## IMMUNOLOGY ORIGINAL ARTICLE

# Interleukin-17A deficiency ameliorates streptozotocin-induced diabetes

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## Summary

Interleukin-17 (IL-17) is a cytokine with critical functions in multiple autoimmune diseases. However, its roles in type I diabetes and the underlying mechanisms remain to be fully elucidated. In the current study, we investigated the impact of IL-17 deficiency on streptozotocin (STZ) -induced diabetes. $Il\text{-}17^{-/-}$ mice exhibited attenuated hyperglycaemia and insulitis after STZ treatment compared with control mice. The $Il\text{-}17^{-/-}$ mice had fewer $CD8^+$ cells infiltrating the pancreas than wild-type controls after STZ injection. Wild-type mice showed increased percentage and number of splenic $CD8^+$ cells and decreased $Gr1^+ CD11b^+$ myeloid-derived suppressor cells (MDSC) after STZ treatment, but $Il\text{-}17^{-/-}$ mice maintained the percentages and numbers of splenic $CD8^+$ cells and MDSC, suggesting that IL-17 is implicated in STZ-induced cellular immune responses in the spleen. We further purified the MDSC from spleens of STZ-treated mice. $Il\text{-}17^{-/-}$ MDSC showed increased ability to suppress $CD8^+$ cell proliferation in vitro compared with wild-type MDSC. Transfer of MDSC to diabetic mice showed that MDSC from $Il\text{-}17^{-/-}$ mice could ameliorate hyperglycaemia. Moreover, recipients with MDSC from $Il\text{-}17^{-/-}$ mice had a decreased percentage of $CD8^+$ cell in the spleen compared with recipients with MDSC from wild-type mice. These data suggest that IL-17 is required in splenic MDSC function after STZ delivery. In summary, our study has revealed a pathogenic role of IL-17 in an STZ-induced diabetes model with important implications for our understanding of IL-17 function in autoimmune diseases.

**Keywords:** diabetes; interleukin-17A; myeloid-derived suppressor cells; streptozotocin.

## Introduction

Interleukin-17 (IL-17) is an inflammatory cytokine secreted by T cells. Six IL-17 family members (IL-17A–F) have been described, and the prototype member of the family is IL-17A, often termed IL-17 in literature. Interleukin-17 mediates pro-inflammatory responses and induces many other cytokines, such as IL-6, tumour necrosis factor-$\alpha$, transforming growth factor-$\beta$, in various cell types.[1] It is important in proliferation, maturation and invasion of neutrophils[2] and is one of the major effectors of T helper type 17 cells, which exhibited pivotal roles in autoimmune disease. Adoptively transferred T helper type 17 cells could stimulate $CD8^+$ cytotoxic T lymphocytes and promote diabetes development.[3] Moreover, increasing evidence showed that other IL-17-producing cells also play important roles in diabetes.[4,5]

However, the roles of IL-17 in different diabetes models remained controversial. In non-obese diabetic (NOD) mouse models, IL-17 neutralization treatment could prevent the development of diabetes.[6] Interleukin-17-deficient ($Il\text{-}17^{-/-}$) NOD mice showed delayed onset of diabetes.[7] On the other hand, an IL-17 knockdown

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Abbreviations: FACS, fluorescence activated cell sorting; MDSC, myeloid derived suppressor cells; NOD, nonobese diabetic; STZ, streptozotocin

NOD line created by directly introducing a short hairpin RNA construct did not show altered diabetes susceptibility.[8] Neutralization of IL-17 *in vivo* did not protect NOD-severe combined immunodeficiency mice against diabetes transferred by diabetic splenocytes.[9] Moreover, IL-17 production was undetectable in CD4^{+} or CD8^{+} T cells in the CD8-driven lymphocytic choriomeningitis virus-induced model of type 1 diabetes.[10] At the same time, *in vitro* data showed that IL-17 markedly promoted induced nitric oxide production and nitric oxide-dependent toxicity in mouse pancreatic islet cells.[11] However, IL-17 may be more involved in affecting immune responses in secondary lymphoid tissues rather than directly mediating beta cell injury, as the IL-17-positive cells were relatively rare in the pancreas during diabetes development.[6]

Myeloid-derived suppressor cells (MDSC) represent a population of immature myeloid cells with the ability to suppress various T-cell functions.[12] MDSC with hallmarks of surface Gr-1 and CD11b in mice accumulate during many pathological conditions including cancer and autoimmunity.[13] MDSC have been shown to play a protective role in diabetes.[14,15] In tumours, IL-17 was reported to promote MDSC recruitment[16] and mediate MDSC-induced tumour immune responses.[17] In diabetes, reports describing the potential link between IL-17 and MDSC in diabetes are currently lacking.

In this study, we examined the effects of IL-17 deficiency on streptozotocin (STZ) -induced diabetes. Our findings indicate that IL-17 is implicated in STZ-induced immune responses in both pancreas and spleen.

## Materials and methods

### Animal models

Six- to eight-week-old *Il-17*^{−/−} mice from Dr Yoichiro Iwakura[18] and wild-type C57BL/6 mice were used for diabetes models. Mice after 4 hr fast were injected intraperitoneally with STZ (50 mg/kg of body weight) in citrate buffer (Sigma, St Louis, MO, USA) or an equivalent volume of citrate buffer daily for five consecutive days. Blood glucose levels were measured before first injection and after final injection between 9 a.m. and 10 a.m. All mice were housed in pathogen-free facilities on a 12-hr light- dark cycle. All protocols and procedures in this study were approved by the Ethics Committee of Wuhan University (Permission No. SYXK2008-0013).

### ELISA

Mouse serum samples were obtained and stored at -20°C until the ELISA. Insulin concentrations were determined using ELISA kits (Millipore, Billerica, MA, USA) as recommended by the assay manufacturer.

### Histology

Pancreatic slides from three mice in each group on Day 35 after injection were analysed. A minimum of 30 islets stained with haematoxylin & eosin from each mouse were examined microscopically by two different observers for the presence of insulitis. The severity of insulitis was scored as follows: 0, no lymphocytic infiltration; 1, lymphocytic infiltration occupying < 25% of the total islet cell area; 2, lymphocytic infiltration occupying 25–49% of the total islet cell area; 3, lymphocytic infiltration occupying 50–75% of the total islet cell area; 4, lymphocytic infiltration occupying > 75% of the total islet cell area, or small retracted islets.

### Flow cytometry

Peripheral blood samples were collected and red cells were removed using Red Blood Cell Lysing Buffer (Sigma). Splenocytes were isolated from freshly obtained spleen specimens after grinding and filtering. After depleting red cells, single splenic cell suspensions were collected for further staining. Single pancreatic cell suspensions were prepared as previous described.[19] For surface staining, cells were stained for 30 min with fluorescently labelled antibodies against surface molecules: CD4 (clone: RM4-5), CD8 (clone: H35-17.2), Gr-1 (clone: RB6-8C5), Ly6G (clone: 1A8), Ly6C (clone: HK1.4), F4/80 (clone: BM8), CD11b (clone: M1/70), and NK1.1 (clone: PK136) (eBioscience, San Diego, CA, USA). For Foxp3 (clone: FJK-16s) staining, cells were fixed, permeabilized using buffers and then stained with labelled antibodies (eBioscience). The cell suspensions were analysed or sorted by FACS (BD Aria III; BD, Franklin Lakes, NJ, USA). Gates were set based on the staining profile of the isotype controls.

### Adoptive transfer

The MDSC (Gr1^{+} and CD11b^{+}) were purified by flow cytometric sorting from the spleens of *Il-17*^{−/−} mice and wild-type mice treated with STZ on Day 24. The MDSC ($2 \times 10^5$) from one mouse were intravenously injected into the tail vein of another wild-type diabetic mouse treated with STZ on Day 13. There were 12 recipients in total, with six in each group.

### CD8^{+} cells and MDSC co-culture

The culture medium is RPMI-1640 plus 10% heat inactivated fetal calf serum (Gibco, Gaithersburg, MD, USA) and 10 mM L-glutamine. 96-well plates were pre-coated with 50 μl PBS per well containing 5 μg/ml LEAF™ purified anti-mouse CD3 and CD28 (Biolegend, San Diego, CA, USA) at 4°C overnight. FACS-sorted splenic CD8^{+} cells from diabetic mice were labelled with 5 μM CFSE

(Sigma) for 5 min, washed and divided into 100 μl ($2 \times 10^5$ cells) per well. FACS-sorted splenic Gr1$^+$ CD11b$^+$ cells (MDSC) from STZ-treated mice were collected and added into CD8$^+$ cells (100 μl, $2 \times 10^5$ cells per well). The cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37°C for 96 hr. Then, 50 μl of supernatant was collected for interferon-γ ELISA (eBioscience). Cells were collected and analysed by FACS. Proliferation Indices were analysed using MODFIT LT software (Verity Software House, Topsham, ME, USA).

### Quantitative RT-PCR

RNA was extracted using Rneasy mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using an RT Kit (Invitrogen, Carlsbad, CA, USA). Quantitative PCR analyses were performed using the SYBR Green PCR Master Mix (Takara, Shiga, Japan). The relative RNA levels of each gene were normalized against β-actin. Primers used were: *Arg1*: 5′-TAC AAG ACA GGG CTC CTT TCA G-3′ and 5′-CCG TTG AGT TCC GAA GCA AG-3′. *iNOS*: 5′-TCC TGG ACA TTA CGA CCC CT-3′ and 5′-CTC TGA GGG CTG ACA CAA GG-3′. *Il-10*: 5′-GGT TGC CAA GCC TTA TCG GA-3′ and 5′-GGG GAG AAA TCG ATG ACA GC-3′. *β-actin*: 5′-TGA AGA TCA AGA TCA TTG CTC CTC-3′ and 5′-CCT GCT TGC TGA TCC ACA TC-3′.

### Statistical analysis

All values are expressed as mean ± SE of the mean. Differences were analysed using Student's *t*-test and $P < 0.05$ was considered statically significant. The severity of the insulitis was analysed by Wilcoxon signed rank test.

## Results

### *Il-17* deficiency attenuates STZ-induced diabetes

To understand the function of IL-17 in diabetes, we established multiple low-dose STZ models of *Il-17*$^{-/-}$ mice and the blood glucose levels were monitored (Fig. 1a). Both *Il-17*$^{-/-}$ and wild-type mice showed comparably increased glucose levels after STZ injection for 15 days. Wild-type control mice maintained a hyperglycaemic state (> 15 mM) from Day 18 to Day 35 before being killed (19·0 ± 3 mM). In contrast, *Il-17*$^{-/-}$ mice treated with STZ exhibited dramatically lower glucose levels from Day 18 to Day 35 before being killed (12·7 ± 2 mM).

In agreement with the blood glucose changes, *Il-17*$^{-/-}$ mice had greatly increased levels of serum insulin compared with wild-type mice on Day 35 after STZ treatment (*Il-17*$^{-/-}$: 203 ± 31 pg/ml versus wild-type: 70 ± 11 pg/ml) (Fig. 1b). We then examined the impact of *Il-17* knockout on the progression of insulitis. Wild-type mice
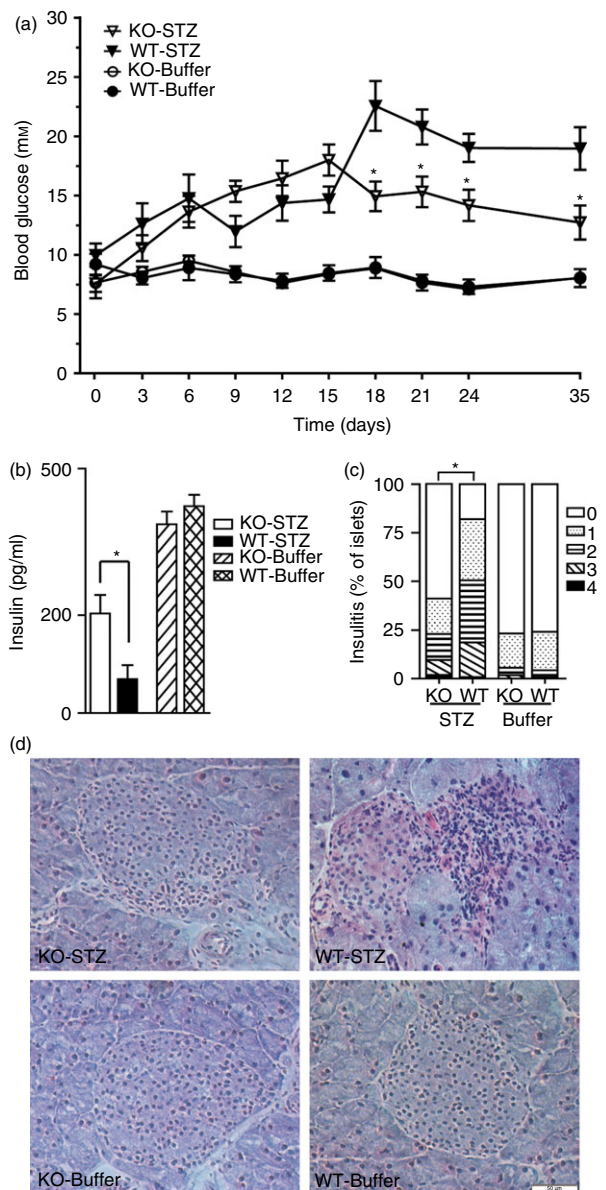


**Figure 1.** *Il-17* deficiency attenuated streptozotocin (STZ) -induced diabetes. Multiple low dose of STZ were injected into *Il-17*$^{-/-}$ (KO) and wild-type (WT) mice; citrate buffer was injected as control. (a) Blood glucose levels were measured before the first injection (Day 0) and after the final injection from Days 1 to 35. (b) The serum insulin levels of four group mice were determined on Day 35. Each group $n = 10$. Student's *t*-tests were performed between the STZ-treated groups. (c) The severity of insulitis was scored as described in the Materials and methods section. Levels of insulitis: 0 (white), 1 (dotted), 2 (horizontal stripes), 3 (diagonal stripes), and 4 (black). Wilcoxon signed rank test analysis was performed to analysis the insulitis between the STZ-treated groups. *$P < 0.05$. (d) Haematoxylin & eosin staining of pancreatic sections of four group mice. Images are representative of major islets in each group. Bar = 50 μm.

showed dramatically increased leucocyte infiltration into the islets after STZ treatment. In contrast, *Il-17*$^{-/-}$ mice exhibited moderate insulitis after STZ treatment (Fig. 1c,

d). Together, *Il-17*$^{-/-}$ mice showed attenuated multiple low doses of STZ-induced diabetes.

## *Il-17* deficiency inhibits CD8$^+$ T-cell infiltration into pancreas after STZ treatment

To characterize the inflammatory cellular response induced by STZ treatment in *Il-17*$^{-/-}$ mice, we quantified several immune cell subsets in the pancreas. Both *Il-17*$^{-/-}$ and wild-type mice showed increased percentages and numbers of CD4$^+$, CD8$^+$, Gr-1$^+$ CD11b$^+$ and NK$^+$ cells on Day 24 after STZ injection (Fig. 2a and c). However, *Il-17*$^{-/-}$ mice had significantly lower percentages and numbers of CD8$^+$ cells than wild-type mice after STZ treatment (Fig. 2a, c and e). We further quantified the immune cell subsets in peripheral blood. The *Il-17*$^{-/-}$ and wild-type mice had similar percentages and numbers of CD4$^+$, CD8$^+$ and NK$^+$ cells in peripheral blood (Fig. 2b and d). *Il-17*$^{-/-}$ mice showed lower percentages and lower numbers of Gr1$^+$ cells and Gr1$^+$ CD11b$^+$ (MDSC) in peripheral blood than wild-type mice. After STZ injection, while *Il-17*$^{-/-}$ mice maintained the levels of Gr1$^+$ cells and MDSC, wild-type mice had dramatically decreased Gr1$^+$ cells and MDSC. In outcome, *Il-17*$^{-/-}$ mice exhibited even higher percentages and numbers of Gr1$^+$ cells and MDSC than wild-type controls after STZ treatment (Fig. 2b, d and f). Together with other reports,[6] we speculated that IL-17 may be more involved in affecting immune responses in the spleen than in the lesion tissue.

## *Il-17* deficiency affects CD8$^+$ cell and MDSC percentages in spleens after STZ treatment

The four groups of mice (*Il-17*$^{-/-}$ mice treated with/without STZ, wild-type mice with/without STZ) had no significant differences in the percentages of CD4$^+$, Gr-1$^+$ and NK$^+$ cells in the spleen (see Supplementary material, Fig. S1). Mice treated with STZ showed increased percentages of CD4$^+$ Foxp3$^+$ (regulatory T) cells after STZ treatment from Day 12, but no significant difference between *Il-17*$^{-/-}$ and wild-type groups (see Supplementary material, Fig. S1). Notably, wild-type mice exhibited gradually increased percentages and numbers of CD8$^+$ cells from Day 15 after STZ injection, which was consistent with published literature on the important roles of CD8$^+$ T cells in the STZ model.[4] At the same time, *Il-17*$^{-/-}$ mice treated with STZ maintained the percentages and numbers of CD8$^+$ cells at the basal levels found in control mice treated with buffer (Fig. 3a and c). On the other hand, wild-type mice showed gradually decreased percentages and numbers of Gr1$^+$ CD11b$^+$ (MDSC) from Day 15 after STZ injection, and *Il-17*$^{-/-}$ mice treated with STZ maintained the percentages and numbers of MDSC at the basal levels of the control mice treated with buffer (Fig. 3b and d). Hence, *Il-17*$^{-/-}$ mice exhibited lower percentages and

numbers of CD8$^+$ cells and higher MDSC than wild-type mice after STZ treatment. These data suggest that IL-17 is implicated in the regulation of splenic CD8$^+$ cells and MDSC during STZ-induced diabetes development.

To further characterize the splenic MDSC from mice treated with STZ, we examined these cells with Ly6G, Ly6C and F4/80 staining. Splenic MDSC from both *Il-17*$^{-/-}$ and wild-type mice had Ly6G$^+$ and Ly6C$^+$ subsets with Ly6C$^+$ cells as a major cell type (Fig. 3e). Hence, CD11b$^+$ Ly6C$^+$ cells contributed mainly to the increased MDSC in *Il-17*$^{-/-}$ mice on Day 15 after STZ treatment. At the same time, there was no significant difference in the percentages of F4/80$^+$ cells in the splenic MDSC between *Il-17*$^{-/-}$ and wild-type mice (61·5 ± 3·2% versus 58·4 ± 2·5%) (Fig. 3e).

## *Il-17*-deficient MDSC ameliorated STZ-induced hyperglycaemia

To characterize the roles of these MDSC, we purified Gr1$^+$ CD11b$^+$ cells from the spleens of *Il-17*$^{-/-}$ and wild-type mice on Day 24 after STZ treatment. As shown in Fig. 4(a), MDSC from STZ-treated *Il-17*$^{-/-}$ mice expressed dramatically higher levels of *Il-10*, and similar levels of *arg1* and *iNOS* compared with those in wild-type mice. We further co-cultured splenic CD8$^+$ cells with the purified MDSC *in vitro*. The proliferation indices of CD8$^+$ cells stimulated by CD3 and CD28 antibodies reached 8·43 ± 0·21. Whereas the proliferation indices of CD8$^+$ cells decreased to 4·88 ± 0·39 after wild-type MDSC were added, they decreased even lower to 4·46 ± 0·31 after *Il-17*$^{-/-}$ MDSC were added (Fig. 4b, and see Supplementary material, Fig. S2). At the same time, the interferon-$\gamma$ production by CD8$^+$ cells showed a slight decrease after MDSC addition, but no significant difference was found among the groups (Fig. 4c). Next, we transferred the isolated MDSC into STZ-treated wild-type mice on Day 13 (Fig. 4d). Both groups (recipients of *Il-17*$^{-/-}$ MDSC, recipients of wild-type MDSC) showed increased blood glucose levels. However, the recipients of *Il-17*$^{-/-}$ MDSC had significantly lower blood glucose levels than recipients of wild-type MDSC (Fig. 4d). We further analysed the spleens of recipients on Day 24 after STZ treatment. The recipients of *Il-17*$^{-/-}$ MDSC exhibited increased expression levels of *iNOS* and *Il-10* (Fig. 4e) and a significantly lower percentage of CD8$^+$ cells than recipients of wild-type MDSC (Fig 4f). These data indicated that *Il-17*$^{-/-}$ MDSC could ameliorate STZ-induced hyperglycaemia with increased *Il-10* expression and suppressed CD8$^+$ cells involved.

## Discussion

Our current study indicates that IL-17 plays a pathogenic role in STZ-induced diabetes. *Il-17*$^{-/-}$ mice exhibited attenuated hyperglycaemia after Day 18 but not
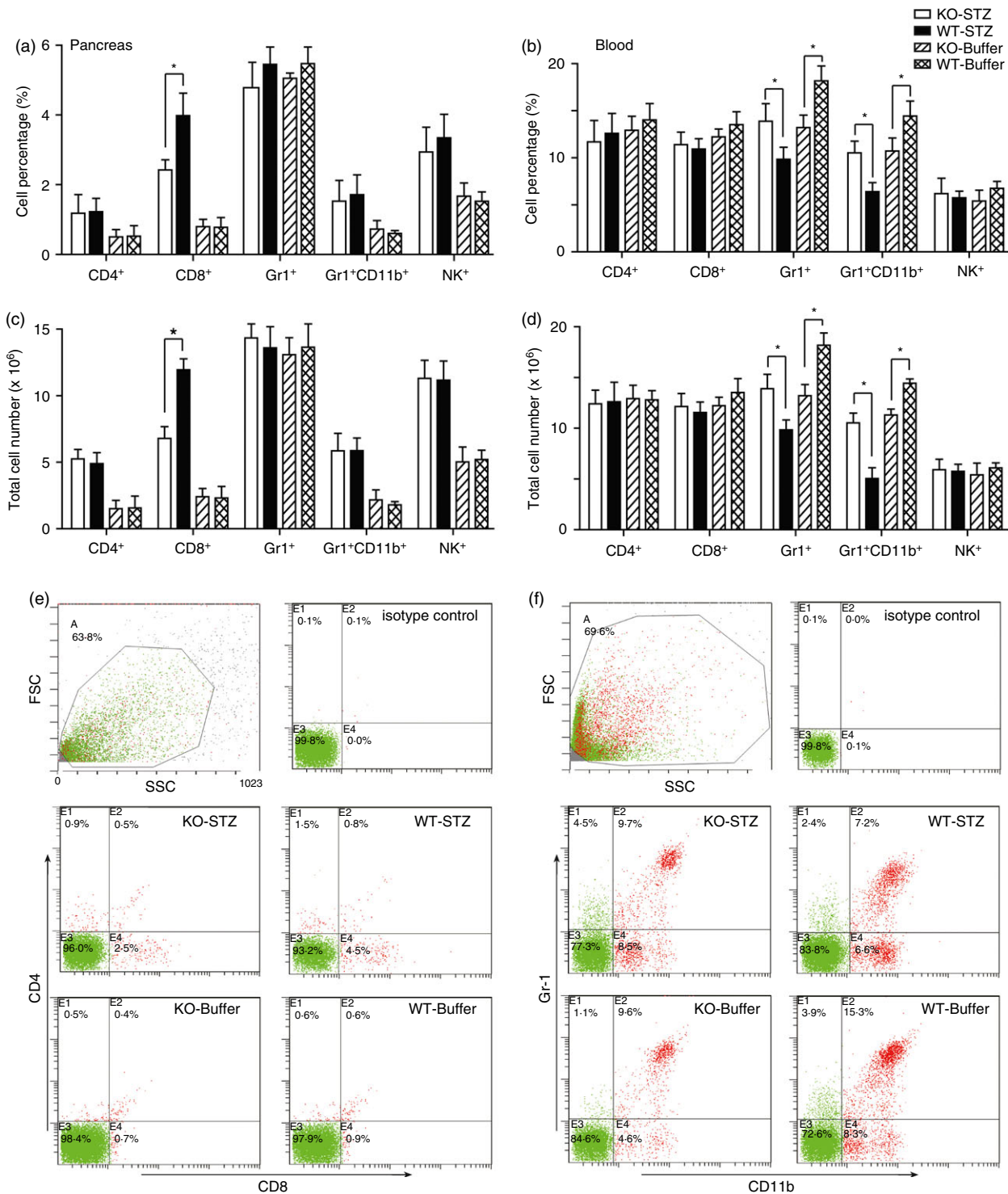
Figure 2. *Il-17* deficiency inhibited CD8^{+} cell infiltration into pancreas after streptozotocin (STZ) treatment. FACS analysis results of pancreatic cells (a and c) and peripheral blood cells (b and d) after CD4, CD8, Gr-1, CD11b, or NK1.1 staining. Single pancreatic cells and peripheral blood cells were prepared from *Il-17*^{−/−} and wild-type mice on Day 24 after STZ or buffer injections. Each group consisted of at least three mice. *$P < 0.05$. Representative plots from FACS analysis of pancreatic cells (e) and peripheral blood cells (f) are shown.

before Day 15 after STZ treatment, suggesting that IL-17 could be more critical in the late phase of STZ induced diabetes development rather than the onset. In support-

ing this statement, IL-17R knockout mice also had decreased levels of blood glucose and islet inflammation in the STZ model.[4] However, IL-17 was reported to
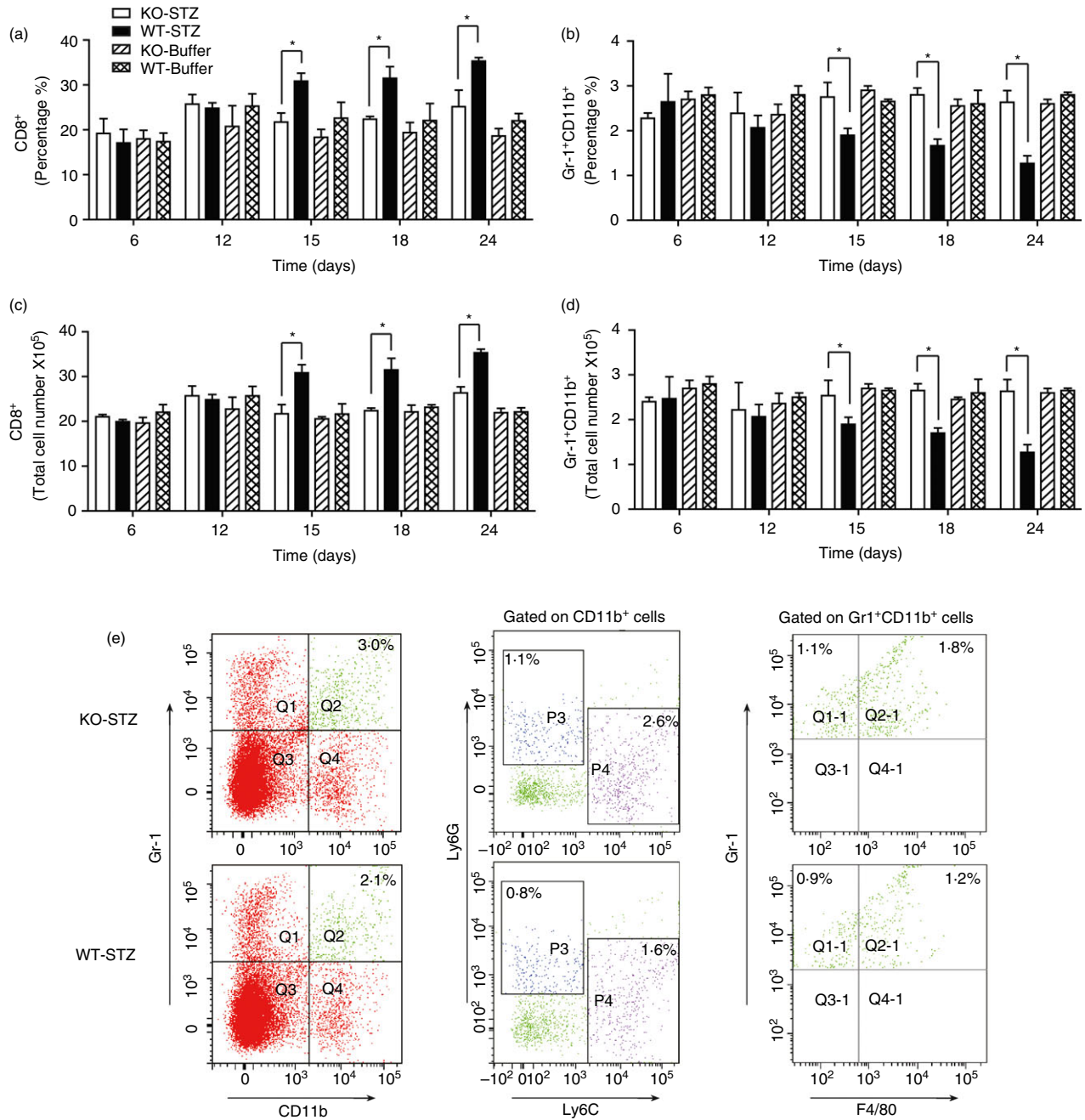
**Figure 3.** *Il-17* deficiency affected numbers of CD8$^+$ cells and myeloid-derived suppressor cells (MDSC) in spleen after streptozotocin (STZ) treatment. FACS analysis results of splenocytes after CD8 (a and c), Gr-1 and CD11b (b and d) staining. Single splenic cells were prepared from *Il-17*$^{-/-}$ and wild-type mice at various time-points after STZ or buffer injection. (e) Representative plots from FACS analysis of the splenic Gr-1$^+$ CD11b$^+$ cells with Ly6C, Ly6G and F4/80. Splenic cells were prepared from *Il-17*$^{-/-}$ and wild-type mice on Day 15 after STZ injections. Each group consisted of at least three individual mice. *$P < 0.05$.

have different roles in NOD mouse models.[6–9] The explanation could be that the NOD model is more complicated, developing spontaneous autoimmune diabetes with the various IL-17-producing T subset cells involved: Th17/Tc17,[4] invariant natural killer T,[5,20] $\gamma\delta$T.[9]

Our findings indicate that IL-17 deficiency inhibits the increase of pancreatic and splenic CD8$^+$ cells after STZ injection. The pancreatic infiltration of T cells is a critical process during the development of diabetes induced by multiple low doses of STZ.[21] Moreover, the splenic immune response including CD8$^+$ cells is also important
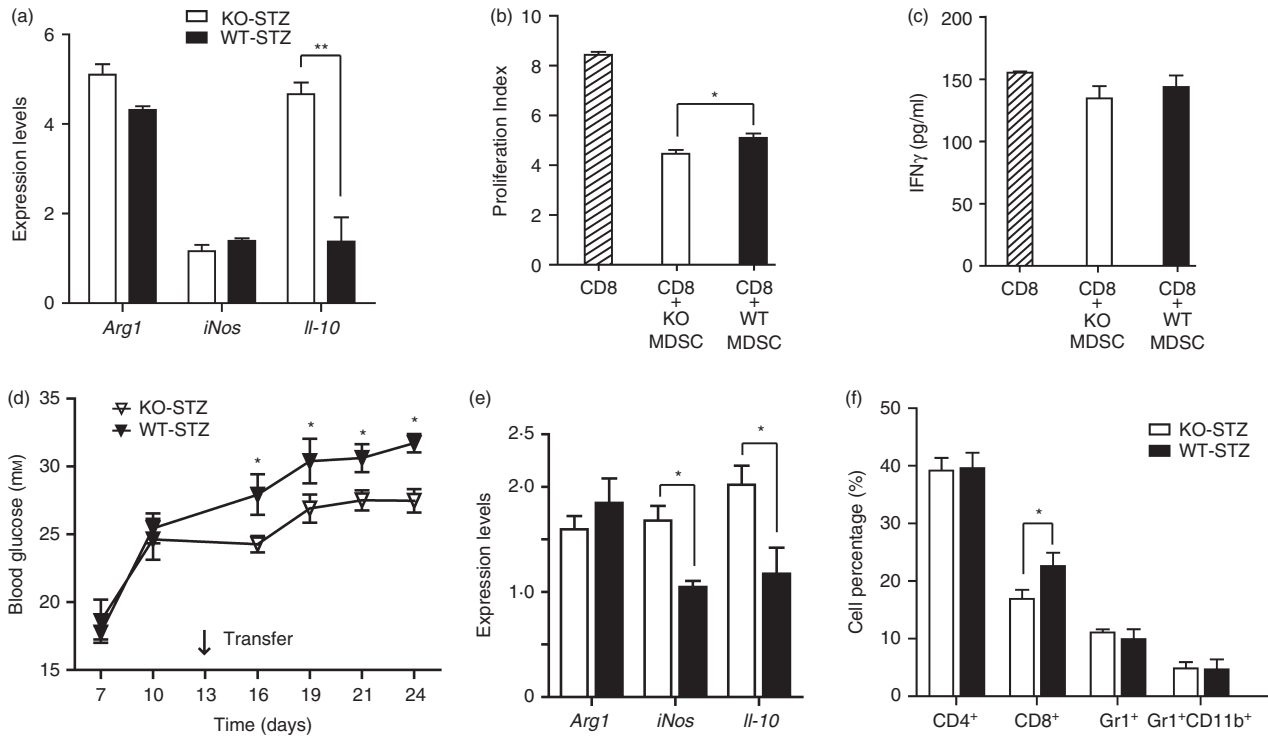
**Figure 4.** *Il-17*-deficient myeloid-derived suppressor cells (MDSC) ameliorated streptozotocin (STZ) -induced hyperglycaemia. (a) Quantitative RT-PCR analysis of the MDSC purified from spleens of *Il-17*$^{-/-}$ and wild-type mice on Day 24 after STZ treatment. Each group $n = 3$. (b) The proliferation indices of CD8 cells co-cultured with MDSC purified from spleens of *Il-17*$^{-/-}$ or wild-type mice on Day 24 after STZ treatment. (c) The concentrations of interferon-γ (IFN-γ) in the co-culture medium. Each group $n = 3$. (d) Blood glucose levels of the recipient mice were measured after final STZ injection from Days 7 to 24. On Day 13, the wild-type mice received MDSC ($2 \times 10^5$/mouse) from *Il-17*$^{-/-}$ or wild-type mice treated with STZ. Each group $n = 6$. (e) Quantitative RT-PCR analysis of spleens from the recipients on Day 24. Each group $n = 3$. (f) FACS analysis results of splenocytes from the recipients on Day 24. Each group $n = 3$. *$P < 0.05$.

in diabetes,[22] and the administration of anti-CD8 antibodies resulted in a protective effect.[23] In our study, the time-point (from Day 15 after STZ injection) of changed splenic CD8$^+$ cell percentages was the same as that of the shift in blood glucose in *Il-17*$^{-/-}$ mice treated with STZ, suggesting that the decreased splenic CD8$^+$ cells of *Il-17*$^{-/-}$ mice may be implicated in diabetes resistance. These data suggested that IL-17 plays important roles in both pancreatic and splenic immune responses during diabetes.

Our study provides evidence that IL-17 plays a role in STZ-induced changes of splenic MDSC. Under normal conditions, IL-17 deficiency alone is not sufficient to change the splenic MDSC (Fig. 3). When STZ is administrated, auto-antigens are released from islets and the splenic MDSC percentage decreases. IL-17 deficiency could completely block this decrease, suggesting that IL-17 has an important role during this process. At the same time, IL-17 deficiency could affect the splenic MDSC function after STZ treatment. *Il-17*$^{-/-}$ MDSC showed increased ability to suppress CD8$^+$ cell proliferation *in vitro* and a protective role after adoptive transfer in diabetic mice (Fig. 4). These results were inconsistent with the fact that

IL-17 is important for MDSC function in the tumour sites[24,25] and infection.[26,27] Further studies are needed, and our study represents a first step towards a better understanding of the connections of IL-17 and MDSC in diabetes.

In summary, our study revealed a pathogenic role of IL-17 in STZ-induced diabetes and the IL-17-dependent splenic immune response involved. Future study of the molecular signalling will shed new light on the systemic understanding of IL-17 function in diabetes.

## Acknowledgements

## Disclosures

The authors declare no commercial or financial conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1**. *Il-17* deficiency did not affect CD4$^+$, Foxp3$^+$ and natural killer (NK) cell percentages in spleen after streptozotocin (STZ) treatment. FACS analysis results of splenocytes after CD4 (a), Foxp3 (b), Gr-1 (c) and NK (d) staining. Single splenic cells were prepared from *Il-17$^{-/-}$* and wild-type mice at various time-points after STZ or buffer injection. Each group consisted of at least three individual mice. *$P < 0.05$.

**Figure S2**. *Il-17* deficiency promoted the ability of myeloid-derived suppressor cells (MDSC) to suppress CD8$^+$ cell proliferation *in vitro*. Representative FACS analysis data of CFSE-labelled CD8$^+$ cells co-cultured with MDSC purified from spleens of *Il-17$^{-/-}$* or wild-type mice on Day 24 after streptozotocin (STZ) treatment.