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RESEARCH ARTICLE

Interleukin 10 Suppresses the Function of Mouse Bone Marrow-Derived Dendritic Cells Infected with Classical Swine Fever Virus C-Strain

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ABSTRACT

Interleukin (IL)-10 inhibits the functions of antigen-presenting cells (APCs), including dendritic cells (DCs), however, the precise mechanism of action of IL-10 has not been fully elucidated. In this work, the effects of IL-10 on classical swine fever virus (CSFV) C-strain-infected mouse bone marrow-derived immature DCs (BM-imDCs) were studied. Additional IL-10 suppressed the maturation of the infected BM-imDCs by down-regulating the expression levels of the surface molecules CD80, CD86 and major histocompatibility complex (MHC) classII, while the autocrine IL-10 had no significant effect on the maturation status of the cells. Both additional and autocrine IL-10 markedly inhibited the secretion production of IL-12P40 derived from the BM-imDCs infected with the C-strain, and reduced the capacity of DCs to promote allogeneic naive T cell proliferation. These results showed that IL-10 may play an important role in the DCs-dependent immune response induced by CSFV C-strain.

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INTRODUCTION

Classical swine fever (CSF) is a highly contagious disease of domestic pigs and wild boars which occurs worldwide. Highly virulent strains of CSF virus (CSFV) cause high mortality and immune suppression (Nandi *et al*., 2011). Efficient attenuated vaccines against CSFV play important role in the control of CSF epidemics in domestic and wild swine (Dong and Chen, 2007; Kaden *et al*., 2010). Among these vaccines, the C-strain may be the most familiar and widely used vaccine in the world on the basis of its efficiency and safety. The C-strain vaccine guaranteed high protection rates and had no virulence in pigs even in young piglets and pregnant sows (Kortekaas *et al*., 2011; Graham *et al*., 2012).

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and were discovered relatively recently (Denning *et al*., 2011; Idoyaga and Steinman, 2011). DCs link innate and adaptive immunity to prevent and coordinate the responses to various diseases, and play important role in the generation of primary T-cell response and immune tolerance (Steinman and Banchereau, 2007; Wu and Liu, 2007). Immature DCs (imDCs) have the potent capacity of antigen uptake, and mature DCs (mDCs) can present antigens efficiently to rare antigen-specific T cells for initiation of immune responses (Mobergslien and Sioud, 2012). The maturation of DCs enhances the expression of surface molecules such as major histocompatibility complex class I (MHC I), MHC II, co-stimulatory molecules (CD80, CD83 and CD86), and cell adhesion molecules (Morelli and Thomson, 2007). DCs control the type of immune responses by releasing different cytokines (Banchereau and Steinman, 1998). The study on the interactions of DCs with virulent viruses and live attenuated vaccines gives an insight into the pathogenesis and immunologic mechanisms utilized by various pathogens.

Interleukin (IL)-10 is the most important cytokine with anti-inflammatory properties, and is known as the cytokine synthesis inhibitory factor (Moore *et al*., 2012). IL-10 inhibits synthesis of T helper (Th) 1 cytokines and proliferation of T cells, and acts as a co-stimulatory signal for mast cells, developing the Th2 response. IL-10 suppresses the maturation and functions of APCs by antagonizing the expression of co-stimulatory molecules (Serebrennikova *et al*., 2012). The cytokine also blocks the maturation of DCs, and the autocrine IL-10 regulates the functions of DCs (Corinti *et al*., 2001).

IL-10 and IL-12 expressed by splenic DCs participated in the induction of the adaptive immune

responses to CSFV (Sanchez-Cordon *et al*., 2005a, 2005b). In vivo, infection with CSFV highly virulent strain significantly increased the production of IL-10 in serum, and the number of DCs expressing IL-10 was high in the spleen of the infected pigs (Jamin *et al*., 2008). Therefore, IL-10 could play a role in the disruption of immune system cells, either inducing apoptosis or impairing DC maturation. Our previous study indicated that CSFV C-strain can infect and replicate in mouse bone marrow-derived immature DCs (BM-imDCs). The infection of BM-imDCs with the C-strain promotes cell maturation and lymphocyte proliferation, and induces a stronger Th1 response and a simultaneous Th2 response (currently unpublished). In this work, in order to explore the role of IL-10 in CSFV infection and the immunologic mechanism of action of the C-strain, the effects of IL-10 and anti-IL-10 on the BM-imDCs infected with the C-strain were studied.

MATERIALS AND METHODS

Mice, CSFV C-strain vaccine and reagents: Five week-old female SPF (specific pathogen free) BALB/c mice were provided by the laboratory animal center of Lanzhou University, China. The freeze-dried CSFV C-strain vaccine was purchased from Qingdao Boite Biopharmaceutical Co., Ltd (Qingdao, China). Fetal bovine serum (FBS) and RPMI medium 1640 were purchased from Gibco Inc. (Australia). Mouse IL-4, IL-10, anti-mouse IL-10, recombinant mouse granulocyte-macrophage colony stimulating factor (rmGM-CSF) and the fluorescent antibodies labeled with PE (anti-mouse CD11c, CD80, CD86 and MHC class II) were obtained from eBioscience Inc. (San Diego, CA, USA). The enzyme linked immunosorbent assay (ELISA) kits for the detection of cytokines were obtained from Dakewe Biotechnology Co., Ltd (Shenzhen, China). Concanavalin A (Con A), dimethyl sulfoxide (DMSO) and 3-(4,5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide (MTT) were bought from Sigma-Aldrich (MO, USA).

Generation of mouse BM-imDCs: BM-imDCs were prepared from the bone marrow of five week-old female specific pathogen free (SPF) BALB/c mice according to a previous report (Li *et al*., 2009). Briefly, the bone marrow cavity of murine femurs and tibias was flushed thoroughly with RPMI 1640 medium, and the erythrocytes were depleted with ammonium chloride solution. The remaining cells were inoculated into six-well culture plates (2 ml/well at 2×10^6/ml) in complete 1640 medium. The medium for imDCs contained 10% FBS, 10 ng/ml rmGM-CSF, 6 ng IL-4/ml, and penicillin–streptomycin at a final concentration of 0.1 mg/ml. The imDCs cultured for five days in a 5% CO_2 incubator were collected and enriched using the StemSep^{TM} Mouse Dendritic Cell Enrichment Kit (StemCell, Vancouver, Canada). The purity of the BM-imDCs was determined by flow cytometry (FCM) analysis of the specific molecular marker CD11c using anti-mouse CD11c-PE.

Infection of BM-imDCs with CSFV C-strain and the addition of IL-10 and anti-IL-10: The enriched BM-imDCs were inoculated into 24-well plates (1 ml/well) or 96-well plates (100 µl/well) at 2×10^6/ml. 24h after culture, the imDCs were infected with CSFV C-strain at 100 rabbit infection doses. The virus was discarded 2h post-incubation, and the complete 1640 medium was subsequently added to each well. Thereafter, mouse IL-10 or anti-mouse IL-10 was added to some of the wells at a final concentration of 10 ng/ml or 20 µg/ml, respectively. Four groups were set up, group 1 (non-infected-BM-imDCs), group 2 (infected-BM-imDCs), group 3 (infected-BM-imDCs with IL-10 added), and group 4 (infected-BM-imDCs with anti-IL-10 added). Five measurements were obtained for each group. The BM-imDCs were sequentially cultured for 72h. Finally, the cells were collected for the detection of CSFV using a reverse-transcription nested polymerase chain reaction (RT-nPCR) assay (Chen *et al*., 2010) and for analysis of the maturation status of the cells by FCM. At the same time, the supernatants were collected for cytokine detection.

The BM-imDCs cultured in 96-well plates were set up and infected with the C-strain similar to the cells in 24-well plates. The BM-imDCs were cultured for 24h after infection, and an MTT test was subsequently carried out.

FCM analysis: The infected BM-imDCs derived from 24-well culture plates were washed once with PBS (pH 7.4), and re-suspended in 200 µl PBS in triplicate. Thereafter, PE-conjugated anti-mouse CD80 was added into the cell suspensions. The samples were incubated for 30 min at 4°C. Following this, the imDCs were collected and washed twice with PBS. Finally, the pellets were re-suspended in 150 µl PBS to provide samples for FCM analysis. The surface marker CD80 was analyzed using a FCM. For each sample, 5,000 to 10,000 cells were acquired routinely. At the same time, CD86 and MHC class II were analyzed using the same procedure as that for the detection of CD80, except for PE-conjugated anti-mouse CD86 and MHC II.

Detection of cytokine production: The supernatants collected from 24-well culture plates were tested for the production of the following ten cytokines, containing mouse IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-10, IL-12P40, IL-12P70, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, using ELISA kits. The procedure was carried out according to the protocols, and two replicates were obtained for each cytokine in each sample. A series of standards were tested on each ELISA plate in order to produce a standard curve. The production of each cytokine was calculated in accordance with its standard curve. The inter-plate and intra-plate coefficients of variation (CVs) of the measurements were lower than 10%.

Mixed lymphocyte reaction (MLR) by MTT assay: The procedure was performed according to a previous report (Lang, 2009). Five replicates were arranged. The mixed lymphocytes were extracted from the spleen of BALB/c mice using the commercial lymphocytes separation medium. The BM-imDCs in 96-well plates were cultured for 24 h post-infection. The media were then thoroughly depleted. The mixed lymphocytes were inoculated into the 96-well plate (100 µl/well) with 2×10^6/ml. Following this,

ConA was added to each well at a final concentration of 20 μg/ml. After 48 h of continuous culture, MTT was added to each well at the final concentration of 150 μg/ml. The culture plate was incubated for 4h at 37°C with 5% CO_2, and the supernatants were thoroughly depleted. DMSO was added with 100 μl/well, and the plate was incubated for 10 min at room temperature. Finally, optical density (OD) 570 values were determined using an ELISA reader. The blank wells (only the media) were included as above containing MTT. At the same time, a control plate without ConA stimulation was subjected to the same process. The stimulation index (SI) was calculated by the following formula:

$$SI = \frac{OD_{570} \text{ value obtained from the well stimulated with ConA} - OD_{570} \text{ value of blank well}}{OD_{570} \text{ value obtained from the well not stimulated with ConA} - OD_{570} \text{ value of blank well}}$$

Statistical analysis: The data obtained from the FCM and MLR assays were analyzed using SPSS 15.0 version software. The values were reported as the mean plus or minus standard deviations (SD). The *t-test* was used to compare the differences among the groups.

RESULTS AND DISCUSSION

A few studies have described the interactions of CSFV with porcine DCs. In vitro, CSFV can infect and replicate in porcine DCs derived from peripheral blood mononuclear cells (Bauhofer *et al*., 2005). The inactivated or rescued CSFV induced a higher degree of DC maturation, but the virulent strain of CSFV suppressed maturation of DCs (Carrasco *et al*., 2004). In vivo, the virulent CSFV infection can enhance the level of IL-10, and IL-10 may disrupt the immune system cells or impair DC maturation (Jamin *et al*., 2008). In the present work, the effects of IL-10 and anti-IL-10 on the C-strain-infected mouse BM-imDCs were studied in vitro for the first time.

The purity of the BM-imDCs obtained was greater than 90% according to FCM analysis of the specific molecular marker CD11c using anti-mouse CD11c-PE (Fig. 1A). Using RT-nPCR detection and sequence analysis, the specific 1140-bp DNA fragments (Lin *et al*., 2008) were obtained from the C-strain-infected BM-imDCs (Fig. 1B), which indicated that the C-strain had successfully infected the BM-imDCs.

A preliminary test was carried out in order to define the optimal concentrations of IL-10 and anti-IL-10. A series of IL-10 (final concentrations of 2.5, 5, 10, 15, 20 ng/ml, respectively) and anti-IL-10 (final concentrations of 5, 10, 15, 20, 25, 30 μg/ml, respectively) were added to the infected BM-imDCs. Then, the surface molecule MHC class II of the infected cells was detected by FCM assay. For the infected cells with added IL-10, the expression level of MHC II decreased with increased IL-10 concentrations of 2.5, 5 and 10 ng/ml, while no persistent decline at the concentrations of 10 ng/ml to 20 ng/ml was observed. Therefore, 10 ng/ml was selected as the optimal concentration of IL-10. These results indicated that lower concentration of IL-10 did not effectively suppress the maturation of the infected BM-imDCs. As such, the level of MHC II, derived from the infected cells with added anti-IL-10, rose slightly with increased anti-IL-10 concentration up to 20 μg/ml, and no increase was detected above this concentration. Thus, the optimal concentration of anti-IL-10 was 20 μg/ml.

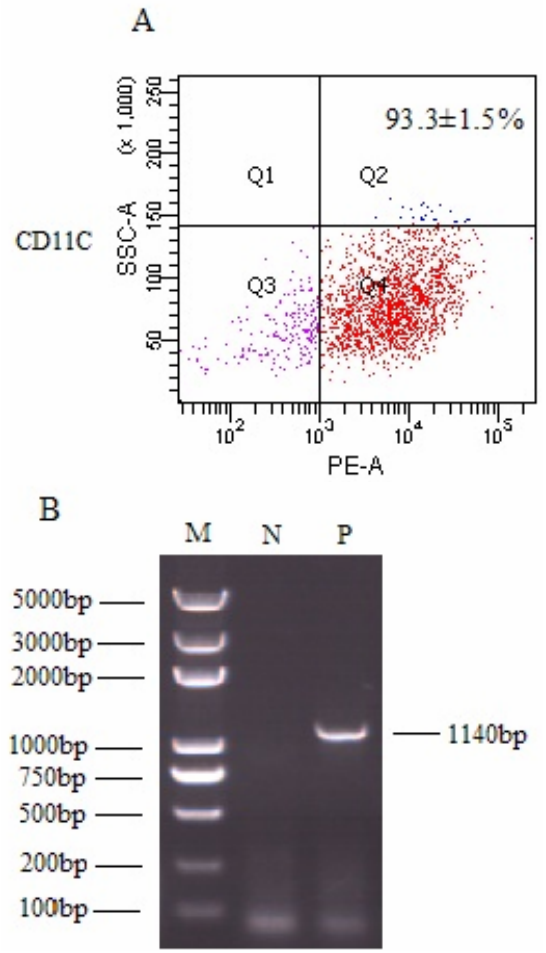

Fig. 1: Generation of BM-imDCs and infection of the C-strain in BM-imDCs. (A) The purity of BM-imDCs was 93.3±1.5% by FCM analysis. (B) The 1140-bp DNA fragments were obtained from the infected BM-imDCs by RT-nPCR amplification and sequence analysis. Lane M: DNA marker. Lane N: No product was obtained using the RNA extracted from the non-infected-BM-imDCs as template. Lane P: the specific DNA fragments were obtained using the RNA extracted from the infected BM-imDCs as templates.

Using FCM analysis, the expression levels of CD80, CD86 and MHC class II in group 2 were obviously higher than those in group 1, which indicated that infection with the C-strain greatly enhanced the degree of maturation of the BM-imDCs. The expression levels of these surface molecules in group 3 down-regulated significantly compared with those in groups 1, 2 and 4, while the expression levels of the molecules in group 4 were similar with those in group 2 (Fig. 2). For the C-strain-infected BM-imDCs, the results showed that IL-10 added at high concentration markedly suppressed the cells maturation, while anti-IL-10 antibody did not significantly enhance the degree of cell maturation. These data also implied that lower autocrine IL-10 had no significant effect on the maturation status of the C-strain-infected DCs. However,

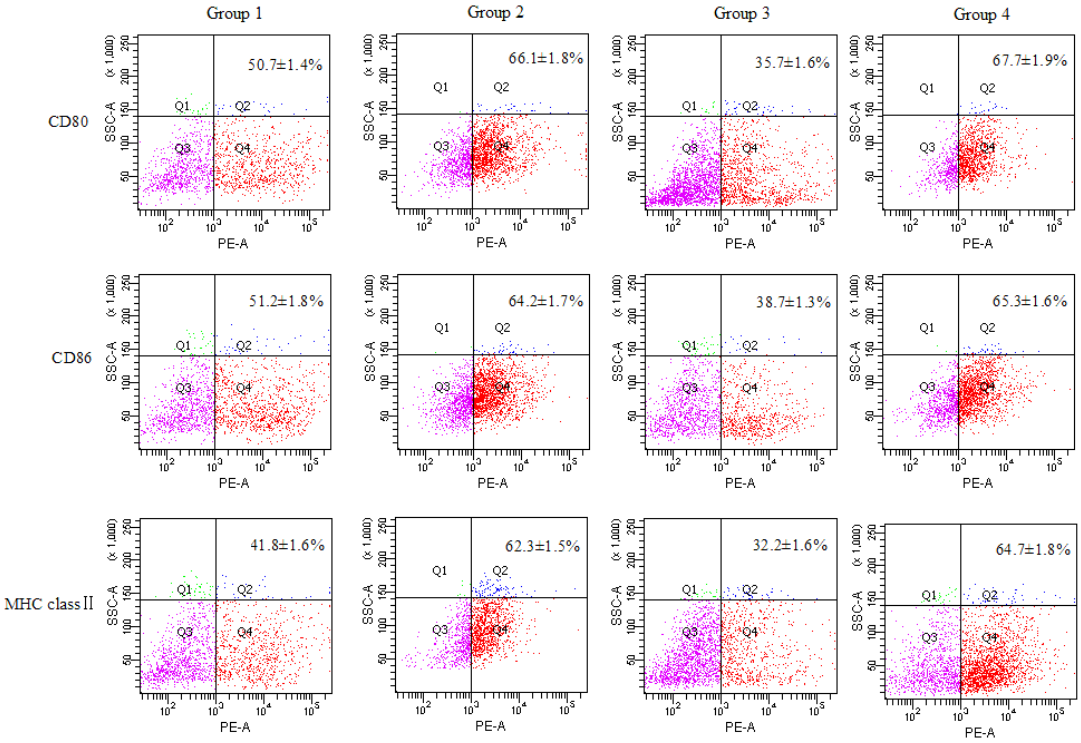

Fig. 2: The expression levels of surface molecules CD80, CD86 and MHC class II in the BM-imDCs in groups 1, 2, 3 and 4. The levels of expression of CD80, CD86 and MHC class II by group 2 were significantly up-regulated compared with those derived from group 1 (P<0.01). The levels of these molecules in group 3 were highly significant lower than those in groups 1, 2 and 4 (P<0.01), and the levels in groups 2 and 4 were not significantly different (P>0.05).

autocrine IL-10 was shown to limit the maturation of DCs prepared from human peripheral blood monocytes, and the capacity of DCs to initiate Th1 response was also limited (Corinti *et al*., 2001; McBride *et al*., 2002), which was different from our results. Mature DCs are no longer sensitive to the inhibitory effects of IL-10 (Gad *et al*., 2011), which supports the results obtained in our study.

It had been reported that IL-10 inhibited the production of TNF-α and IL-12P70 by human DC as reported (McBride *et al*., 2002), while the activities of IL-10 differ between various cell types and even with different sub-populations of the DCs (Moore *et al*., 2001). In this study, the levels of IL-2, IL-5 and IL-12P70 in the four groups were undetectable using their respective kits. The productions of IL-1α and IL-1β had no significant differences among the four groups, and the concentrations of the two cytokines were about 62.1±2.8 and 74.5±3.8 pg/ml, respectively. It is worth mentioning that the infection with the C-strain did not enhance or inhibit the secretion of IL-10 based on the similar levels of IL-10 (42.5±3.4 pg/ml) in group 1 and group 2. No IL-10 was detected in group 4 using the kit. The production of IL-6, IL-12P40, TNF-α and IFN-γ by group 2 were significantly higher than that produced by group 1, which indicated that the infection with the C-strain markedly enhanced the expression levels of the four cytokines. The production of IL-6, TNF-α and IFN-γ in groups 2, 3 and 4 was similar, which showed that IL-10 and anti-IL-10 antibody had no significant effect on the secretion of the three cytokines in the infected BM-imDCs. The secretion production of IL-12P40 by group 3 was significantly lower than that by group 2 and the level of IL-12P40

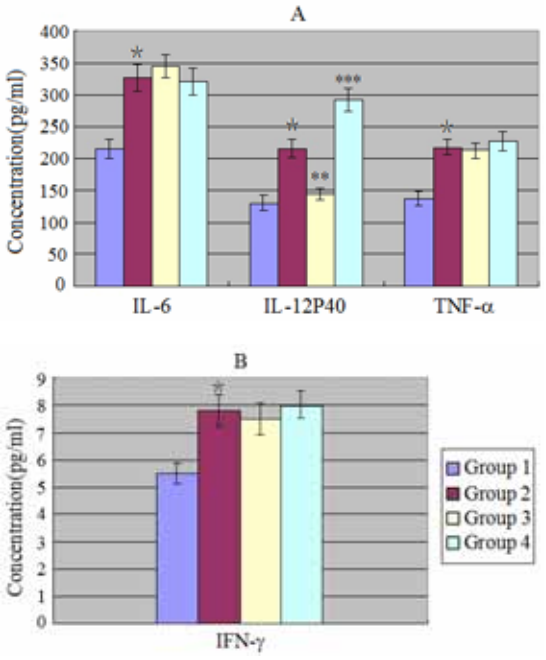

Fig. 3: The effects of IL-10 on the production of IL-6, IL-12P40, TNF-α (A) and IFN-γ (B) derived from the C-strain-infected BM-imDCs. * The production of these cytokines in group 2 was significantly up-regulated compared with that in group 1 (P<0.01for IL-6, IL-12P40 and TNF-α and P<0.05 for IFN-γ). The differences in the production of IL-6, TNF-α and IFN-γ were not significant in groups 2, 3 and 4. **Group 3 showed a significant down-regulation in the production of IL-12P40 compared with group 2 (P<0.01), ***while group 4 showed significant up-regulation in the level of IL-12P40 compared with groups 1, 2 and 3 (P<0.01).

Table 1: The SIs of T lymphocyte of mice by the means of MTT

Groups	SIs obtained from the five replicates of each group					Means±SD
	1	2	3	4	5	
1	0.72	0.81	0.77	0.71	0.78	0.76±0.04
2	1.85	1.86	1.90	1.92	1.87	1.88±0.03
3	0.58	0.61	0.55	0.53	0.60	0.57±0.03
4	2.22	2.14	2.19	2.33	2.26	2.23±0.07

derived from group 4 was significantly higher than that in groups 1, 2 and 3. Therefore, it can be confirmed that additional or autocrine IL-10 greatly suppressed the secretion of IL-12P40, and anti-IL-10 antibody effectively prevented this suppression. The results are intuitively shown in Fig. 3 (A and B).

The ability of the C-strain-infected BM-imDCs to induce allogeneic naive T cell proliferation was tested using the MLR assay. The SIs obtained from the five replicates arranged for each group was shown in Table 1. The SIs obtained in groups 1, 2, 3 and 4 were 0.76±0.04, 1.88±0.03, 0.57±0.03, 2.23±0.07, respectively. These data showed that the C-strain-infected BM-imDCs effectively promoted the proliferation of allogeneic naive T cells, and additional or autocrine IL-10 inhibited this proliferation in the infected BM-imDCs. Naturally, the infected BM-imDCs treated with anti-IL-10 antibody showed an increased capacity to activate naive T cells.

Conclusion: The infection of BM-imDCs with CSFV C-strain enhanced cell maturation and the production of IL-6, IL-12P40, TNF-α and IFN-γ, and promoted T lymphocytes proliferation. Additional IL-10 significantly inhibited maturation of the infected BM-imDCs and the capacity of DCs to stimulate naive T lymphocytes proliferation, and diminished the production of IL-12P40 by the DCs. Autocrine IL-10 had the same effects as additional IL-10 on the C-strain-infected BM-imDCs, although it did not inhibit maturation of the cells. These data demonstrated that IL-10 plays an important role in the DCs-dependent immune response triggered by the C-strain vaccine.

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