Cell. Mol. Life Sci. 65 (2008) 3851 – 3860
1420-682X/08/233851-10
DOI 10.1007/s00018-008-8540-1

Cellular and Molecular Life Sciences

Research Article

The pathogenic role of interleukin-27 in autoimmune diabetes

R. Wang[a,†], G. Han[a,†], J. Wang[a], G. Chen[a], R. Xu[a], L. Wang[a], X. Li[b], B. Shen[a] and Y. Li[a,*]

[a] Department of Molecular Immunology, Institute of Basic Medical Sciences, Taiping Road, No. 27, Beijing 100850 (China)
[b] Institute of Immunology, Medical School of Henan University, Kaifeng (China), Fax: +8610 – 68159436, e-mail: liyan62033@yahoo.com.cn

Received 02 September 2008; received after revision 27 September 2008; accepted 01 October 2008
Online First 20 October 2008

Abstract. Interleukin (IL)-27 is an IL-12-related cytokine that can promote both anti- and pro-inflammatory immune responses. This study investigated the potential role of IL-27 in autoimmune diabetes. We detected a high level of IL-27 in diabetic NOD mice. In addition, blockade of IL-27 significantly delayed the onset of diabetic splenocyte-transferred diabetes, while IL-27-treated diabetic splenocytes promoted the onset of the disease, compared with untreated controls. Furthermore, IL-27 up-regulated pro-inflammatory cytokines IFN-γ and IL-17 and down-regulated anti-inflammatory cytokines IL-4, TGF-β, and IL-10 secreted by diabetic splenocytes. These results demonstrate a pathogenic role of IL-27 in T cell-mediated autoimmune diabetes.

Keywords. IL-27, autoimmune diabetes, IFN-γ, IL-17, IL-4, TGF-β, IL-10.

Introduction

The non-obese diabetic (NOD) strain of mouse is an increasingly useful and important model of autoimmune type 1 diabetes (T1D) [1]. Inflammatory autoreactive T cells mediate the autoimmune status. Investigators generally believe that a Th1 effector response is associated with disease progression in NOD mice, and that Th2-like and Tregs responses can help suppress the development of diabetes [2–4]. Autoantigen-derived imbalance of Th1 and Treg cell differentiation from naïve $CD4^+$T cells is a major cause of diabetes [1]. The discovery of a new Th cell lineage, an IL-17-secreting Th17 cell, led to some confusion over the role of Th1 cells in autoimmunity [5, 6]. To date, the role of Th17 cells in autoimmune diabetes is still unclear.

The formation of distinct lineages of effector and regulatory T cells (Tregs) from naive $CD4^+$T precursors in response to antigen stimulation is a hallmark of the adaptive immune system [7–9]. Intrinsic changes have been researched in cytokine production that may alter Th1 polarization in NOD mice. A number of studies have shown that antigen-presenting cell populations from NOD mice may produce an array of cytokines that promote Th1 responses when compared to other strains of mice [10, 11]. The cytokine IL-12 is regarded as one of the most important inducers of Th1 responses. In fact, administration of IL-12 can promote diabetes incidence and onset [12]. But, the IL-12 knockout NOD mouse develops diabetes normally [13]. These studies suggest there may be another factor like IL-12.

IL-27 is a member of the IL-12 family that plays a role in the development of the CD4 T cell cytokine phenotype [14]. Thus, we propose that IL-27 plays a potential role in autoimmune diabetes. As expected,

† These authors contributed equally to this work.
* Corresponding author.

our results demonstrate that IL-27 has an important effect on autoimmune diabetes.

Materials and methods

Mice. NOD, NOD-scid mice, Balb/C and C57BL/6J mice, were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in our animal facilities under specific pathogen-free conditions. In female NOD mice, spontaneous diabetes began to appear by 12-weeks of age (increasing to 80–90 % incidence at 30 weeks of age), while there was about a 20 % incidence at 30 weeks of age in male NOD mice. Mice were screened for glucose levels every week and considered diabetic when glucose levels were $\geq$ 13.8 mMol/L. This study was approved by the Animal Care and Use Committee of the Beijing Institute of Basic Medical Science.

Preparation of lymphocytes from spleens and pancreatic lymph nodes. Lymphocytes from the spleen and pancreatic lymph nodes were collected under sterile conditions, obtained with mouse Ficoll separation liquid, washed twice with an incomplete RPMI medium and then re-suspended at 5×10^6 cells/ml in 10 % fetal calf sera (Atlanta Biologicals, Atlanta, GA) in an RPMI1640 medium containing 1 mM nonessential amino acids (ICN Pharmaceuticals, Costa Mesa, CA), 2 mM L-glutamine (ICN), and 2-mercaptoethanol (50 mM; ICN).

Cell isolation. Dendritic cells (DCs), $CD4^+$T, $CD4^+CD25^+$T or γσT cells were isolated from mixed splenocytes according to the manufacturer's protocol. Briefly, spleen cells were first collected from 12-week-old NOD mice and lymphocytes were enriched using Ficoll isolation. The lymphocytes were washed twice with PBS buffer containing 0.5 % BSA, and 2mM EDTA. For depleting CD11c+DCs, the cells were stained with anti-CD11c microbeads. For depleting CD4+CD25+T cells, enriched on T cell enrichment columns (R&D Systems), T cells were then stained with biotin-conjugated anti-CD25 antibody (7D4) followed by PE-conjugated streptavidin (Rockland Immunochemicals) and anti-PE microbeads (Miltenyi Biotec). For depleting γσT cells, PE-conjugated anti-γσ antibody (eBiosciences, GL3) and anti-PE-Microbeads (Miltenyi Biotec) were added. Cells were washed twice and then isolated using MACS Separator columns (Miltenyi Biotec Inc). FACS analysis showed CD11c+ in DCs was >90 % and CD11c+ cells in DC-depleted splenocytes, CD4+ cells in CD4+T-depleted splenocytes, CD4+CD25+ cells in CD4+CD25+T-depleted splenocytes, γσT and in γσT-depleted splenocytes were <2 %.

Cell cultures. Generally, cells (4×10^5 cells/well) were cultured in a 1.5 ml splenocyte culture in 12-well plates without any treatment or stimulation. In some conditions (described in figure legend), cells were also cultured in the presence of 10 ng/ml rmIFN-γ (BD Pharmingen, San Diego, CA), 10 ng/ml rmIL-27 (R&D Systems, Minneapolis, MN), 10 μg/ml rat anti-mouse IFN-γ antibody (XMG1.2, eBioscience, San Diego, CA), 0.67 μg/ml goat anti-mouse-IL-27 P28 antibody (AF1834, R&D Systems), or isotype control antibody (Santa Cruz, San Diego, CA). The cells were then incubated at 37 °C, 5 % CO_2 for 3 days. On the third day, we collected the supernatant and cells. The supernatant was used for determination of cytokines and the cells were transferred into recipient mice.

Relative mRNA measurement by real time-PCR. RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNAs were prepared by mixing 1 μg of RNA with 1 μM olidodeoxy-thymidine, 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 20U of RNAse inhibitor, and 525U of MMLV reverse transcriptase (Invitrogen) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$. After incubation at 42 °C for 90 min followed by 94 °C for 5 min, one fiftieth of the cDNA was mixed with primer pairs. Genes of interest were: p28 sense primer 5'-AGCCTGTTGCTGC-TACCCTTGC-3', antisense primer 5'-GTGG ACA-TAGCCCTGAACCTCA-3'; EBI3 sense primer 5'-TCTTCCTGTCACTTGCCCTC TG-3', antisense primer 5'-AGTTGGGAGCCTG GAGAGGAGT-3'; GAPDH 5' sense primer 5'-TTGTCAGCAATG-CATCCTGCAC-3'; antisense primer 5'-ACAGCT-TTCCA GAGGGGCCATC-3'. Real-time PCR reactions were run on an ABI Prism 7000 thermal cycler at 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s/60 °C for 30 s. Relative levels of mRNA for each factor were normalized to GAPDH, determined using the Ct value and the formula: $2^{-\Delta\Delta}$Ct.

Measurement of cytokine secretion. Measurement of cytokines in both serum and cell culture supernatants was performed by ELISA using a commercially available ELISA kit in accordance with the manufacturer's instructions. TGF-β, IL-27 and IL-17A ELISA kits were obtained from R&D Systems, USA. IFN-γ, IL-4 and IL-10 ELISA kits were purchased from BD Pharmingen. Results are expressed as pg/ml.

Adoptive transfer of diabetes. *In vitro* culture of 5×10^5 cells/ml splenocytes or $CD4^+$T cells-depleted spleno-

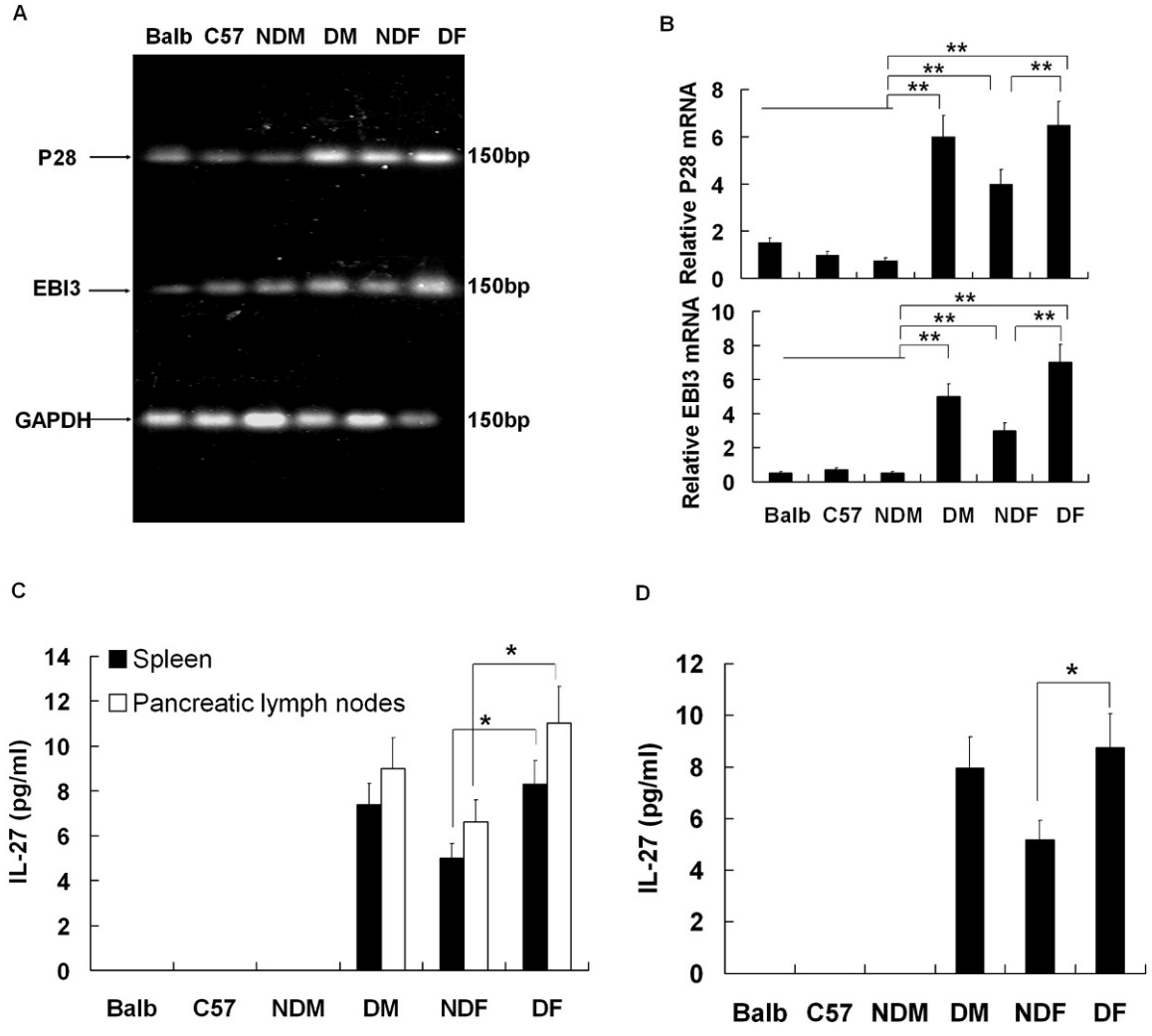

Figure 1. Expression of IL-27 protein and mRNA in diabetic NOD mice. Total RNA was isolated from splenocytes in 12-wk Balb/C (Balb), C57BL/6J (C57), non-diabetic male NOD (NDM, glucose levels 5.8 ~ 8.6 mMol/L), diabetic male NOD (DM, glucose levels 15.1 ~ 21.8 mMol/L), non-diabetic female NOD (NDF, glucose levels 6.2 ~ 9.4 mMol/L), and diabetic female NOD (DF, glucose levels 16.7 ~ 19.5 mMol/L) mice, and subjected to both semi-quantitative (*A*) and quantitative real-time (*B*) PCR analyses for IL-27p28 and EBI3 mRNA expression. Data was normalized relative to GAPDH mRNA expression levels in each respective sample (*B*). (*C*) Lymphocytes from the spleen and pancreatic lymph nodes in the above-mentioned mice were cultured *in vitro* without any stimulation for three days. On the 3rd day, cell-free supernatant was collected. The production of IL-27 was measured by ELISA from cell-free supernatants. (*D*) The production of IL-27 in serum from the above-mentioned mice was measured by ELISA. The results are representative of four separate experiments, with three mice per group (**$P < 0.01$ and *$P < 0.05$).

cytes from 12-wk non-diabetic or diabetic (described in figure legend) NOD mice were carried out in 50 ml bottles for three days without any treatment/stimulation, in the presence of 0.67 μg/ ml anti-rat-IL-27 monoclonal antibody (R&D Systems), 10 ng/ml rmIL-27 (R&D Systems), or isotype control antibody. Recipient mice (described in figure legend) were given 1×10^7 cells/mouse by i. v. injection into the tail veins at six- to eight weeks of age. For the anti-rat-IL-27 monoclonal antibody-treated group, mice were injected with 50 μg anti-IL-27 monoclonal antibody or isotype control antibody three times per week after cell transfer until completion of experiments. For the IL-27-treated group, mice were not injected with IL-27 after cell transfer. Recipient mice were monitored for the development of diabetes up to four weeks after the cells were transferred.

Statistical analysis. Results of blood glucose and cytokine ELISA were analyzed by the Student′s *t*-test. Differences in diabetes incidence were analyzed by χ quadrate test. The cumulative diabetes onset was compared by Kaplan–Meier analysis. All differences reported in the results were significant ($P < 0.05$).

Results

IL-27 expression is high in diabetic NOD mice. We measured messenger RNA (mRNA) expression in the splenocytes from Balb/c, C57BL/6J, male non-diabetic NOD, male diabetic NOD, female non-diabetic NOD, and female diabetic NOD mice. The data showed that the levels of mRNA expression in splenocytes from male diabetic NOD, female non-diabetic NOD, and female diabetic NOD mice were obviously higher than that from Balb/c, C57BL/6J, and male non-diabetic NOD as measured by regular RT-PCR (Fig. 1A) or by real-time quantitative PCR (qPCR; (Fig. 1B). Diabetic NOD mice expressed a significantly higher level of IL27 mRNA than gender-matched non-diabetic mice (Fig. 1A, 1B). The results suggest that IL-27 expression is up-regulated on the mRNA level in diabetic NOD mice. By regular ELISA, we could detect IL-27 protein expression in the supernatant of cultured splenocytes and pancreatic lymph node cells (Fig. 1 C) and serum (Fig. 1D) from male diabetic NOD, female non-diabetic NOD, and female diabetic NOD mice but not from Balb/c, C57BL/6J, and male non-diabetic NOD. Diabetic NOD mice expressed significantly higher levels of IL27 protein than gender-matched non-diabetic mice (Fig. 1C, 1D). We also determined IL-27 expression in 30-wk mice and found similar results (data not shown). These results suggest that IL-27 expression is up-regulated in diabetic NOD mice. However, non-diabetic female NOD mice expressed a high level of IL-27, although the level was significantly lower than that in diabetic female NOD mice. This may be associated with a high, spontaneous diabetes incidence in female NOD mice.

IL-27-secreting cells are mainly DCs in diabetic splenocytes. IL-27 is produced early by activated antigen-presenting cells (APCs) in response to microbial infection. DCs are one of the important APCs. By removing DCs from diabetic splenocytes, we found that the DC-depleted splenocytes expressed lower levels of IL-27 mRNA (Fig. 2A, 2B) and protein (Fig. 2C), while DCs expressed higher levels of IL-27 mRNA (Fig. 2A, 2B) and protein (Fig. 2C), compared with that of splenocytes. A previous study has shown that IFN-γ can promote IL-27 expression in APCs such as macrophages [15]. Here, we found a higher level of IFN-γ in the supernatant of three-day cultured splenocytes from diabetic NOD mice than that from non-diabetic gender-matched NOD mice (Fig. 2D). $CD4^+$T cell-depletion significantly suppressed the level of IFN-γ in three-day cultured splenocytes, suggesting that IFN-γ-secreting cells are mainly $CD4^+$T cells in diabetic splenocytes (Fig. 2D). IL-27 expression obviously decreased when neutral anti-IFN-γ antibody was added to cultured splenocytes, while IFN-γ up-regulated IL-27 expression in DCs (Fig. 2A-C). We also determined IL-27 expression in DCs from 30-wk mice and found similar results (data not shown). These results suggest that IFN-γ-stimulated DCs are mainly IL-27-secreting cells in diabetic NOD mice. Recent reports showed that $Foxp3^+$Tregs modified DCs are the main source of IL-27 from DCs [16]. However, $CD4^+CD25^+$T cell-depleting could not significantly suppress the level of IL-27 in three-day cultured splenocytes. The results suggest $Foxp3^+$Tregs modified DCs are not the main source of IL-27.

Blockade of IL-27 delays diabetic splenocyte-transferred diabetes. Next, we ascertained the role of IL-27 in T cell-mediated diabetes. To examine the effect of IL-27 on diabetic onset and incidence, neutral anti-IL-27-treated diabetic splenocytes were used to transfer diabetes into female NOD-scid mice. Subsequently, mice were injected with anti-IL-27 neutral antibody three times per week until five weeks after the cells were transferred. At the second week after the cells were transferred, only two (20%) of the 10 mice in the anti-IL-27-treated group developed diabetes, whereas six (60%) of the 10 mice in the untreated group developed diabetes. At the third week, only three (30%) of the 10 mice in the anti-IL-27-treated group, developed diabetes, whereas nine (90%) of the 10 mice in the untreated group developed diabetes. At the fourth week, 10 (100%) of the 10 mice in the untreated group developed diabetes, compared to five (50%) of the 10 mice in the anti-IL-27-treated group. At the fifth week, only six (60%) of the 10 mice in the anti-IL-27-treated group developed diabetes (Fig. 3). Kaplan–Meier analysis showed that the cumulative diabetes onset significantly decreased in the anti-IL-27-treated splenocyte-transferred group, compared with that in the untreated splenocyte-transferred group (Fig. 3, $^{**}P < 0.01$). To determine which cell population was responsible for the delay in splenocyte-transferred diabetes, $CD4^+$T cells were depleted from diabetic splenocytes before transfer into NOD-scid mice. Kaplan–Meier analysis showed that the cumulative diabetes onset significantly decreased in the $CD4^+$T-depleted group, compared with that in the undepleted group ($^{**}P < 0.01$), suggesting that $CD4^+$T cells were responsible for splenocyte-transferred diabetes. To test whether blockade of IL-27 reduces diabetes incidence by affecting the $CD4^+$T cells, neutral anti-IL-27-treated $CD4^+$T-depleted splenocytes were used to transfer diabetes into female NOD-scid mice. Subsequently, mice were injected with anti-IL-27 neutral antibody three times per week until five weeks after the cells were transferred.

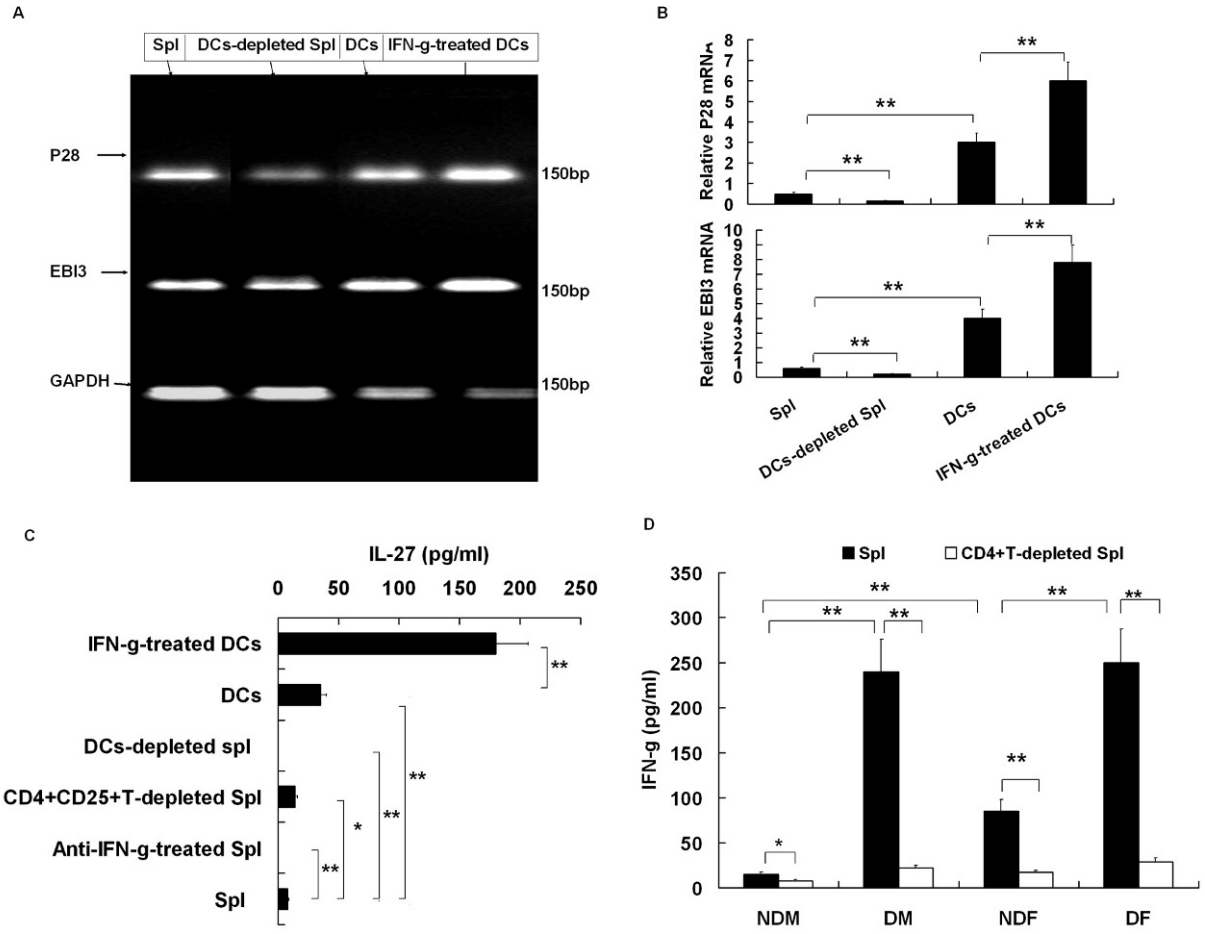

Figure 2. Role of DCs in IL-27 expression. Splenocytes (Spl), DCs-depleted Spl, DCs, and $CD4^+CD25^+$T-depleted Spl were collected from 12-wk diabetic female NOD mice and cultured *in vitro* for three days. In the process of culture, DCs and Spl were treated with 10 ng/ml IFN-γ and 10 μg/ml rat anti-mouse IFN-γ antibody, respectively. On the third day, total RNA was isolated and the cell-free supernatants were collected from these cells. Total RNA was subjected to both semi-quantitative (*A*) and quantitative real-time (*B*) PCR analyses for IL-27 p28 and EBI3 mRNA expression. Data was normalized relative to GAPDH mRNA expression levels in each respective sample. (*C*) The production of IL-27 was measured by ELISA from cell-free supernatants. (*D*) The production of IFN-γ in the supernatant of three-day-cultured Spl and $CD4^+$T cells-depleted Spl from 12-wk non-diabetic male NOD (NDM, glucose levels 5.8 ~8.6 mMol/L), diabetic male NOD (DM, glucose levels 15.1 ~ 21.8 mMol/L), non-diabetic female NOD (NDF, glucose levels 6.2 ~ 9.4 mMol/L), and diabetic female NOD (DF, glucose levels 16.7 ~ 19.5 mMol/L) mice was measured by ELISA. The results are representative of three separate experiments, with four mice per group (**$P < 0.01$ and *$P < 0.05$).

Kaplan–Meier analysis showed that blockade of IL-27 had no effect on $CD4^+$T-depleted diabetic splenocyte-transferred diabetes onset in NOD-scid (Fig. 3, $P > 0.05$). The results suggest that blockade of IL-27 delays diabetic splenocyte-transferred diabetes onset in NOD-scid by affecting $CD4^+$T cells.

IL-27 promotes diabetic splenocyte-transferred diabetes. To prove further the pathogenic role of IL-27 in T cell-mediated diabetes, a high level of IL-27 was used to treat non-diabetic splenocytes and then 1×10^7 splenocytes were transferred into seven-week-old male NOD mice. Mice were monitored until four weeks after the cells were transferred. Blood glucose was first up-regulated in the second week, and then decreased after the mice received IL-27-treated splenocytes or untreated splenocytes in the second week (Fig. 4A). Student's *t*-test analysis showed that the level of blood glucose was higher in the mice that received IL-27-treated splenocytes than that in the mice that received untreated splenocytes at the second week and third week after treatment (**$P < 0.01$). These results demonstrate that IL-27 promotes non-diabetic splenocytes to up-regulate blood glucose but not to result in diabetes in male NOD mice. To determine which cell population was responsible for IL-27 up-regulating glucose, IL-27-treated $CD4^+$T-depleted non-diabetic splenocytes were used to transfer diabetes into male NOD mice. Kaplan–Meier analysis showed that IL-27 had no effect on $CD4^+$T-

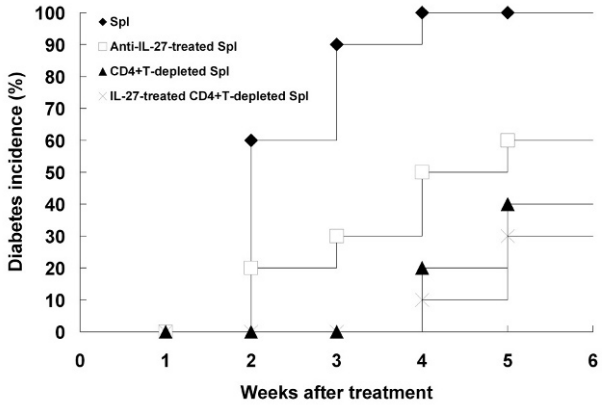

Figure 3. Blockade of IL-27 delays diabetic splenocytes-transferred diabetes in NOD-scid. Splenocytes (Spl) and $CD4^+$T cell-depleted Spl from 12-wk diabetic female NOD mice were cultured *in vitro* for three days in the presence of 0.67 μg/ml goat anti-mouse-IL-27 P28 antibody. On the third day, 1×10^7 cells (per mouse) were injected into the tail veins of six- to eight-wk-old female NOD-scid mice. Recipient mice (10 mice per group) were monitored for the development of diabetes up to diabetes onset in all recipient mice. Kaplan–Meier analysis showed that the cumulative diabetes onset was different between the anti-IL-27-treated Spl-transferred group and the untreated Spl-transferred group ($P < 0.01$) and not obviously different between the anti-IL-27-treated $CD4^+$T cell-depleted Spl-transferred group and the untreated $CD4^+$T cell-depleted Spl-transferred group ($P > 0.05$). Results of diabetes incidence (number of diabetic out of total mice) represent three separate experiments.

depleted non-diabetic splenocyte-transferred diabetes onset in male NOD mice (Fig. 4A, $P > 0.05$). The results suggest that IL-27 promote non-diabetic splenocytes to up-regulate blood glucose in male NOD mice by affecting $CD4^+$T cells.

Diabetic splenocytes were treated with a high level of IL-27 and then transferred into seven-week-old male NOD mice. Mice were monitored until four weeks after the cells were transferred because the level of blood glucose in non-diabetic mice returned to normal. We found that zero (0%) of the 10 mice that received diabetic splenocytes developed diabetes, whereas three (30%) of the 10 mice that received IL-27-treated diabetic splenocytes developed diabetes at four weeks after therapy (Fig. 4B). The χ quadrate test analysis showed that diabetes incidence was high in the IL-27-treated splenocyte-transferred group, compared with the untreated splenocyte-transferred group (Fig. 4B, $^{**}P < 0.01$). The data suggests that IL-27 promotes diabetic splenocytes to induce diabetes in male NOD mice. To determine which cell population was responsible for the IL-27 inducing diabetes, IL-27-treated $CD4^+$T-depleted diabetic splenocytes were used to transfer diabetes into male NOD mice. Kaplan–Meier analysis showed that IL-27 had no effect on $CD4^+$T-depleted diabetic splenocyte-transferred diabetes in male NOD mice (Fig. 4B, $P > 0.05$). The results suggest that IL-27 induces diabetic splenocytes-transferred diabetes in male NOD mice by affecting $CD4^+$T cells.

IL-27-treated diabetic splenocytes were also transferred into seven-week-old female NOD-scid mice. At the second week after the cells were transferred, seven (70%) of the 10 mice that received IL-27-treated diabetic splenocytes developed diabetes, whereas only three (30%) of the 10 mice that received untreated diabetic splenocytes developed diabetes. At the third week, 10 (100%) of the 10 mice that received IL-27-treated diabetic splenocytes developed diabetes, whereas only six (60%) of the 10 mice that received untreated diabetic splenocytes developed diabetes (Fig. 4C). Kaplan–Meier analysis showed that the diabetes onset was higher in the IL-27-treated splenocyte-transferred group than that in the untreated splenocyte-transferred group (Fig. 4C, $^{**}P < 0.01$). These results suggest that IL-27 promotes diabetic splenocytes to accelerate diabetes onset in female NOD-scid mice. To determine which cell population was responsible for IL-27 accelerating diabetes, IL-27-treated $CD4^+$T-depleted diabetic splenocytes were used to transfer diabetes into female NOD-scid mice. Kaplan–Meier analysis showed that IL-27 had no effect on $CD4^+$T-depleted diabetic splenocyte-transferred diabetes in female NOD-scid mice (Fig. 4C, $P > 0.05$). The results suggest that IL-27 accelerates diabetic splenocyte-transferred diabetes in female NOD-scid mice by affecting the $CD4^+$T cells.

The effect of IL-27 on the profiles of cytokines in diabetic splenocytes. Finally, we detected the mechanisms by which IL-27 enhanced the ability of diabetic splenocytes to induce diabetes in the recipient. Because Th cell differentiation has an important role in the pathology of diabetes, the effect of IL-27 on the profile of Th cytokines was studied. IL-27 could significantly promote diabetic splenocytes to secrete Th1-related IFN-γ and Th17-related IL-17 which were higher in diabetic splenocytes than that in non-diabetic splenocytes, and reduce diabetic splenocytes to secrete Th2-related IL-4, Treg-related TGF-β and Tr1-related IL-10 which were lower in diabetic splenocytes than that in non-diabetic splenocytes (Fig. 5). These results suggest that IL-27 may promote the ability of diabetic splenocytes inducing diabetes by enhancing pro-inflammatory IFN-γ and IL-17 production and reducing anti-inflammatory IL-4, TGF-β, and IL-10 production. The finding that IL-27 up-regulated IL-17 and reduced IL-10 in diabetic splenocytes is apparently not consistent with previous studies that IL-27 suppressed Th17 cells [17] and interleukin 27 induced interleukin 10–producing anti-inflammatory T cells [16]. To determine which cell

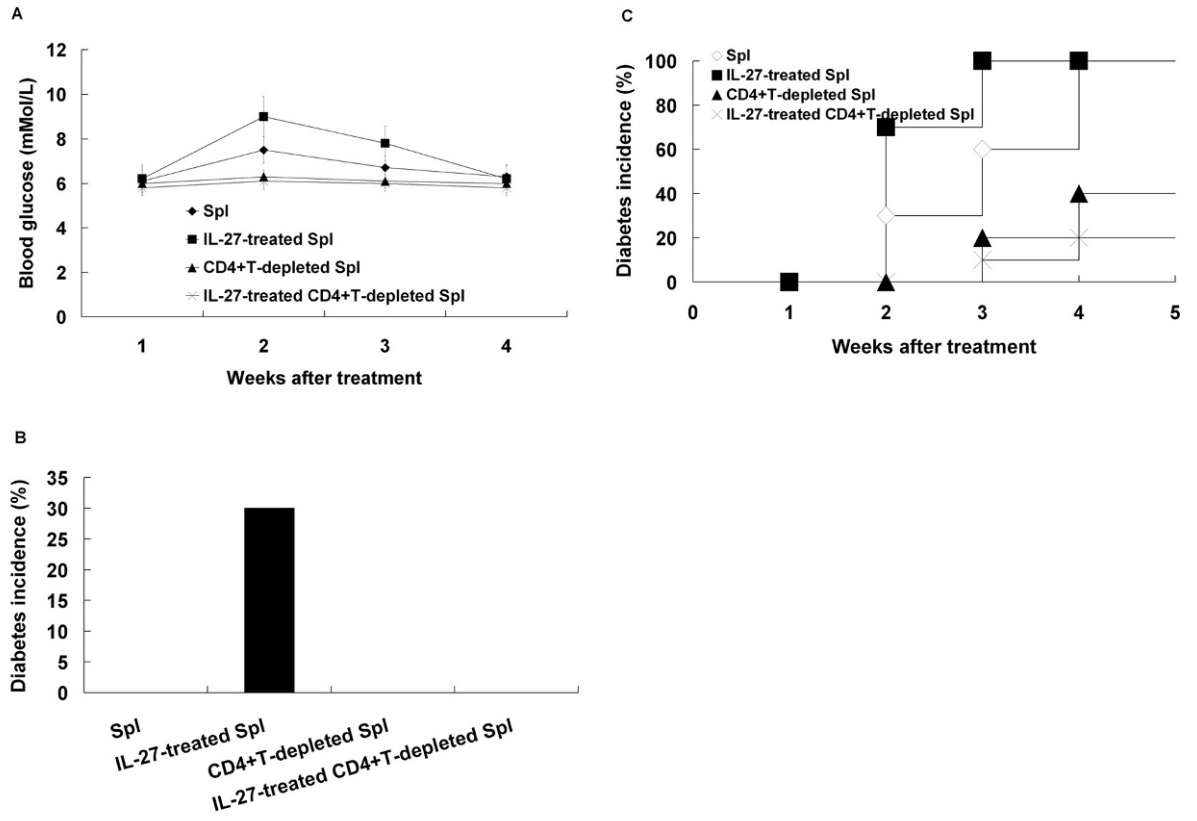

Figure 4. IL-27 promotes diabetic splenoycte-transferred diabetes in NOD-scid or female NOD mice. (*A*) Splenocytes (Spl) and $CD4^+T$ cell-depleted Spl from 12-wk non-diabetic female NOD mice were cultured *in vitro* for three days without, or in the presence of 10 ng/ml IL-27 and then injected into the tail veins of six- to eight-wk-old male NOD mice. Student's *t*-test analysis showed that the level of blood glucose was significantly different between the untreated Spl-transferred group and the IL-27-treated Spl-transferred group at two and three weeks after transfer ($P < 0.05$) and not obviously different between the IL-27-treated $CD4^+T$ cell-depleted Spl-transferred group and the $CD4^+T$ cell-depleted Spl-transferred group ($P > 0.05$). Spl and $CD4^+T$ cell-depleted Spl from 12-wk diabetic female NOD mice were cultured *in vitro* without, or in the presence of 10 ng/ml IL-27 for three days and injected into the tail veins of six- to eight-wk-old male NOD mice (*B*) or female NOD-scid mice (*C*). Recipient mice (1×10^7 cells per mouse, 10 mice per group) were monitored for the development of diabetes up to 4 wk. The χ quadrate test analysis showed that diabetes incidence in (*B*) (showed the data at 4 weeks after transfer) was significantly different between the Spl-transferred group and the IL-27-treated Spl-transferred group at two and three weeks after transfer (**$P < 0.01$) and not significantly different between the IL-27-treated $CD4^+T$ cell-depleted Spl-transferred group and the $CD4^+T$ cell-depleted Spl-transferred group ($P > 0.05$). Kaplan–Meier analysis showed that the cumulative diabetes onset in (*C*) was significantly different between the untreated Spl-transferred group and the IL-27-treated Spl-transferred group at two and three weeks after transfer ($P < 0.01$) and not significantly different between the IL-27-treated $CD4^+T$ cell-depleted Spl-transferred group and the $CD4^+T$ cell-depleted Spl-transferred group ($P > 0.05$). Results of diabetes incidence (number of diabetic out of total mice) represent three separate experiments.

population is responsible for IL-27 up-regulating IL-17, $CD4^+T$ or γσT cells were depleted from diabetic splenocytes before culture. We found that IL-17 levels in IL-27-treated γσT-depleted splenocytes is significantly lower than that in IL-27-treated splenocytes (Fig. 5), while IL-17 levels in IL-27-treated $CD4^+T$-depleted splenocytes is similar to that in IL-27-treated splenocytes (data not shown). The results suggest that IL-27 up-regulates IL-17 mainly by affecting γσT cells but not Th17 cells in diabetic splenocytes. We propose that IL-27 may indirectly reduce the IL-10 level in diabetic splenocytes. We found that the IFN-γ level in IL-27-treated $CD4^+T$-depleted splenocytes was significantly lower than that in IL-27-treated splenocytes (Fig. 5) suggesting that IL-27 up-regulated IFN-γ mainly by affecting $CD4^+T$ cells in diabetic splenocytes. IFN-γ-secreting $CD4^+T$ cell production may suppress the development of IL-10-secreting $CD4^+T$ cells. Once IFN-γ was blocked, IL-27 promoted IL-10 secretion (Fig. 5).

Discussion

IL-27, a novel IL-12 family cytokine, is a heterodimeric molecule composed of an Epstein-Barr virus–induced gene 3 (EBI3) and p28 [14]. EBI3, which is related to IL-12/IL-23 p40 subunit, has been

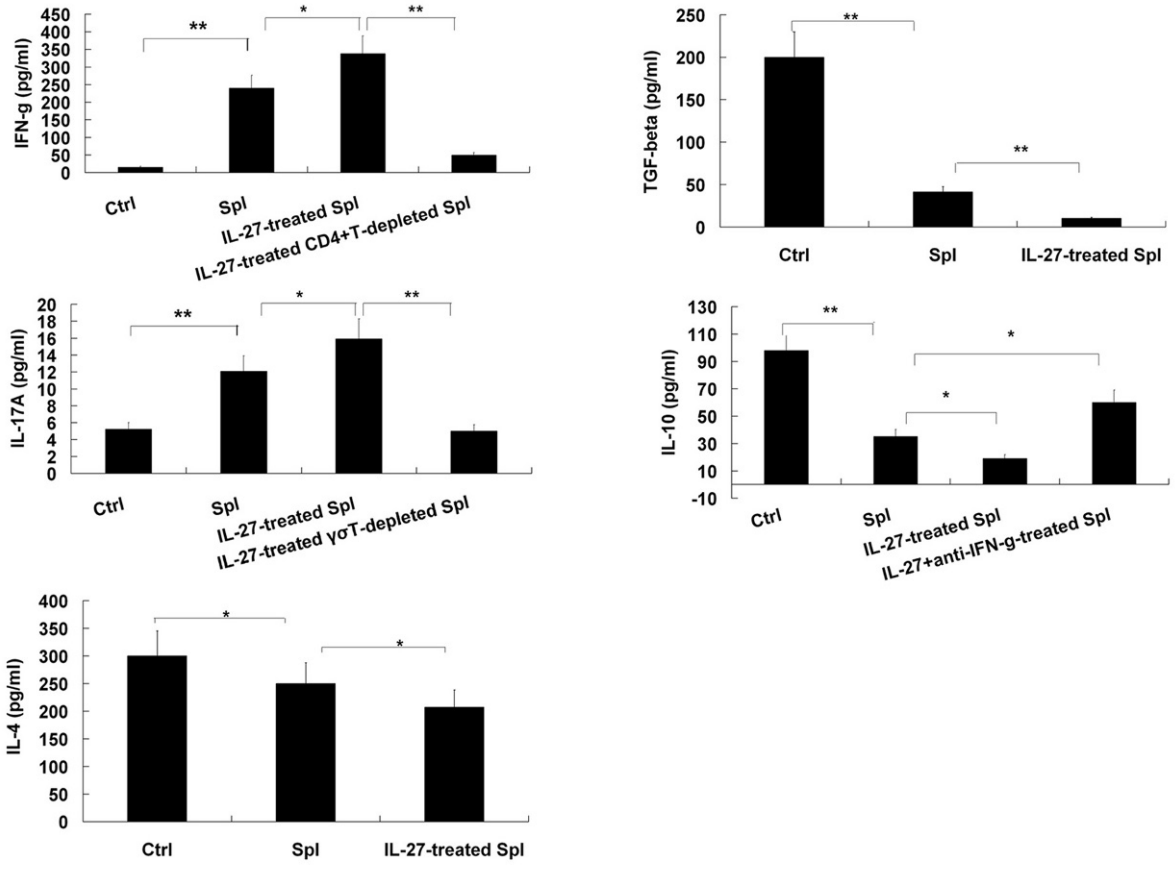

Figure 5. The effect of IL-27 on the production of cytokines in diabetic splenocytes. Splenocytes (Spl), $CD4^+$T-depleted Spl and γσT-depleted Spl from 12-wk diabetic female NOD mice were cultured *in vitro* for three days without, or in the presence of 10 ng/ml IL-27, or 10 ng/ml IL-27 and 10 μg/ml rat anti-mouse IFN-γ antibody. Spl from 12-wk nondiabetic male NOD mice were used as a control (Ctrl).The production of IFN-γ, IL-17A, IL-4, TGF-β and IL-10 was measured by ELISA from cell-free supernatants. The results are representative of three separate experiments, with three mice per group per experiment (*$P < 0.05$ and **$P < 0.01$).

shown to be expressed in EBV-transformed B cells, tonsils, spleens, and placental trophoblasts [18, 19]. In normal mice, IL-27 expression could not be detected. We also could not detect IL-27 expression in Balb/C or C57BL/6J (Fig. 1). However, IL-27 expression could be induced under some conditions [20]. IL-27 is also highly expressed in inflammatory bowel diseases [15, 21] and experimental autoimmune encephalomyelitis [22]. Here, we also detected IL-27 expression in autoimmune diabetic NOD mice (Fig. 1).

IL-27 is produced early by activated antigen-presenting cells in response to microbial infection. DCs are the relevant APCs which provoke an autoimmune response in NOD mice and not in other strains. We found that DCs could secrete IL-27 in diabetic NOD mice (Fig. 2). IL-27 synergizes with IL-12 in IFN-γ production by naive $CD4^+$ T cells [23]. We also found that the level of IFN-γ was high in diabetic NOD mice (Fig. 2). On the other hand, it also showed that IFN-γ could promote IL-27 production. In addition, we also found that IL-27 could up-regulated IL-23 (not shown). Thus, these pro-inflammation cytokines might be induced by each other.

Previous studies have shown that neutralizing the p28 subunit suppressed the ongoing adjuvant-induced arthritis [24]. To detect the role of IL-27 in diabetes, we used diabetes-transfer model. Blockade of IL-27 could reduce the incidence of diabetes (Fig. 3), while IL-27-treated diabetic splenocytes could promote diabetes onset or incidence (Fig. 4). This suggests that IL-27 is the most important key for diabetes in NOD mice. In addition, we also found that $CD4^+$T cells were critical for splenocyte-transferred diabetes and IL-27 promoted diabetes by affecting $CD4^+$T cells (Fig. 3, 4).

Generally, $CD4^+$Th cell differentiation was affected by the profile of Th cytokines. Therefore, we wanted to know which cytokine IL-27 could cause the effect. Previous studies also showed that rIL-27 could augment proliferation and secretion of IFN-γ by naive $CD4^+$ T cells [20]. We found IL-27 could up-regulate

IFN-γ secreted by $CD4^+$ T cells in diabetic splenocytes (Fig. 5). IL-27 might affect diabetes in NOD mice by promoting Th1 cell differentiation. The fact that the IL-12 knockout NOD mouse develops diabetes normally [13], makes us propose that while the IL-27 knockout NOD mouse may develop diabetes normally, IL-27 promotes the incidence of diabetes. This suggests that the mechanisms by which diabetes in NOD mice is induced are very complicated.

A new type IL-17-secreting Th cell, namely Th17 has shown a pathogenic role in other autoimmune diseases such as those of the central nervous system (CNS). We found that IL-17 was high and IL-27 could up-regulate the production of IL-17 in diabetic splenocytes (Fig. 5). However, these findings disagree with previous reports that IL-27 negatively regulates the development of Th17 cells during inflammation in the CNS [17]. These findings apparently disagree. We found that mainly γδT cells but not Th17 secreted IL-17 in NOD mice (unpublished data). IL-17-secreting γδT cells in NOD mice are currently under study. We here found that IL-27 could up-regulate the production of IL-17 secreted by γδT cells but not Th17 in diabetic splenocytes (Fig. 5). However, the mechanisms by which IL-27 regulated the secretion of IL-17 in γδT cells need further study.

The absence of regulatory Th2 cytokines such as IL-4 might have resulted in exacerbating diabetes in the NOD setting. We also found that the production of IL-4 decreased, and IL-27 could reduce the production of IL-4 in diabetic splenocytes (Fig. 5). Our results showed that IL-27 was an intrinsic change in cytokine production that altered Th1/Th2 polarization in NOD mice. This might be a mechanism by which IL-27 promoted diabetes in NOD mice. Based on the fact that IL-27 suppresses Th2 cell development and Th2 cytokines production from polarized Th2 cells, a novel therapeutic way for Th2-mediated allergic inflammation has been studied [23].

Previous studies have shown that IL-27 can suppress the differentiation of $CD4^+Foxp3^+$ Treg cells which suppress immune reactivity to self-antigens [25]. Now we know that TGF-β can induce the differentiation of $CD4^+Foxp3^+$ Treg cells. We found that IL-27 reduced the production of TGF-β in diabetic splenocytes. Compared with non-diabetic splenocytes, diabetic splenocytes secreted a low level of TGF-β. Thus, IL-27 might promote diabetes by suppressing the production of TGF-β.

Previous studies have shown that IL-27 can induce IL-10 secretion [16]. However, our findings disagree, as we found that IL-27 reduced the production of IL-10 in diabetic splenocytes (Fig. 5). IL-27 may mainly induce IFN-γ secretion in diabetic splenocytes. IFN-γ induced the differentiation of $CD4^+$T cells and suppressed the differentiation IL-10-secreting Tr1 in diabetic splenocytes. When IFN-γ was blocked, IL-27 promoted IL-10 secretion in diabetic splenocytes (Fig. 5). Thus, IL-27 mainly reduced IL-10 levels by indirectly up-regulating IFN-γ, although IL-27 to some extent may induce the IL-10 secretion in diabetic splenocytes.

In summary, IL-27 plays a pathogenic role in T-cell-mediated diabetes by suppressing anti-inflammatory cytokines and promoting pro-inflammatory cytokines.

Acknowledgements. This work was supported by National Nature and Science Funds 30571732 and National "973" Fund Grant (2007CB512406 and 2009CB522408). Conflicts of interest: The authors have no conflicts of interest.

1 Anderson, M. S. and Bluestone, J. A. (2005) The NOD mouse: a model of immune dysregulation. Annu. Rev. Immunol. 23, 447–485.
2 Delovitch, T. L. and Singh, B. (1997) The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunity 7, 727–738.
3 Rabinovitch, A. (1994) Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? Diabetes 43, 613–621.
4 Salomon, B., Lenschow, D. J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A. and Bluestone, J. A. (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12, 431–440.
5 Furuzawa-Carballeda, J., Vargas-Rojas, M. I. and Cabral, A. R. (2007) Autoimmune inflammation from the Th17 perspective. Autoimmun. Rev. 6, 169–175.
6 Bailey, S. L., Schreiner, B., McMahon, E. J. and Miller, S. D. (2007) CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4(t) T(H)-17 cells in relapsing EAE. Nat. Immunol. 8, 172–180.
7 Glimcher, L. H. and Murphy, K. M. (2000) Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes. Dev. 14, 1693–1711.
8 Murphy, K. M. and Reiner, S. L. (2002) The lineage decisions of helper T cells. Nat. Rev. Immunol. 2, 933–944.
9 Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. and Murphy, K .M. (2006) Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 24, 677–688.
10 10. Marleau, A. M. and Singh, B. (2002) Myeloid dendritic cells in non-obese diabetic mice have elevated costimulatory and T helper-1-inducing abilities. J. Autoimmun. 19, 23–35.
11 Liu, J. and Beller, D. I. (2003) Distinct pathways for NF-κB regulation are associated with aberrant macrophage IL-12 production in lupus- and diabetes-prone mouse strains. J. Immunol. 170, 4489–4496.
12 Trembleau, S., Penna, G., Bosi, E., Mortara, A., Gately, M. K. and Adorini, L. (1995) Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice. J. Exp. Med. 181, 817–821.
13 Trembleau, S., Penna, G., Gregori, S., Chapman, H. D., Serreze, D. V., Magram, J. and Adorini, L. (1999) Pancreas-infiltrating Th1 cells and diabetes develop in IL-12-deficient nonobese diabetic mice. J. Immunol. 163, 2960–2968.
14 Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Bazan, J. F., de-Waal-Malefyt, R., Rennick, D. and Kastelein, R. A. (2002) IL-27, a heterodimeric cytokine composed of EBI3 and p28

protein, induces proliferation of naive CD4(+) T cells. Immunity 16, 779–790.
15 Schmidt, C., Giese, T., Ludwig, B., Mueller-Molaian, I., Marth, T., Zeuzem, S., Meuer, S. C. and Stallmach, A. (2005) Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease:elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. Infl. Amm. Bowel. Dis. 11, 16–23.
16 Awasthi, A., Carrier, Y., Peron, J. P. S., Bettelli, Estelle., Kamanaka, M., Flavell, R. A., Kuchroo, V. K., Oukka, M. and Weiner, H. L. (2007) A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat. Immunol. 8, 1380–1389.
17 Colgan, J. and Rothman, P. (2006) All in the family: IL-27 suppression of T(H)-17 cells. Nat. Immunol. 7, 899–901.
18 Devergne, O., Birkenbach, M. and Kieff, E. (1997) Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. Proc. Natl. Acad. Sci. USA 94, 12041–12046.
19 Jianguo, L., Xiuqin, G. and Ma, X. (2007) Regulation of IL-27 p28 gene expression in macrophages through MyD88- and interferon-γ–mediated pathways. J. Exp. Med. 204, 141–152.
20 Devergne, O., Hummel, M., Koeppen, H., Le-Beau, M. M., Nathanson, E. C., Kieff, E. and Birkenbach, M. (1996) A novel interleukin-12 p40-related protein induced by latent Epstein-Barr virus infection in B lymphocytes. J. Virol. 70, 1143–1153.
21 Honda, K., Nakamura, K., Matsui, N., Takahashi, M., Kitamura, Y., .,Mizutani, T., Harada, N., Nawata, H., Hamano, S. and Yoshida, H. (2005) T helper 1-inducing property of IL-27/WSX-1 signaling is required for the induction of experimental colitis. Infl. Amm. Bowel. Dis. 11, 1044–1052.
22 Li, J., Gran, B., Zhang, G. X., Rostami, A. and Kamoun, M. (2005) IL-27 subunits and its receptor (WSX-1) mRNAs are markedly up-regulated in infl ammatory cells in the CNS during experimental autoimmune encephalomyelitis. J. Neurol. Sci. 232, 3–9.
23 Yoshimoto, T., Yoshimoto, T., Yasuda, K., Mizuguchi, J. and Nakanishi, K. (2007) IL-27 suppresses Th2 cell development and Th2 cytokines production from polarized Th2 cells: a novel therapeutic way for Th2-mediated allergic inflammation. J. Immunol. 179, 4415–4423.
24 Goldberg, R., Wildbaum, G., Zohar, Y., Maor, G. and Karin, N. (2004) Suppression of ongoing adjuvant-induced arthritis by neutralizing the function of the p28 subunit of IL-27. J. Immunol. 173, 1171–1178.
25 Huber, M., Steinwald, V., Guralnik, A., Brüstle, A., Kleemann, P., Rosenplänter, C., Decker, T. and Lohoff, M. (2008) IL-27 inhibits the development of regulatory T cells via STAT3. Int. Immunol. 20, 223–234.