $\alpha$  or IL-32 $\beta$  was detected by both reverse transcription (RT)-PCR and Western blotting (Fig. 1a,b). Although not clearly understood, the multiband pattern of IL-32 on the Western blot is thought to be caused by modifications of the IL-32 peptide according to sequence analysis.<sup>3</sup> In addition, we could not detect any secreted IL-32 in the culture medium using Western blotting (data not shown). We investigated the genes that were modulated by IL-32 using these stable cell lines. One of the genes that was upregulated by IL-32 $\beta$  after PMA treatment was that for IL-10. The IL-10 transcripts were increased in IL-32 $\beta$ -expressing K562 cells upon PMA stimulation compared with mock vector-transfected K562 cells (Fig. 1c). The effect of IL-32 $\alpha$  on IL-10 induction was not clear (data not shown). However, we could not detect IL-10 by



**Figure 1.** Construction of interleukin-32 $\alpha$  (IL-32 $\alpha$ ) or IL-32 $\beta$  stable cell lines in K562 cells, and IL-10 induction by IL-32 $\beta$  upon phorbol 12-myristate 13-acetate (PMA) treatment. Stable cell lines for IL-32 $\alpha$  or IL-32 $\beta$  were constructed in K562 cells and confirmed by reverse transcription-polymerase chain reaction (RT-PCR) (a) and Western blotting (b). K562-IL-32 $\beta$  cells were treated with 50 nM PMA for 24 hr, then total RNA was extracted for RT-PCR. Interleukin-10 transcripts were induced upon PMA stimulation of IL-32 $\beta$  expressing cells (c). A concomitant reduction in IL-10 transcripts with IL-32 knock-down by small interfering RNA transfection was detected (e). IL-32 $\beta$  suppression was confirmed by Western blotting (d).

ELISA or Western blotting. This may be the result of an effect of PMA-induced differentiation on translation because PMA causes erythroleukaemia K562 cells to differentiate into megakaryocytic cells.<sup>17–19</sup> To confirm that IL-32 $\beta$  induces IL-10, we transfected IL-32 $\beta$ -expressing K562 cells with IL-32 siRNA. Non-targeting control siRNA was included in experiments as a control. Interleukin-32 silencing caused the specific decrease of IL-32 expression as well as a concomitant reduction in IL-10 transcripts (Fig. 1e). The extent of IL-32 silencing was more drastic at the protein level than at the level of transcription (Fig. 1d,e).

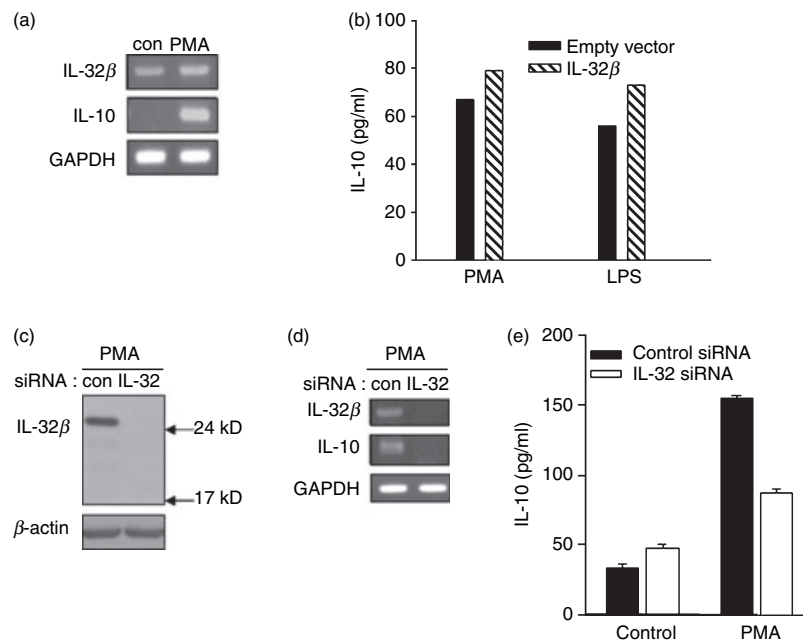
### Silencing of IL-32 $\beta$ induces downregulation of IL-10 in U937 cells

When we performed RT-PCR for IL-32 with total RNA from the promyelomonocytic cell line U937, the putative IL-32 $\beta$  band (567 base pairs for cDNA size) was observed (Fig. 2a). We cloned this band into a sequencing vector and confirmed its identity as IL-32 $\beta$  by sequencing (data not shown). Interleukin-32 is very abundant in U937; U937 cells have been used as a model system for monocyte or macrophage-like cells that produces IL-10 upon stimulation.<sup>20–24</sup> We also observed that IL-10 transcription was

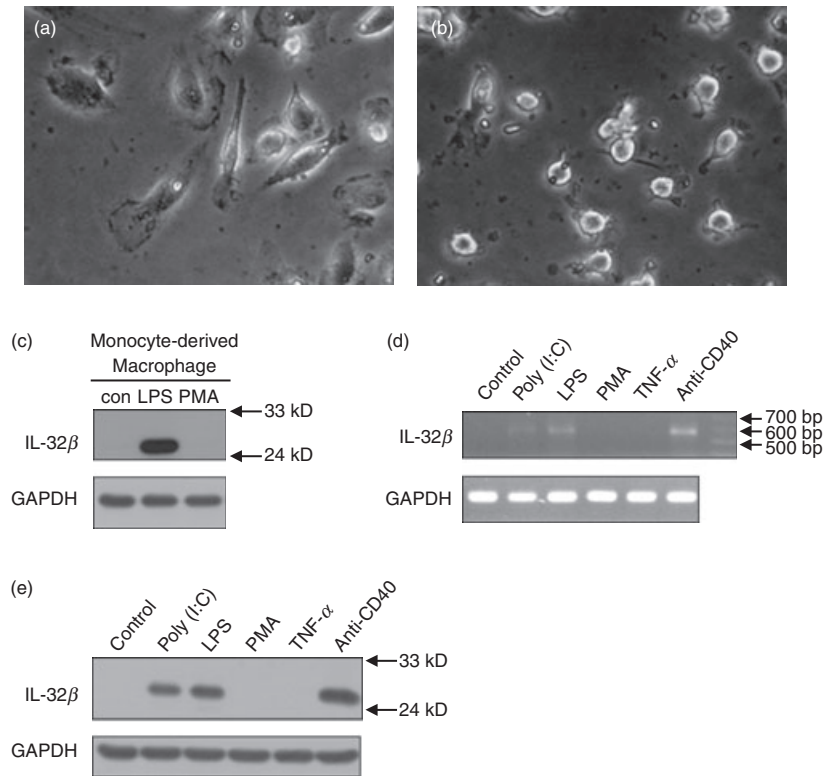
induced by PMA treatment (Fig. 2a). Therefore, we tried to determine the relevance of IL-32 $\beta$  in the induction of IL-10 in U937 cells treated with PMA and LPS. Production of IL-10 was further increased by ectopic overexpression of IL-32 $\beta$  in U937 cells (Fig. 2b). We speculated that IL-32 $\beta$  might have a positive role in IL-10 induction. To test this, we tried to knock-down IL-32 $\beta$  by transfecting U937 cells with IL-32 siRNA. With IL-32 $\beta$  suppression, PMA did not induce IL-10 transcription (Fig. 2d). We confirmed that the IL-32 siRNA worked effectively (Fig. 2c). We further confirmed the effect of IL-32 $\beta$  on IL-10 induction using ELISA. When U937 cells were transfected with IL-32 siRNA then treated with PMA for 24 hr, a significant reduction in IL-10 production compared with control cells was observed (Fig. 2e). These observations imply that IL-32 $\beta$  upregulates IL-10 production.

### IL-32 $\beta$ is induced in both monocyte-derived macrophages and monocyte-derived DC by LPS, poly(I:C) and anti-CD40 antibody treatment, but not by PMA treatment

Interleukin-32 is abundantly expressed in the spleen.<sup>3</sup> We investigated the expression pattern of IL-32 in myeloid lineage cells from human PBMC. Monocytes were



**Figure 2.** Regulation of interleukin-10 (IL-10) expression by IL-32 $\beta$  in U937 cells. U937 cells expressing endogenous IL-32 $\beta$  were treated with 50 nM phorbol 12-myristate 13-acetate (PMA) for 24 hr, then IL-10 transcription was measured (a). U937 cells were transfected with 1  $\mu$ g pcDNA3.1+IL-32 $\beta$  and pcDNA3.1+ empty vector by electroporation followed by PMA treatment for 24 hr. Additional increase of IL-10 by ectopic expression of IL-32 $\beta$  was analysed by enzyme-linked immunosorbent assay (ELISA) (b). This experiment was performed three times in duplicate. Mean values of duplicate are presented. U937 cells were transfected with 1  $\mu$ g of IL-32 small interfering (si) iRNA or control siRNA. Twenty-four hours later, cells were treated with 50 nM PMA for 20 hr. A decrease in IL-10 expression by IL-32 suppression was detected by reverse transcription–polymerase chain reaction (d). Knock-down of IL-32 $\beta$  protein was checked by Western blotting (c). A reduction in secreted IL-10 after IL-32 $\beta$  silencing was measured using an ELISA kit (e). All values of this experiment are shown as the mean  $\pm$  SEM of duplicates.



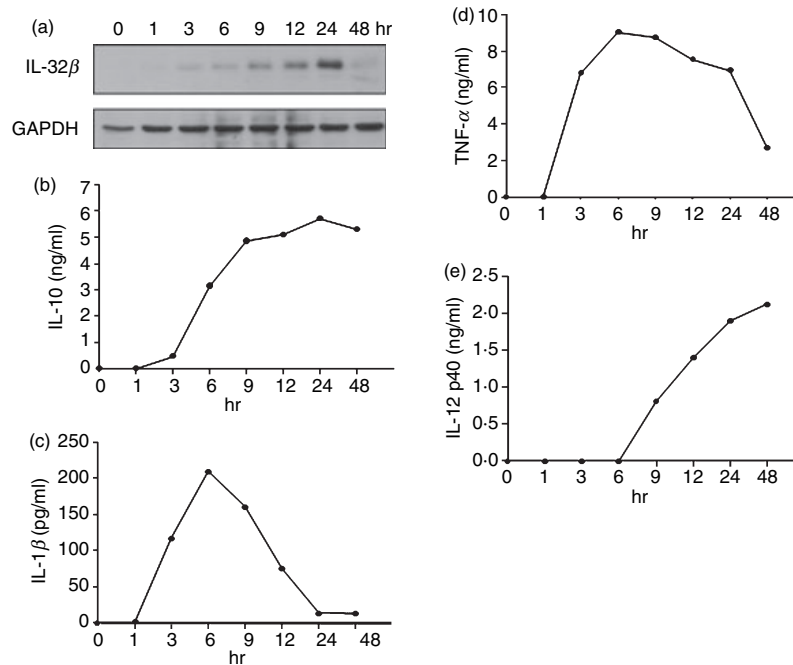
**Figure 3.** Interleukin-32 $\beta$  (IL-32 $\beta$ ) induction in monocyte-derived macrophages or monocyte-derived dendritic cells (DC). Phase contrast images of monocyte-derived macrophages (a) and monocyte-derived DC (b) obtained from human peripheral blood mononuclear cells. Macrophages were treated with 1  $\mu$ g/ml lipopolysaccharide (LPS) or 20 nM phorbol 12-myristate 13-acetate (PMA) for 24 hr, then analysed by Western blotting for IL-32 (c). Monocyte-derived DC were treated with 10  $\mu$ g/ml poly(I:C), 1  $\mu$ g/ml LPS, 20 nM PMA, 100 ng/ml tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), or 1  $\mu$ g/ml of anti-CD40 for 24 hr, then total RNA or cell lysates were prepared for reverse transcription–polymerase chain reaction (RT-PCR) (d) or Western blotting (e), respectively. Untreated negative controls were analysed simultaneously (c–e).

purified from human PBMC, and then differentiated into macrophages or DC for 6 days. The phenotypes of differentiated cells were ensured by detecting cell-type-specific surface markers like CD1a, CD14, CD80 and CD86 (data not shown), and the cells were photographed (Fig. 3a,b). We detected IL-32 $\beta$  by Western blotting, which was induced in monocyte-derived macrophages by LPS treatment but not by PMA treatment (Fig. 3c). Interleukin-32 $\beta$  was strongly induced in monocyte-derived DC upon treatment with LPS, poly(I:C) and the anti-CD40 antibody. There was little effect of PMA or TNF- $\alpha$  on IL-32 $\beta$  induction (Fig. 3d,e). These data indicate that IL-32 $\beta$  is induced by microbial products that activate the Toll-like receptor 3 (TLR3) or TLR4 signalling pathways. The bands in Fig. 3(d) were confirmed as IL-32 $\beta$  cDNA by sequencing but we did not detect IL-32 $\beta$  in the culture medium by ELISA (data not shown).

#### IL-10 production coincides with IL-32 $\beta$ expression in a time-dependent manner

We tried to reveal the relationship between IL-32 $\beta$  and IL-10, or other proinflammatory cytokines, in primary

cells using human PBMC. Monocytes were purified from human PBMC and differentiated into DC. To analyse the time-course expression patterns of IL-32 $\beta$ , IL-10 and proinflammatory cytokines, LPS-treated monocyte-derived DC and culture media were prepared at the indicated time-points (Fig. 4). Expression of IL-32 $\beta$  gradually increased until 24 hr, then decreased (Fig. 4a). The production pattern of IL-10 was identical to that of IL-32 $\beta$ , which was peaked at 24 hr (Fig. 4b). On the other hand, IL-1 $\beta$  and TNF- $\alpha$  induction peaked at 6 hr, and then declined steeply (Fig. 4c,d). This implies that IL-1 $\beta$  and TNF- $\alpha$  were induced earlier, before IL-10 was available in the culture medium, then those cytokines decreased sharply as IL-10 was increased. Interleukin-12 p40 was induced at around 9 hr, and continued to be mildly increased until 48 hr, at which time IL-32 $\beta$  and IL-10 production was no longer increased (Fig. 4e). Much later induction of IL-12 p40 than of IL-1 $\beta$  and TNF- $\alpha$  might be the result of IL-10, which is already effective enough at this time. The expression pattern of IL-12 p40 did not correspond to the pattern of IL-32 $\beta$  expression. It means that IL-32 $\beta$  is irrelevant to IL-12 induction. These results suggest that IL-10 production is



**Figure 4.** Time-course expression patterns of interleukin-10 (IL-10) and proinflammatory cytokines. Monocytes purified from human peripheral blood mononuclear cells (PBMC) were differentiated into dendritic cells (DC). Cells and culture media were prepared at the indicated time-points. Seventy micrograms of cell lysates were used for Western blotting for IL-32 $\beta$  (a) and culture supernatants were analysed for IL-10, IL-1 $\beta$ , tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-12 p40 using the respective enzyme-linked immunosorbent assay (ELISA) kits (b, c, d, e). The ELISA were performed twice in duplicate with two independent sources of PBMC and the mean values of duplicate were presented.

promoted by IL-32 $\beta$ , but other proinflammatory cytokines are suppressed.

#### IL-32 $\beta$ increases IL-10, but decreases IL-12 in monocyte-derived DC

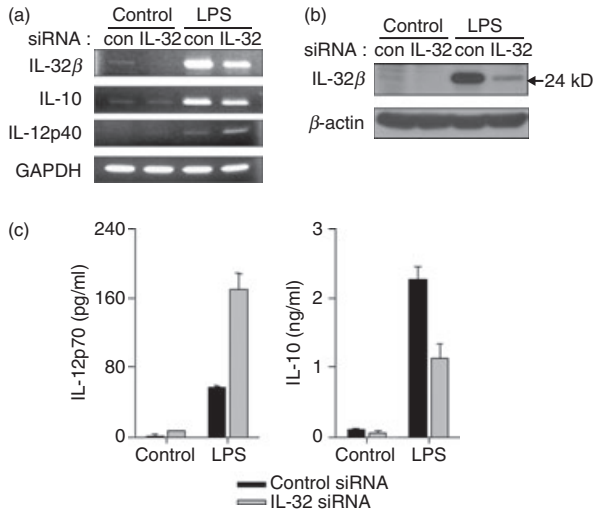
We tried to confirm the relationship between IL-32 $\beta$  and IL-10 in monocyte-derived DC. Monocytes purified from human PBMC were transfected with IL-32 siRNA and differentiated into DC by incubation with rhIL-4 and rhGM-CSF for 6 days. In contrast to the observed reduction in IL-10 transcription, IL-12 p40 transcription was increased by LPS treatment when IL-32 $\beta$  was suppressed (Fig. 5a). We verified, using Western blotting, that IL-32 siRNA worked effectively in the transfected monocytes (Fig. 5b). The protein levels of IL-10 and IL-12 p70 in the culture medium were measured using ELISA kits. Consistent with our transcriptional analysis, we observed a substantial reduction in IL-10 and an increase in IL-12 p70 as a result of IL-32 $\beta$  silencing (Fig. 5c). These observations strongly suggest that IL-32 $\beta$  induces IL-10 production, but suppresses IL-12 production. Alternatively, the inhibitory effect of IL-32 $\beta$  on IL-12 induction may be mediated by the increased IL-10, because IL-10 has been shown to limit the inflammatory response by inhibiting IL-12 production.<sup>13</sup>

#### IL-10 neutralization increases proinflammatory cytokines production such as IL-12 p40, IL-1 $\beta$ and TNF- $\alpha$

Previous data showed that IL-32 $\beta$  promotes IL-10 production but suppresses proinflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$  and IL-12. However, it is ambiguous whether the suppression of proinflammatory cytokines is by IL-32 $\beta$  or IL-10. Therefore, we performed an IL-10 neutralization experiment to analyse whether the suppression of proinflammatory cytokines is the result of the induction of IL-10 or not. Interleukin-10 neutralizing antibody was administered to monocyte-derived DC in the presence or absence of LPS for 24 hr. Expression of IL-32 $\beta$  and the levels of proinflammatory cytokines were analysed. Interleukin-32 $\beta$  was expressed in the LPS-treated cells (Fig. 6a); IL-12 p40, IL-1 $\beta$  and TNF- $\alpha$  production were increased in the IL-10 neutralizing antibody-treated cells even though IL-32 $\beta$  was expressed in those cells (Fig. 6b-d). Our results suggest that the suppression of proinflammatory cytokines was the result of IL-10 induction.

#### Discussion

When confronted with an infectious agent, a host organism mounts inflammatory responses that initiate an adequate immune response. However, this inflammatory



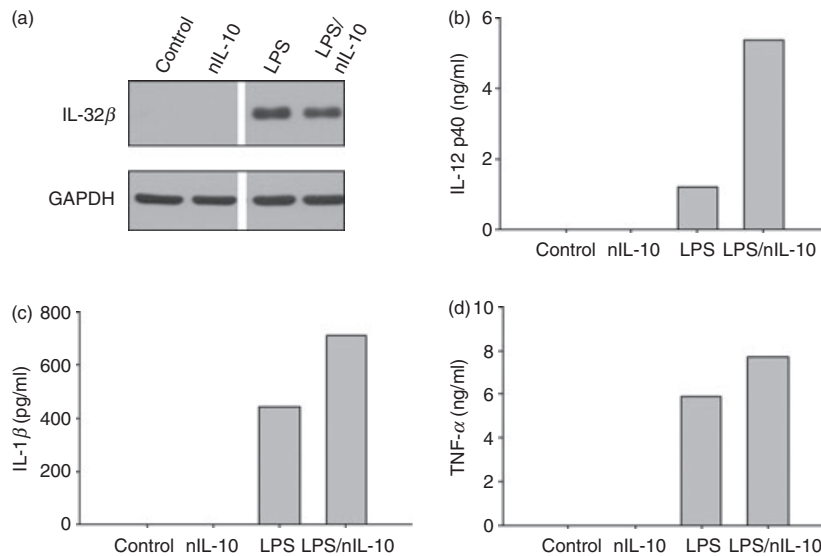
**Figure 5.** Interleukin-10 (IL-10) induction by IL-32 $\beta$  upon lipopolysaccharide (LPS) stimulation in monocyte-derived dendritic cells (DC). Non-targeting control small interfering (si) RNA or IL-32 siRNA was transfected into monocytes, which were then differentiated into DC. Cells were treated with 1  $\mu$ g/ml LPS for 24 hr, then total RNA was purified, cell lysates were prepared, and the culture medium was collected. Interleukin-10 transcripts were induced by IL-32 $\beta$ , but IL-12 p40 transcription was decreased (a). IL-32 $\beta$  expression was drastically reduced by IL-32 siRNA transfection (a, b). Culture media were assayed for IL-12 p70 and IL-10 by enzyme-linked immunosorbent assay (c). All values represent the mean  $\pm$  SEM of duplicates.

immune response must be limited to avoid harmful effects to the host. There must be mediators that balance the pro- and anti-inflammatory responses. In fact, the

proinflammatory cytokines IL-12 and TNF- $\alpha$  are known to induce the anti-inflammatory cytokines IL-10 and IFN- $\gamma$ . Interleukin-10 inhibits the induction of proinflammatory cytokines, including IL-12, and as a result protects the host from excessive inflammatory reactions.<sup>25</sup>

Interleukin-32 is a proinflammatory cytokine the function of which has recently been redefined. Unlike most cytokines, IL-32 has six isoforms as a result of alternative splicing and the cell surface receptor for IL-32 has not yet been elucidated, despite considerable effort. Moreover, IL-32 $\beta$  seems to function intracellularly rather than as a soluble factor because we could not detect IL-32 $\beta$  by Western blotting with K562-IL-32 $\beta$  culture medium before or after treatment with PMA (data not shown). The culture media of U937 cells or DC did not contain IL-32 $\beta$ , either. Interleukin-32 $\beta$  was also produced by T cells but was not secreted into the culture medium,<sup>6</sup> and intracellular accumulation of IL-32 $\alpha$  protein was detected in human pancreatic periacinar myofibroblasts but was not detected in culture supernatants.<sup>4</sup> It is possible that the pathway for IL-10 induction includes IL-32 $\beta$ -interacting molecules. The issues regarding IL-32 secretion are still controversial.

Interestingly, IL-32 $\beta$  was not induced by PMA in monocyte-derived macrophages or monocyte-derived DC. This, however, does not necessarily mean that IL-10 is not induced by PMA in these cells. It implies that there is another pathway for IL-10 induction mediated by IL-32 $\beta$ . Although we confirmed IL-32 $\beta$  cDNA by sequencing, there may be roles for other isoforms because we also identified IL-32 $\gamma$  cDNA in U937 cells (data not shown).



**Figure 6.** The effect of interleukin-10 (IL-10) neutralization on proinflammatory cytokine production;  $1.5 \times 10^6$  monocyte-derived dendritic cells were treated with 10  $\mu$ g IL-10 neutralizing antibody (nIL-10) in the absence or presence of 1  $\mu$ g/ml lipopolysaccharide (LPS) for 24 hr, then cells and culture media were prepared and analysed for IL-32 $\beta$  by Western blotting (a) and proinflammatory cytokines by enzyme-linked immunosorbent assay (ELISA). Interleukin-10 neutralization increases the IL-12 p40, IL-1 $\beta$  and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (b–d). The ELISA were performed twice in duplicate with two independent sources of peripheral blood mononuclear cells and the mean values of duplicates were presented.

There are several reports showing the kinetics of cytokine production in THP-1 promonocytic cells. Anti-inflammatory cytokine IL-10 production precedes IL-12 and IL-6 production upon treatment with bacterial lipoprotein, whereas the concentration of TNF- $\alpha$  reached its peak 2 hr after stimulation then declined sharply.<sup>15,26</sup> We confirmed IL-32 $\beta$  expression in THP-1 cells by Western blotting and cDNA sequencing after RT-PCR, as in U937 promonocytic cells (data not shown). This suggests that the endogenous IL-32 $\beta$  might induce IL-10 earlier than IL-12 or IL-6 in THP-1 cells. It would appear that TNF- $\alpha$  is produced much earlier, before IL-10 can be available in the culture medium.<sup>26</sup>

We demonstrated that IL-32 $\beta$  was induced by poly(I:C) or LPS, indicating that the TLR3 and TLR4 signalling pathways induce IL-32 $\beta$ . Therefore, we hypothesize that microbial products induce some isoforms of IL-32 which then trigger proinflammatory responses. However, other isoforms, including IL-32 $\beta$ , which may be induced with time gaps from the induction of proinflammatory isoforms, can suppress inflammation by inducing anti-inflammatory cytokines as a regulatory loop.

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