Collagen I-induced dendritic cells activation is regulated by TNF-α production through down-regulation of *IRF4*

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Previously we have shown that collagen I enhances the maturation and function of dendritic cells (DCs). Inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and lipopolysaccharide (LPS) are also known to activate DCs. Here we investigated the involvement of TNF-α on the collagen I-induced DCs activation. TNF-a neutralization inhibited collagen I-induced IL-12 secretions by DCs. Additionally, we observed suppression of collagen I-induced costimulatory molecules expression along with down-regulation of genes involved in DCs activation pathway. Furthermore, TNF-α inhibition upon collagen Istimulation up-regulated the expression of interferon regulatory transcription factor *IRF4*, when compared to collagen I only treated cells. Collectively, our data demonstrate that collagen I induce TNF-α production, which is crucial for the activation and function of DCs, through down-regulation of *IRF4*, and implicates the importance in development of anti- TNF-α therapeutics for several inflammatory diseases.

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1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that are present in peripheral tissues and lymphoid organs (Lee *et al.* 2007). To elicit immune response, immature DCs undergo a differentiation process called maturation and then migrate to lymph nodes, to present captured antigen to T-cells. This process constitutes changes in morphology, such as, cytoplasmic reorganization, loss of adhesion molecules, increased cellular migration, and production of various cytokines and chemokines (Lee *et al.* 2007; Poudel *et al.* 2012). A crucial component of this maturation process is their expression of costimulatory molecules such as CD80, CD83, CD86 and major histocompatibility complex (MHC) class I and II (Lee *et al.* 2007; Hu *et al.* 2008). On the other hand, DCs produce interleukin (IL)-12 and TNF-α during the maturation process which are important in inflammatory conditions and differentiation of T-helper type 1 (Th1) cells, and also in the induction of costimulatory molecules expression (Winzler *et al.* 1997; Iwasaki 2007; Njau *et al.* 2009). Lack of adequate activation of DCs may thus lead to an unbalanced immune response.

Early work with murine and human DCs demonstrated that DCs maturation can be induced by collagen I, a major extracellular matrix (ECM) component (Lee *et al.* 2007; Poudel *et al.* 2012). DCs interact with collagen present at the inflammatory sites and regulate the function of DCs. This interaction is known to enhance the expression of costimulatory molecules

Keywords. Anti-TNF-α; collagen I; interleukin-12; *IRF4*; maturation; monocyte-derived dendritic cells

Abbreviations used: DC, dendritic cells; GMCSF, granulocyte-macrophage colony stimulating factor; *IRF4*, interferon regulatory factor 4; ECM, major extracellular matrix; MHC, major histocompatibility complex; Th1, T-helper type 1

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expression and secretion of cytokines such as IL-12 and TNF-α (Mahnke *et al.* 1996; Brand *et al.* 1998).

TNF-α has a vital role in inflammation and immune responses. It initiates the immunological response to local injury, recruits leukocytes and enhances the release of other pro-inflammatory cytokines (Boks *et al.* 2014). In several inflammatory diseases such as rheumatoid arthritis, ankylosing spondylytis, psoriatic arthritis, inflammatory bowel diseases and multiple scelerosis, use of TNF-α inhibitors is an important treatment strategy (Feldmann and Steinman 2005; Wong *et al.* 2008; van Vollenhoven 2009). Members of TNF superfamily have been shown to regulate DCs differentiation. TNF-α activity neutralization within 48h of granulocyte-macrophage colony stimulating factor (GMCSF)/ TNF-α treated CD34+ progenitors halted DCs development (Santiago-Schwarz *et al.* 1998), suggesting a role for TNF-α during DCs differentiation.

This study was performed to examine the role of TNF-α upon collagen I-induced DCs activation. We investigated whether TNF-α inhibition influences the activation and functions of collagen I-activated DCs. We also analysed whether TNF-α inhibition affects the release of IL-12 which is secreted by collagen I-treated DCs. Furthermore, we examined if TNF-α inhibition exhibited any effect on expression of interferon regulatory factor 4 (*IRF4*), a transcription factor of IRF family which is expressed by immune cells including DCs and is known to have essential role in DCs activation (Akbari *et al.* 2014; Vander Lugt *et al.* 2014).

2. Materials and methods

2.1 *Animals and reagents*

Female C57BL/6 mice (age, 6–10 weeks) were purchased from Samtaco (Seoul, Korea). All animal studies were performed in accordance with the protocol approved by the Institutional Animal Care and Use of Committee of Chonbuk National University Medical School. Recombinant murine GMCSF, TNF-α, and IL-4 were obtained from Biosource (Camarillo, CA). Collagen I was purchased from Advanced Biomatrix Inc. (San Diego, USA). TNF-α neutralizing antibody and control IgG were from eBioscience (San Diego, USA). All reagents were from Sigma.

2.2 *Isolation of bone-marrow derived-DCs*

DCs were generated from the bone marrow of mice as previously described (Lee *et al.* 2007). Briefly, Bone marrow cells were harvested from the femur and tibia of mice, then laid carefully on Histopaque-1119. After centrifugation (500*g*) for 20 min, the cells collected from the interface were cultured in RPMI1640 containing 10% FBS, 10 ng/mL GMCSF and 10 ng/mL IL-4. Medium was changed every 2 days and immature DCs were harvested on day 6. The cells were examined for CD11c and were assayed for subsequent experiments.

2.3 *Reverse transcription PCR and quantitative real-time PCR analysis*

Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA synthesis was performed using SuperScript III First Strand Synthesis System (Invitrogen). Forward and reverse primers used are listed in Table 1. Cycling conditions were as follows: denaturing at 95°C for 30 s; annealing at 55°C for 60 s; and extension at 72°C for 90 s (Marecki *et al.* 2009). Amplified products were run on a 1.2% gel and photographed. Quantitative PCR was performed using primers previously described (Martin *et al.* 2007; Besin *et al.* 2011; Seth *et al.* 2011).

2.4 *Flow cytometric analysis*

Cells were washed in 0.1% sodium azide and incubated with anti-FcgRIIb to block specific binding of IgG on ice for 10 min and then with specific antibodies (Bioleged, San Diego, USA) on ice for 30 min. FITC or PE conjugated antibodies towards CD11c, CD80, CD86 and MHC-II were used. As a control, cells were also stained with isotype-matched control antibodies. Data acquisition was performed using FACS Calibur (Becton-Dickinson, San Diego, USA) and data were analysed with FlowJo software (Tree Star, Inc. Ashland, OR).

2.5 *Cytokine release analysis by ELISA*

Levels of TNF-α and IL-12 were measured using a commercial ELISA kit (Biosource) according to the manufacturer's protocol. Briefly, capture antibody was coated in 96-well plates for overnight at 4°C and then blocked with ELISA buffer containing 50 mM Tris, 0.5% BSA, 2 mM EDTA, 150 mM NaCl and 0.05% Tween-20 for 1 h. Then, the medium (100 μL) was added to the wells and incubated for 2h at room temperature. After this, the plates were incubated with biotinylated detection antibodies and strepatvidin-horseradish peroxidase for 1h, and washed with ELISA buffer. After washing, tetramethylbenzidine substrate (BD Pharmingen) and 0.04% hydrogen peroxide in PBS was added to the plates. Finally, the reaction was stopped by adding 2 M sulfuric acid, and optical density was measured at 405 nm using a micro-plate reader.

2.6 *Statistical analysis*

All data were expressed as the mean ± SEM. The statistical analysis of data was performed using the Student's *t*-test, and $p<0.05$ was considered statistically significant.

Table 1. Primers for qPCR and RT-PCR

Gene	Forward	Reverse	Size (bp)	Methods
CCR7	CCAGCAAGCAGCTCAACATT	GCCGATGAAGGCATACAAGA	92	qPCR
CCL17	GGATGCCATCGTGTTTCTGA	GCCTTCTTCACATGTTTGTCTTTG	75	qPCR
CCL19	ATGCGGAAGACTGCTGCC	AGCGGAAGGCTTTCACGAT	69	qPCR
IRF4	AATGGGAAACTCCGACAGTG	TAGGAGGATCTGGCTTGTCG	214	qPCR
GAPDH	CATGGCCTTCCGTGTTC	CCTGGTCCTCAGTGTAGC	152	qPCR
IRF4	GCTGCATATCTGCCTGTATTACCG	GTGGTAACGTGTTCAGGTAACTCGTAG	555	RT-PCR
GAPDH	GAAGGGCTCATGACCACAGTCCATG	TGTTGCTGTAGCCGTATTCATTGTC	450	RT-PCR

3. Results

3.1 *Collagen I induces TNF-α production by DCs*

Firstly, we assayed surface marker of bone marrow derived DCs (BMDCs) using PE-conjugated CD11c antibody and an isotype-matched control antibody. Figure 1A shows that >90% cells were CD11c positive, suggesting that DCs are differentiated from mice bone marrow cells. Next, to determine the effect of collagen I on TNF-α production by DCs, we plated the cells on collagen coated or non-coated dishes for 24 h and culture soup was assayed for TNF-α production. Figure 1b shows that collagen I significantly induced TNF-α production by DCs.

3.2 *TNF-α inhibition suppresses IL-12 production by DCs*

As collagen I has been reported to enhance cytokine production by DCs (Lee *et al.* 2007), we investigated the role of TNF-α neutralization to IL-12 production by DCs. We used lipopolysaccharide (LPS) as positive control. To rule out the possibility of LPS contamination to the collagen I solution, we treated collagen I with polymyxin B at 50 mg/L (PB) for 1 h. As shown in figure 2, collagen I treated cells released significant levels of IL-12. Moreover, we found no significant difference in IL-12 production between collagen I only and PB treated-collagen I treated cells, suggesting no LPS contamination in the collagen I solution. However, the IL-12 production was significantly reduced in TNF-α neutralizing antibody treated cells when compared to control. These results indicate that collagen I induced TNF-α is important for the IL-12 production by DCs.

3.3 *Effect of TNF-α neutralization on the DCs phenotype*

To investigate the effect of collagen I-induced TNF-α on DCs maturation and the effects of TNF-α neutralization, DCs were treated with collagen I and left treated with TNF-α neutralization

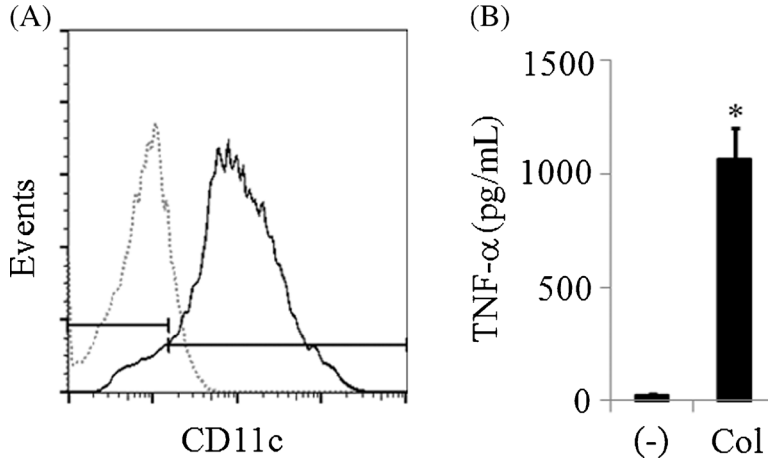

Figure 1. Collagen I induces TNF-α production by DCs. Bone marrow cells were harvested from the femur and tibia of mice and were cultured in RPMI1640 containing 10% FBS, 1% penicillin-streptomycin, 10 ng/mL GM-CSF and 10 ng/mL IL-4. After day 6, non-adherent and loosely attached cells were screened for CD11c expression. Faint line represent isotype control and dark line represents corresponding antibody staining (**A**). The cells were cultured in the collagen (Col) coated (10.5 μg/cm^2) or non-coated dishes for 24 h, and culture supernatant was analysed for TNF-α production by commercial ELISA kit (**B**). The results are expressed as the mean ± S.E.M. (n=3), *p<0.05.

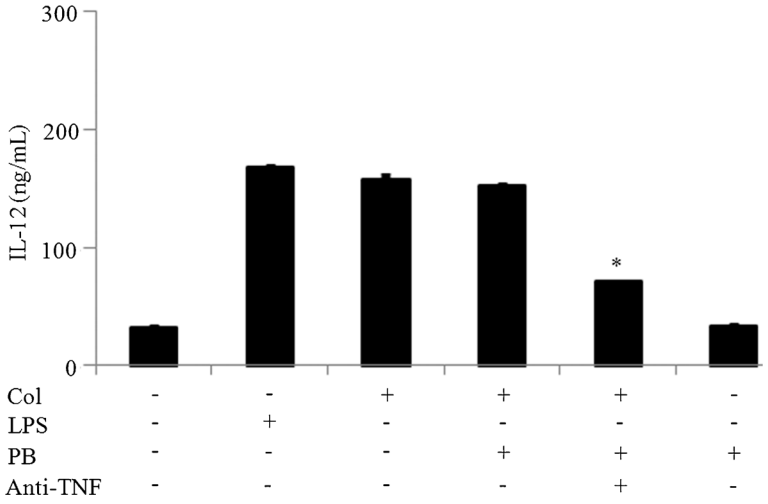

Figure 2. TNF-α inhibition suppresses IL-12 production by DCs. Immature DCs were cultured in the presence or absence of collagen I (Col) as indicated, and after 24 h, culture supernatant was analysed for the IL-12 production using commercial ELISA kit. LPS (10 ng/mL) was used as a positive control. Polymyxin B (PB) (50 mg/L) was used to eliminate the possibility of LPS contamination in Col solution. 1 μg/mLl of PB alone was treated to the cells in non-coated dish. Anti-TNF-α antibody and control antibody were used at 20 μg/mL. The results are expressed as the mean ± S.E.M. (n=3), *p<0.05.

antibody or an isotype control for 48 h. Non-treated cells were used as negative control. Collagen I treated cells showed significant enhancement in surface expression of the CD80, CD86, and MHC-II molecules (figure 3A and B). When TNF-α activity was neutralized, expression of the costimulatory molecules was significantly inhibited (figure 3A and B).

3.4 *TNF-α neutralization suppresses the expression of genes involved in DCs activation/maturation*

To examine the effect of neutralization of TNF-α on the expression of DCs activation pathway genes during collagen I treatment, RNA was isolated from the collagen I treated or untreated cells with or without TNF-α neutralizing antibody for quantitative PCR analysis. Collagen I induced significant expression of *CCR7*, *CCL17* and *CCL19* (figure 4). Interestingly, the expression levels of *CCR7*, *CCL17* and *CCL19* were significantly inhibited when TNF-α was neutralized during collagen I treatment.

3.5 *IRF4 gene expression after collagen I treatment and inhibition of TNF-α*

Previous reports indicate that *IRF4* has crucial role in DCs development (Akbari *et al.* 2014; Vander Lugt *et al.* 2014). We hypothesized that *IRF4* could modulate the collagen I-induced DCs activation upon TNF-α activity inhibition. Therefore, to determine the effect of TNF-α neutralization in *IRF4* expression in DCs treated with or without TNF-α inhibitor, at 6 h after collagen I treatment with or without TNF-α neutralization, RNA was isolated from the cells and reverse-transcription and quantitative PCR analysis was performed. Results showed that TNF-α inhibition significantly enhanced the expression of *IRF4* when compared to collagen I only treated cells (figure 5A–C), suggesting the role of *IRF4* in collagen I-induced DCs when TNF-α is inhibited.

4. Discussion

Immature DCs are transformed to mature DCs upon given stimulus and are characterized by up-regulation of cell surface costimulatory molecules expression, and secretion of a variety of cytokines that can shape both innate and adaptive immunity (Pan *et al.* 2013). The present study demonstrated that TNF-α plays a crucial role in the maturation and function of DCs stimulated with collagen I. It was evident from the results, which show that inhibition of TNF-α during collagen I stimulation impairs the production of cytokine, chemokines and expression of the costimulatory molecules.

Previous studies have shown that inflammatory molecules such as TNF-α lead to DCs maturation (Roake *et al.* 1995). During chronic inflammation, maturation of DCs by endogenous inflammatory molecules could lead to local inflammation and potential autoimmunity (Thomas *et al.* 1994; Steinman *et al.* 2003). Additionally, collagen accumulation at the inflammatory sites has been reported to be crucial in

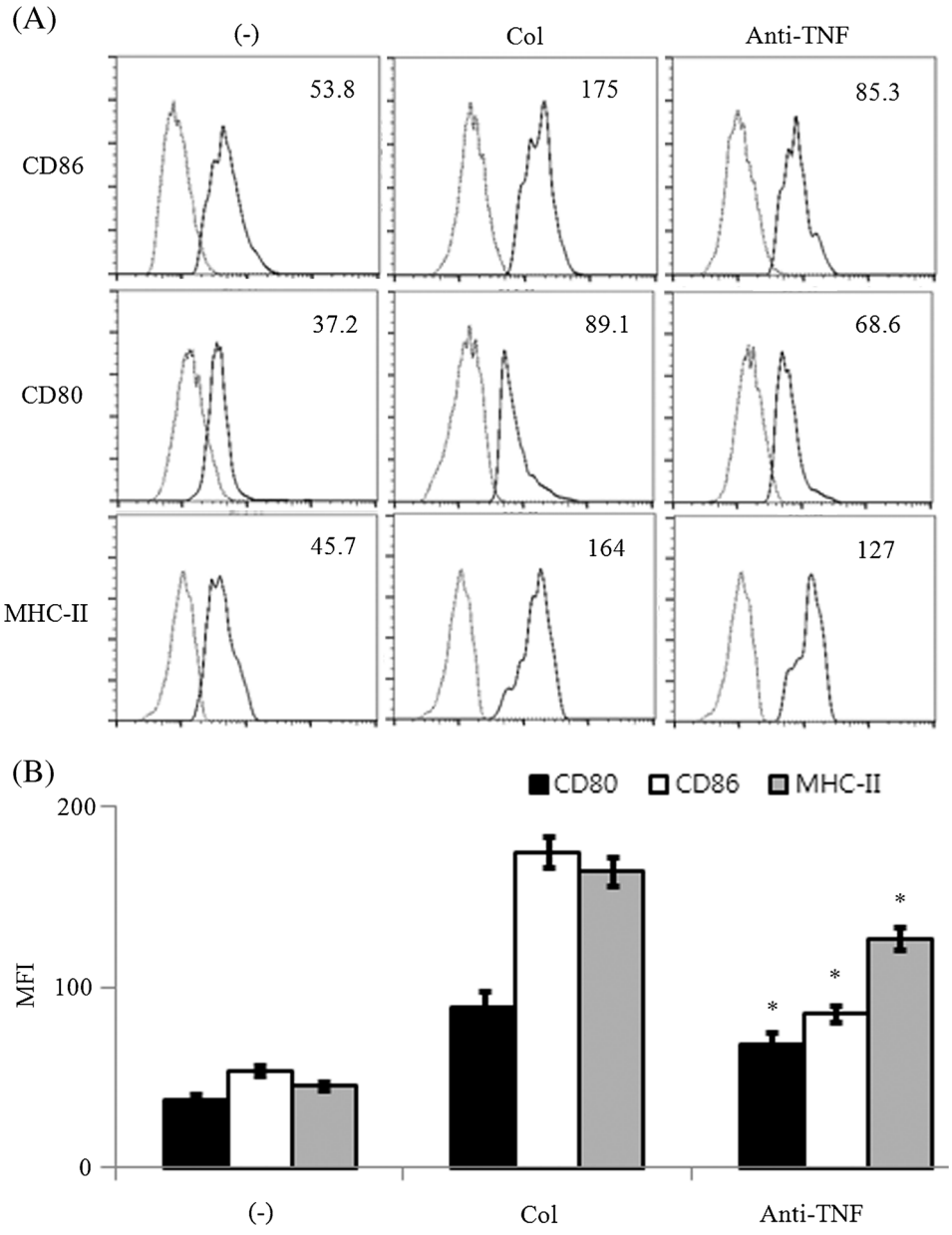

Figure 3. Effect of TNF-α neutralization on the DCs phenotype. Immature DCs were cultured in the presence or absence of collagen I with or without TNF neutralizing antibody (20 μg/mL) for 48 h and expression of CD86, CD80, and MHC-II was analysed by flow cytometry. Faint line represent isotype control and dark line represents corresponding antibody staining (**A**). Bar diagram represents the mean fluorescence intensity (**B**). The results are expressed as the mean ± S.E.M. (n=3), *p<0.05.

regulating the maturation and function of DCs (Lee *et al.* 2007). The role of TNF-α in collagen I-activated DCs is less studied. Our data shows that neutralizing TNF-α during collagen I treatment to the DCs significantly inhibited IL-12 production when compared to control. This could have been mediated via *IRF4* up-regulation upon TNF-α neutralization. Similar result has been demonstrated by a study using *IRF4*-deficient mice. The study showed that DCs from *IRF4* deficient mice released higher levels of IL-12 upon *Leishmania major* infection when compared to control mice (Akbari *et al.* 2014). Moreover, our data showed that the collagen I-induced costimulatory molecules expression was inhibited upon TNF-α neutralization. Previous reports have shown that soluble factors such as TNF-α and IL-1 produced

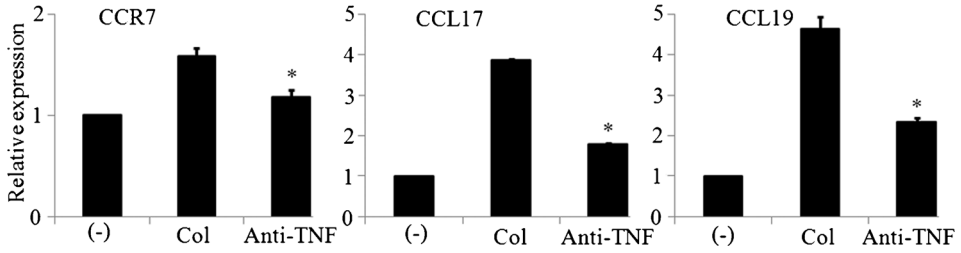

Figure 4. TNF-α neutralization suppresses the expression of genes involved in DCs activation/maturation. Immature DCs were cultured in the presence or absence of collagen I with or without TNF-α neutralizing antibody (20 μg/mL) and murine TNF-α (10 ng/mL) for 6 h, RNA was isolated, cDNA was synthesized, and quantitative PCR was performed with gene specific primers of above indicated genes. The results are expressed as the mean ± S.E.M. (n=3), *p<0.05.

by monocytes and DCs are potent autologous or heterologous inducers of DCs maturation (Njau *et al.* 2009). However, a study reported that neutralization of TNF-α during the differentiation and maturation of DCs did not alter the phenotype of cells in the rheumatoid patients and healthy controls (van Lieshout *et al.* 2005). Furthermore, a study demonstrated that anti-TNF-α treatment within 2 days of cytokine exposure, impaired the DCs development and promoted the myelogranulocytic hematopoiesis in CD34+ progenitor cells (Santiago-Schwarz *et al.* 1998). Similar to our results, data from one group of researchers show that TNF-α inhibition suppressed the expression of CD83, CD86 and MHC-II, but that of CD80 was not affected when DCs were infected with *Chlamydia pneumoniae* (Njau *et al.* 2009). In addition, activated DCs are known to express higher levels of chemokines. We examined whether this process was TNF-α mediated. We investigated the effect of TNF-α neutralization on the expression of several DCs specific chemokines during collagen I treated or untreated conditions. Our data demonstrated that TNF-α neutralization during maturation of DCs stimulated with collagen I resulted in decreased expression of DCs activation pathway genes (*CCR7*, *CCL17* and *CCL19*). Similar to our results, other studies have reported that inhibition of TNF-α down-regulates the expression of chemokines expression during DCs maturation (van Lieshout *et al.* 2005; Njau *et al.* 2009). The

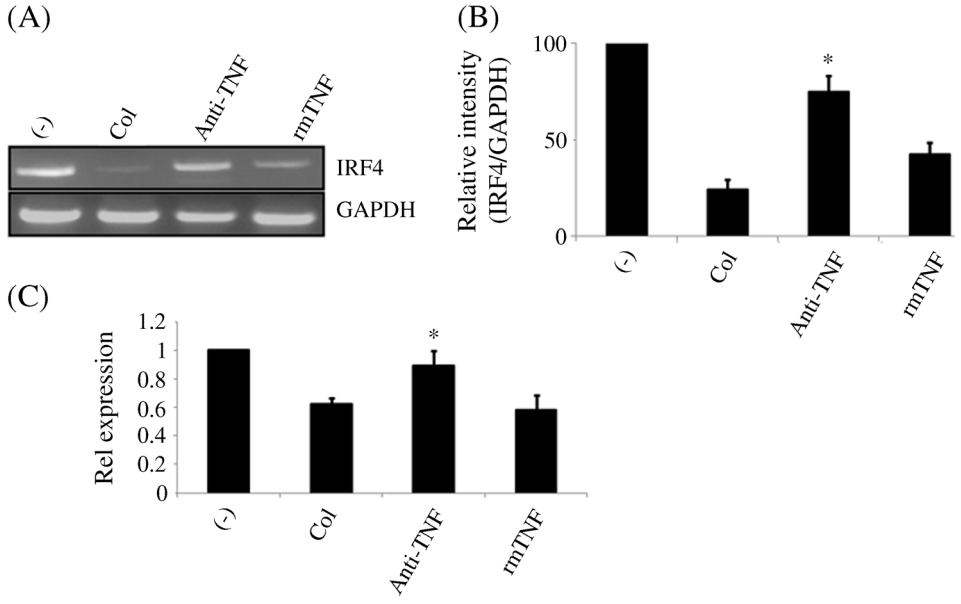

Figure 5. IRF4 gene expression after collagen I treatment and inhibition of TNF-α. Immature DCs were cultured in the presence or absence of collagen I with or without TNF-α neutralizing antibody (20 μg/mL) for 6 h, RNA was isolated, cDNA was synthesized, and RT-PCR (**A**). Bar diagram represents the band intensity to that of GAPDH (**B**). Quantitative-PCR was performed with gene specific primers of *IRF4* (C). The results are expressed as the mean ± S.E.M. (n=3), *p<0.05.

decreased expression of chemokines by DCs after TNF-α neutralization suggests a critical role of TNF-α in the control of cell migration *in vivo*. A previous report suggests that TNF-α blockade reduces the synovial cell influx in patients after initiation of anti-TNF-α administration (van Lieshout *et al.* 2005).

Early works have reported that *IRF4* is crucial for DCs development and functional diversity (Suzuki *et al.* 2004; Tamura *et al.* 2005). It has been shown that mice lacking *IRF4* exhibit a diminished capacity for LPS-activated up-regulation of CD80 and CD86 (Suzuki *et al.* 2004). However, in our study, collagen I seems to inhibit the expression of *IRF4* in DCs after 6h incubation with anti-TNF-α antibody. Moreover, IRF-4 has been shown to negatively regulate IRF-5 promotor reporter activities and bind to IRF-5 promoters in vivo and in vitro (Xu et al., 2011). Additionally, IRF-5 has been reported to play a crucial role in TNF secretion in DCs (Krausgruber *et al.*, 2010), suggesting that IRF-4 and IRF-5 might regulate the cytokines expression by DCs upon collagen stimulation. Similarly, other transcription factors such as *IRF8* and *PU.1* are also known to regulate the development of DCs (Kanada *et al.* 2011), further studies are required to address this issue.

To sum up, the potential role of TNF-α in DCs biology, and huge application of anti-TNF-α therapeutics, motivated us to examine the effects of TNF-α neutralization on DCs development and function. We speculated that TNF-α neutralization would interfere with DCs maturation and reduce the production of cytokines and chemokines upon collagen I stimulation. We show here that neutralization of TNF-α during collagen I-stimulation of DCs leads to the impaired maturation and reduction in cytokine and chemokines expression and potential up-regulation of *IRF4,* when compared to collagen I only stimulated cells. These data suggest an importance in development of anti-TNF-α therapeutics for the several inflammatory disorders.

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