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Original Article

Silencing c-Kit expression in human DCs suppresses Th2, Th17 response but enhances Th1 response

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Abstract: Dendritic cells (DCs) are integral to the differentiation of T helper cells into T helper type 1 TH1, TH2 and TH17 subsets. RNA interference (RNAi), which causes the degradation of any RNA in a sequence specific manner, is a posttranscriptional gene silencing mechanism. Targeting the c-Kit in DCs has been used as an approach to enhance antitumor immunity. Here, we shwed that transfection of DCs with siRNA specific for c-Kit gene can significantly knock down c-Kit. When exposed to TNF-α, immature DCs transfected with c-Kit siRNA can differentiate into mature DCs without reducing viability or IL-12p70 production. The c-Kit siRNA-treated DCs exhibited an increased allostimulatory capacity in a lymphocyte proliferation assay. Furthermore, c-Kit siRNA-transfected DCs enhanced TH1 responses by increasing IFN-γ and decreasing IL-4 production, and much stronger cytotoxic activity was observed when DCs were co-transfected with c-Kit siRNA and an endogenous tumor antigen in vitro. Our findings indicate that silencing the c-Kit gene in DCs with siRNA may offer a potential approach to enhance antitumor immunotherapy.

Keywords: Small interfering RNA, c-Kit, dendritic cells, anti-tumor immunity

Introduction

Dendritic cells (DCs) are highly specialized antigen presenting cells with the unique capacity to initiate and control primary immune responses. DCs are efficient antigen-presenting cells and can prime naive CD4+T cells toward a TH1 or TH2 response or the recently discovered TH17 response [1-3]. Considerable progress has been made in the last decade in our understanding of the molecular basis of T helper responses. Previous studies have identified the transcription factor GATA-3 as the master regulator of TH2 differentiation [4-7]. The transcription factors T-bet and ROR-γT are now recognized as crucial regulators of TH1 and TH17 differentiation, respectively [8, 9]. However, there have been a few studies that have addressed the role of c-Kit expressed by DCs in modulating immune responses [10]. Although c-Kit has been best studied in the context of mast cells, administration of SCF in vivo has been shown to have mast cell independent effects [11]. c-Kit expressing DCs have been shown to modulate NK cell function by increasing the cytolytic and IFN-γ secreting ability of NK cells, thereby defining the consequence of this receptor in at least one aspect of DC function [10]. The importance of c-Kit signaling via PI3 kinase has also been explored in previous study by using mutant mice [10-13].

RNA interference (RNAi) is a remarkable experimental tool that has emerged in recent years. RNAi is triggered by double stranded RNAs (dsRNAs) that cause selective gene silencing. It is a conserved mechanism that pervades the biological world and was first discovered by Fire and his colleagues in 1998 [14]. Introduction of dsRNAs into cells can elicit at least four different types of responses that can selectively suppress target gene expression. dsRNAs can induce inhibition of protein translation, degradation of mRNAs [15-17], transcriptional inhibition, and cause chromosomal rearrangements [18]. The RNAi approach has several advantages over conventional methods, e.g. antisense oligonucleotide technology, including high efficacy and specificity [19]. In addition, since small interfering RNA (siRNA) is quite stable, no

chemical modifications are required to achieve a sufficient half-life in cell culture media [20].

In this study, siRNA specific for the c-Kit gene was transfected into DCs derived from healthy donor and its effect on the viability, differentiation, and function of DCs was investigated. In addition, the in vitro effect of c-Kit silenced DCs prime T helper responses was also explored.

Materials and methods

Generation of DCs

Blood samples were obtained from healthy donor following informed consent approved by Wuhan General Hospital of Guangzhou Command. DCs were generated from CD14+ monocytes cultured in Iscove's Modified Dubecco's Medium (IMDM) with 10% FBS and presence of GM-CSF and IL-4 as described. On day 5, immature DCs were matured overnight by an inflammatory cytokine cocktail (IL-1b, IL-6, TNF-α and prostaglandin E2; Cyt-DCs) as described [21]. Cells were collected for siRNA transfection on day 6.

RNAi mediated inhibition of c-Kit in dendritic cells

ON-TARGETplus SMARTpool siRNAs (Dhamacon, Lafayette, CO) were used to inhibit human c-Kit (X06182.1). CD14+ cells separated from magnetic column were resuspended at 10^5 cells/μl in phenol red free Opti-MEM reduced-serum medium (Invitrogen, Carlsbad, CA). Cells were electroporated with 20 ng siRNA in 0.4 mm gap electroporation cuvettes using BTX ECM830 (Harvard Apparatus, Holliston, MA) at 500 v, 2 pulses of 500 ms. Immediately after transfection, DCs were further matured with TNF-α at a concentration of 50 ng/ml and incubated at 37°C with 5% CO_2 for 48 h. The annexinV-propidium iodide method of determining apoptosis/necrosis was used as previously described. Inhibition of target genes was confirmed by western blot. The maturation of DCs partially characterized by their IL-12p70 production using the Quantikine ELISA sets according to the manufacturer's instructions (R&D systems, Wiesbaden, Germany) , and partially characterized by flow cytometry after staining with antibodies against CD1a, CD80, CD8, CD14, CD86 and HLA-DR (all from BD). Data were analyzed with Flowjo software (Tree Star, Ashland, OR).

Allogeneic lymphocyte proliferation

Allogeneic lymphocyte activation was set up by culturing 48 h siRNA treated DCs (5×10^4 cells/well) in triplicate with various concentrations of allogeneic lymphocytes isolated from the same donor's PBMCs by using T cell isolate kit (stemcell technoligies). The mitogenic activity of the growth factors was determined using a cell counting kit-8 (Dojindo), in which 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) was used as a substrate. After incubating cells for 7 days, 10 ml cck-8 solution was added to each well of the plate. The plate was incubated for 4 h in an incubator (37°C, 5% CO_2). The OD value at 450 nm was measured with a microplate reader using 600 nm as the internal reference.

Antigen presentation assay

To investigate the antigen presentation and T cell stimulatory capacity of c-kit silenced DCs, allogeneic lymphocytes were isolated from peripheral blood. DCs were co-transfected with siRNA and total RNA of MFC using Gene Silencer. After transfection, DCs were incubated in IMDM with 10% FBS and 50 ng/ml TNF-α to mature DCs for 48 h, then DCs were resuspended and seeded in triplicate in round-bottomed 96-well plates for use as stimulator cells at 5×10^4 cells/well. These cells were co-cultured with allogeneic lymphocytes (5×10^5 cells) in 200 ml IMDM supplemented with 10% FBS and 25 Units/ml IL-2 for 6 days. The supernatants were then harvested and IFN-γ, IL-4 and IL-17 in the medium were measured by ELISA using the Quantikine ELISA sets according to the manufacturer's instructions (R&D systems, Wiesbaden, Germany). The expression level of GATA-3, T-bet and ROR-γT were confirmed by western blot.

Induction of tumor-specific cytotoxic lymphocyte using c-kit silenced DCs

DCs were co-transfected with C-kit siRNA and total RNA of MFC using Gene Silencer. After transfection, DCs were incubated in IMDM complete medium containing 50 ng/ml TNF-α to mature DCs for 48 h. For induction of cytotoxic lymphocyte (CTL), 2×10^5 DCs (co-transfected with c-kit siRNA and total RNA of MFC) were incubated with 2×10^6 allogeneic lympho-

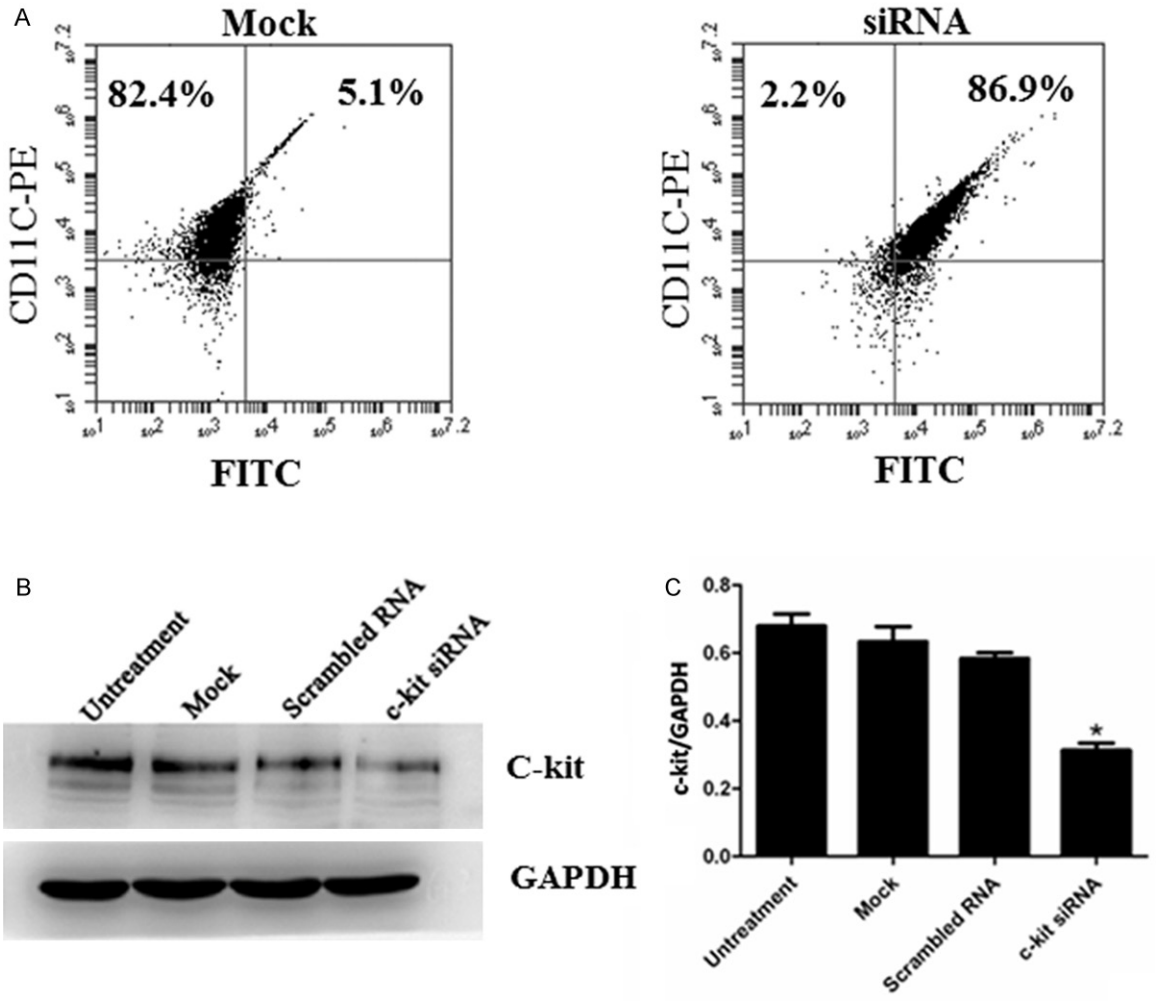


Figure 1. Efficacy of siRNA transfection of DCs and specific inhibition of c-kit expression. A. DCs were transfected with FITC-labeled or FITC unlabeled siRNA (200 nM) via Gene Silencer reagent. The transfection efficacy was observed using a flow cytometer 24 h later (left, Mock: FITC-unlabeled siRNA; right, c-kit siRNA: FITC-labeled siRNA). The purity of DCs was also assessed (left, 85%). B, C. The c-kit expression of DCs was analyzed with western blot. Data are representative of three independent experiments.

cytes in IMDM medium supplemented with 10% FBS and 25 Units/ml IL-2. Additional gene modified DCs were stored at -80°C to be used later for re-stimulation. After 7 days of culture, cells were restimulated. Cells were further cultured for one week, and then the cytolytic activity of induced CTL was analyzed using a cytotox 96 non-radioactive cytotoxicity assay kit (Promega).

CTL assay

To determine the cytolytic activity of induced CTL, we used the cytotox 96 non-radioactive cytotoxicity assay based on the calorimetric detection of the released enzyme LDH. Briefly, target cells (tumor cell lines) were harvested, washed, counted, and diluted to 2×10^5 cells/ml then 50 ml/well were seeded in a 96 well plate. Effector cells (lymphocytes) were washed, counted, diluted, and added at an effector: target cell ratio of 10:1. All of the conditions were assayed in triplicate. Cells were incubated at 37°C for 4 h, and then 50 ml of supernatants were assayed for lactic acid dehydrogenase (LDH) activity following the manufacturer's protocol. Controls for spontaneous LDH release in effector and target cells, as well as target maximum release, were prepared. The calculation of cytotoxicity percentage was as follows: cytotoxicity% = (experimental-effector spontaneous-target spontaneous)/(target maximum-target spontaneous) ×100.

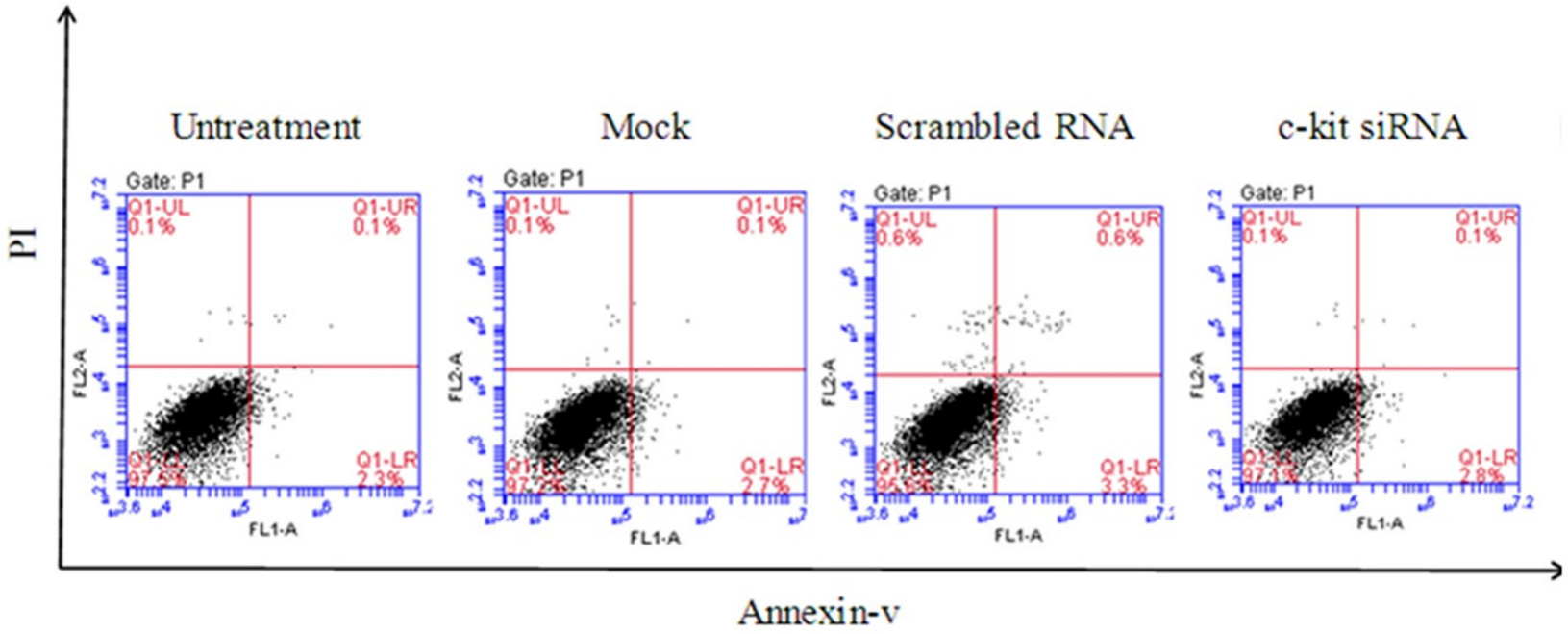
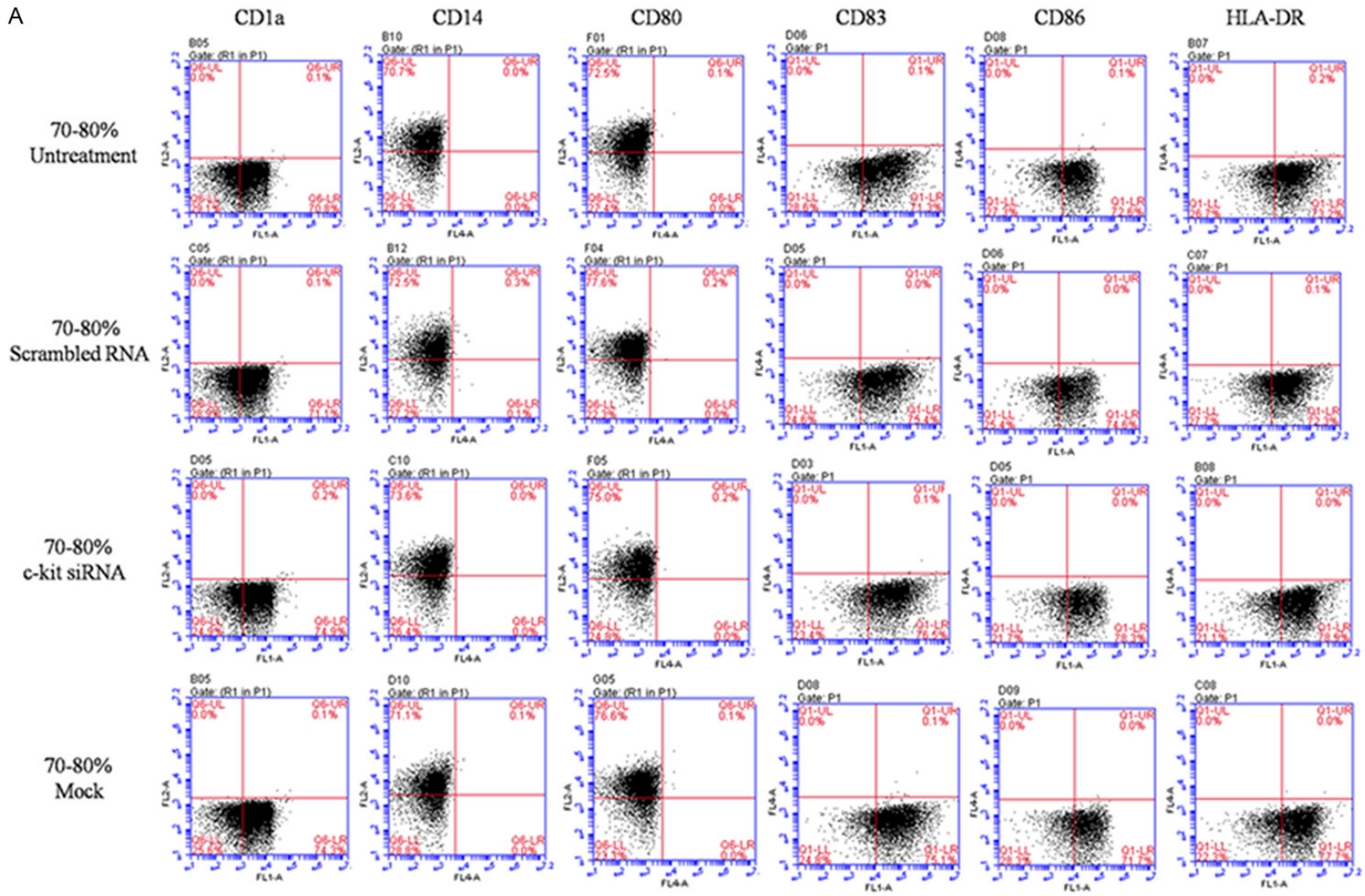


Figure 2. siRNA transfection does not affect the viability of DCs. DCs were cultured and treated as indicated in materials and methods. Percentage apoptosis and necrosis was evaluated using annexin V and propidium iodide by flow cytometry. Data are representative of three independent experiments.


A
CD1a
CD14
CD80
CD83
CD86
HLA-DR
70-80%
Untreatment
70-80%
Scrambled RNA
70-80%
c-kit siRNA
70-80%
Mock

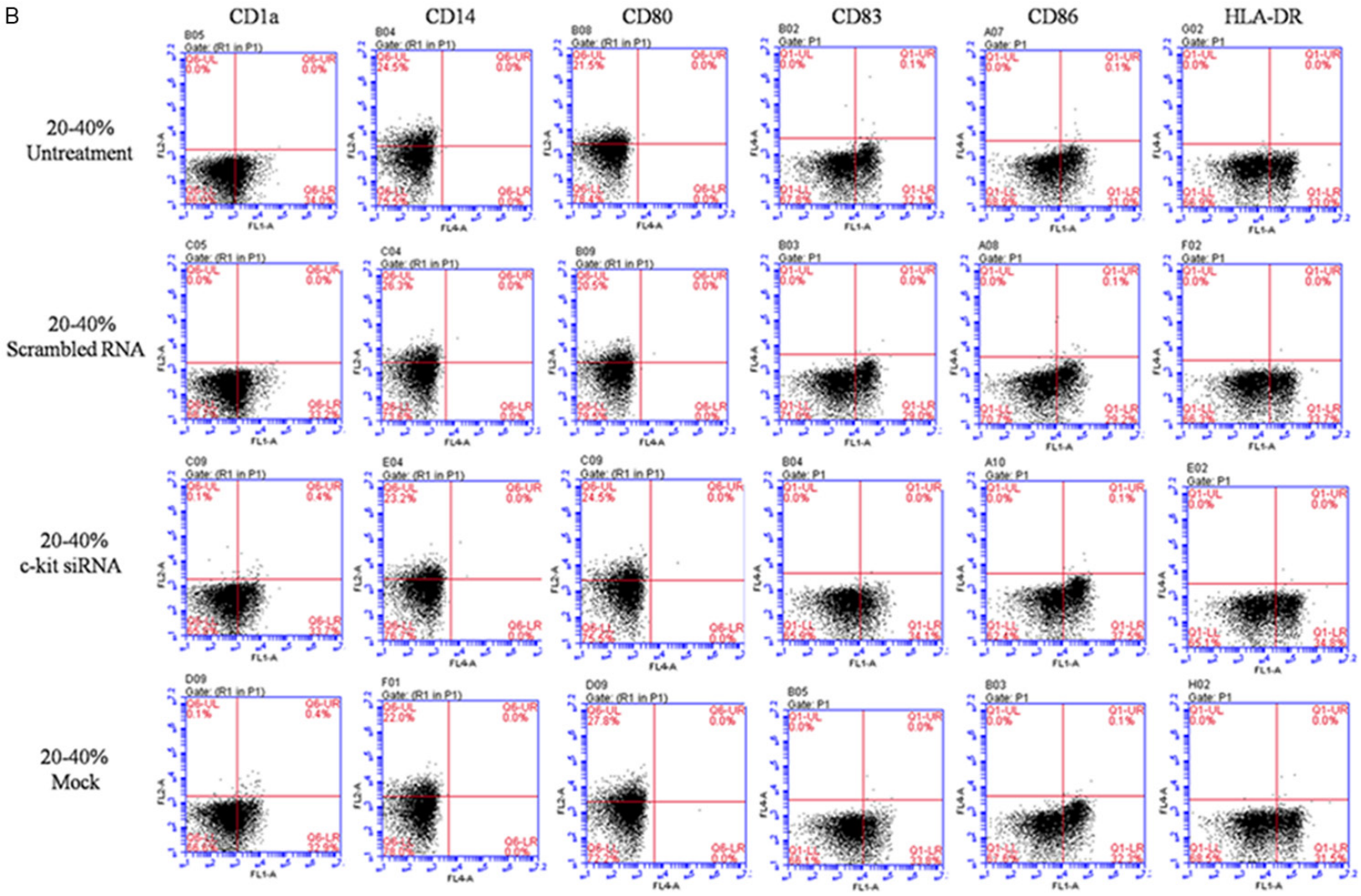


Figure 3. siRNA transfection neither alters nor induces DC maturation. DCs were treated as indicated in materials and methods. A. Mature DCs. B. Immature DCs. Data are representative of three independent experiments.

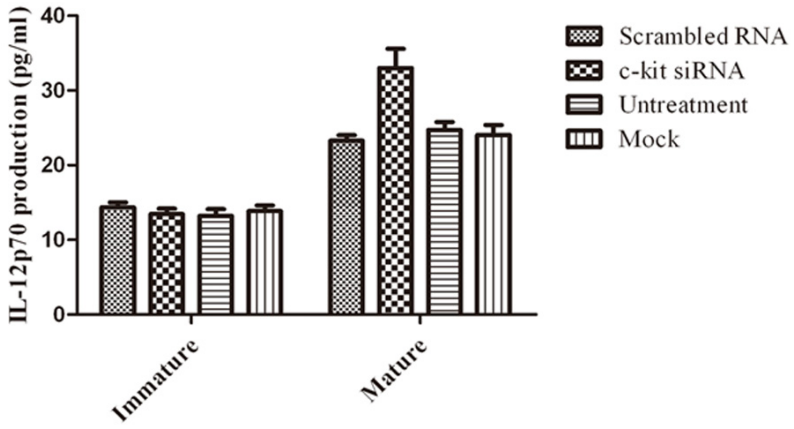


Figure 4. siRNA transfection does not influence IL-12p70 production by DCs. DCs were treated as indicated in materials and methods. Supernatants were harvested from cultures and analyzed for IL-12p70 production using ELISA. The results are the mean ± SD values obtained in three independent experiments ($P > 0.05$, by one-way ANOVA and NewmaneKeuls test).

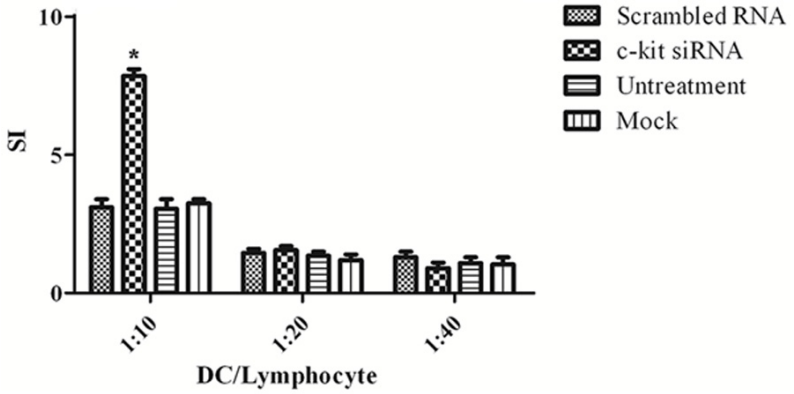


Figure 5. C-kit siRNA transfection increases DC-induced allogeneic lymphocyte proliferation. Proliferation was determined using the CCK-8 assay kit. The results are the mean ± SD values obtained in three independent experiments (*$P < 0.05$, P values was less than 0.001 for the c-kit siRNA group relative to the untreatment group, the scrambled RNA group, and the Mock group, respectively, by one-way ANOVA and NewmaneKeuls test).

Western blot

About 5×10^6 cells were lysed. Protease inhibitor mixture and pellets were kept at -80°C until use. Protein amounts were determined from the Bio-Rad protein assay. For each condition, 25 mg of protein was separated on 12% polyacrylamide gels and transferred to PVDF sheets. Monoclonal specific for c-Kit, agged-2, GATA-3, T-bet, ROR-γ and GAPDH (Santa Cruz Biotechnology, 1/100 dilution) was revealed with HRP-conjugated goat anti-Mouse Abs (Santa Cruz Biotechnology, 1/2000 dilution) using the ECL western blotting analysis system (Amersham Pharmacia Biotech).

Statistical analyses

We used Student's unpaired two-tailed t-test for all statistical analyses. Differences between groups were considered significant when $P < 0.05$. All statistical analyses (except those performed for microarray data) were performed with GraphPad Prism software.

Results

DCs are efficiently transfected with siRNA and c-Kit expression is significantly down-regulated

The transfection efficiency was measured using a flow cytometer (**Figure 1A**). More than 80% of DCs were efficiently transfected. The specificity of siRNA inhibition in DCs after transfection was investigated. Immature DCs were collected on culture day 6 and were transfected with 200 nM c-Kit siRNA or 200 nM control siRNA. After transfection, DCs were matured by adding 50 ng/ml TNF-α for 48 h, and then cells were collected to analyze c-Kit expression by western blot. It was observed that c-Kit siRNA could significantly knock down c-Kit.

siRNA transfection does not reduce the viability of DCs

To assess the toxicity of siRNA and the transfection reagent, the viability of DCs was measured. On day 6 of culture, DCs were treated with transfection reagent alone (Mock), transfected with non-silencing siRNA, or with c-kit siRNA. After 48 h of transfection, apoptosis and necrosis of DCs were evaluated using

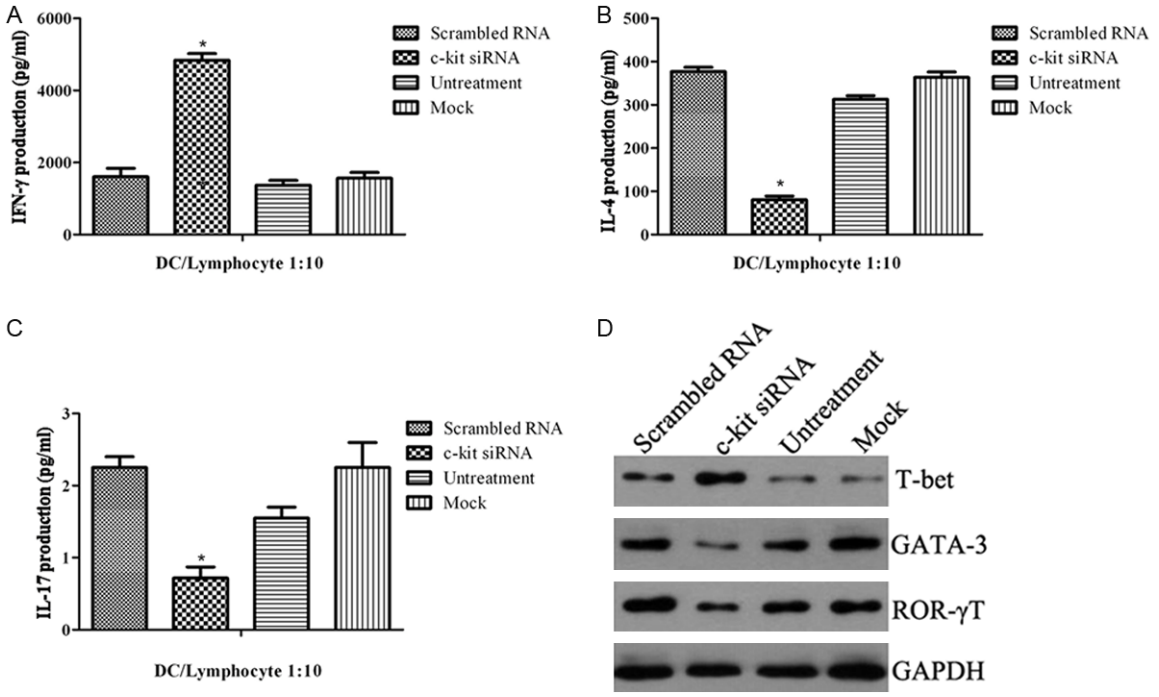


Figure 6. C-kit siRNA-treated DCs promote Th1 polarization. After 6 days of stimulation in the presence of c-kit siRNA-treated DCs, IFN-γ (A), IL-4 (B) and IL-17 (C) levels in the culture medium were determined using ELISA. The results are the mean ± SD values obtained in three independent experiments (*$P < 0.05$, P values were less than 0.001 for the c-kit siRNA group relative to the untreatment group, the scrambled RNA group, and the Mock group, respectively, by one-way ANOVA and NewmaneKeuls test). The expression level of T-bet in c-kit siRNA treated DCs stimulate allogeneic lymphocytes is up-regulation but the expression level of GATA-3 and ROR-γT is down-regulation (D).

annexin V and propidium iodide staining (**Figure 2**). Compared with untreated DCs, neither the transfection reagent alone nor the transfection reagent in combination with siRNA affected cell viability.

Cell surface phenotype analysis after c-kit siRNA transfection

To evaluate the effects of c-kit siRNA transfection on DC phenotype, a homogenous population of immature DCs were cultured with GM-CSF and IL-4 for 6 days and were matured with 50 ng/ml TNF-α for 48 h after siRNA transfection. Then DCs were collected to assess their phenotype by flow cytometry. Maturation of DCs led to dramatic phenotype changes, which is shown by the up-regulation of CD1a, CD80, CD83, CD14, CD86 and HLA-DR on the surface, shown in **Figure 3**.

IL-12p70 production of DCs after siRNA transfection

The maturation of DCs could be partially characterized by their IL-12p70 production after antigen or TNF-α stimulation. Thus, the IL-12p70 concentration in the culture medium of immature and mature DCs treated with transfection reagent alone, non-silencing siRNA, or c-kit siRNA after 48 h was evaluated. No alteration of IL-12p70 production was detected. These data indicate that transfection of H-2K DCs with c-kit siRNA does not affect their cytokine release after maturation **Figure 4**.

Lymphocyte stimulatory ability of DCs after c-kit siRNA transfection

DCs were transfected with c-kit siRNA, non-silencing control siRNA, transfection reagent alone, or were left untreated on culture day 6. These DCs were matured with 50 ng/ml TNF-α for 48 h. After that, allogeneic lymphocytes were cultured with these cells at various concentrations for 6 days before the lymphocyte proliferation assay was carried out. Compared with the other three groups, the allostimulatory activity of DCs transfected with c-kit siRNA was similar when the ratio of DC: lymphocyte was 1:40 and 1:20. However, c-kit siRNA-treated DCs significantly promoted the induction of

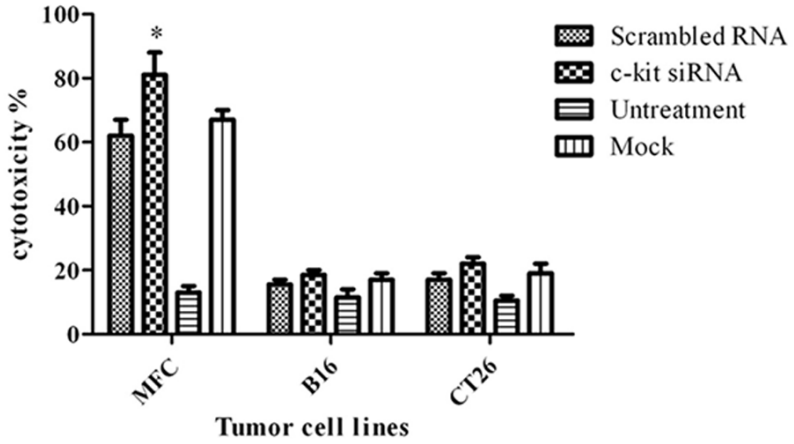


Figure 7. DCs transfected with c-kit siRNA and MFC RNA enhance the tumor-specific CTL response in vitro. The results are the mean ± SD values obtained in three independent experiments (*P < 0.05, MFC: P values were 0.001 for the c-kit siRNA group relative to the no treatment group, 0.01 for the c-kit siRNA group relative to the scrambled RNA group, and 0.01 for the c-kit siRNA group relative to the Mock group. by one-way ANOVA and NewmaneKeuls test).

lymphocyte proliferation in comparison with the other three groups when the ratio of DC: lymphocyte increased to 1:10 (**Figure 5**). This demonstrated that silencing the c-kit of DCs can enhance their allostimulatory ability.

C-kit siRNA-treated DCs polarize native T cells toward a Th1 response

To examine the function of c-kit siRNA transfected DCs, DCs were co-transfected with c-kit siRNA and total RNA of MFC initially. Allogeneic lymphocytes were stimulated with these DCs. After 6 days of stimulation, the IFN-γ concentration in the culture medium was obviously increased in the presence of c-kit siRNA treated DCs (**Figure 6A**). IL-4 and IL-17 production in the cultures was very low compared with the other groups (**Figure 6B** and **6C**). Furthermore, the expression level of T-bet in c-kit siRNA treated DCs stimulate allogeneic lymphocytes is up-regulation but the expression level of GATA-3 and ROR-γT is down-regulation (**Figure 6D**). The results indicated that c-kit siRNA-treated DCs polarized allogeneic lymphocytes toward the Th1 response.

DCs transfected with c-kit siRNA can enhance the specific CTL response

The cytotoxic activity of CTL induced by c-kit siRNA-treated DCs was analyzed 7 days after restimulation using a cytotox 96 non-radioactive cytotoxicity assay kit. Specific lysis of MFC target cells could be detected in the c-kit siRNA group, the scrambled RNA group, and the Mock group. However, much stronger cytotoxic activity was exhibited in the c-kit siRNA group. In addition, MFC RNA-transfected DCs had an increased cytotoxic effect compared to untreatment group when the CT26 and B16 cell lines were used (**Figure 7**).

Discussion

The discovery of DCs has enhanced our knowledge of the divergent pathways of the TH1 and TH2 responses, as well as the recently discovered TH17 response [1-3]. In this study, we show that silencing the c-kit gene did not affect the viability and maturation of DCs, and significantly increased allogeneic lymphocyte proliferation and polarized allogeneic lymphocytes toward the Th1 response. Although c-Kit has been best studied in the context of mast cells, administration of SCF in vivo has been shown to have mast cell-independent effects. c-Kit-expressing DCs have been shown to modulate NK cell function by increasing the cytolytic and IFN-γ secreting ability of NK cells, thereby defining the consequence of this receptor in at least one aspect of DC function [10]. Mast cells are known to finely tune T cell responses because of their ability to secrete a variety of cytokines, DCs, being professional antigen-presenting cells, have a quite essential role in the orchestration of the adaptive immune response. Our experiment of adoptive transfer of DCs demonstrated that using the intact mast cell c-Kit signaling that is present in the recipients to cripple c-Kit signaling only in adoptively transferred DCs blunts the TH2 and TH17 response. On the basis of these results, it seems probable that inhibit c-Kit expression in DCs may be reduce the susceptibility of allergic disease.

It is well accepted that a successful vaccine should induce antigen specific CD4+Th1 as well as CD8+T cells. The antigen specific CD4+Th1 cells are needed for the induction and maintenance of CD8+T cell responses and may direct-

ly or indirectly contribute to tumor cell destruction [22-24]. The activation of the c-Kit-PI3 kinase axis in DCs is a key regulatory mechanism that promotes TH2 and TH17 responses and limits the TH1 response. Because of the pivotal role of c-kit in endogenous antigen cross-presentation aforementioned, one of the effective approaches to simultaneously activate CD4+ and CD8+T cells is to knock c-kit down.

IL-12p70 is an inducible cytokine which is crucial to promoting the development of TH1 cells and cell-mediated immunity [25-27]. It is mainly released by DCs. Although silencing c-kit gene in DCs cannot directly enhance IL-12p70 production in our study, it may enhance cross-presentation of an endogenous antigen, such as total RNA of tumor cells, to CD4+T cells. Activated CD4+T cells will greatly increase their interaction with conditioned DCs. This interaction via CD40 and CD40 ligand can trigger DC production of IL-12p70 and is critical for generating TH1 cell help for cytotoxic responses. After antigen stimulation, CD4+T cells differentiate into at least two types of effector cells that differ as to the pattern of cytokines they produce upon re-stimulation. Th1 cells are defined by the production of IFN-γ and mediate predominantly cellular immune responses, whereas the signature cytokines of TH2 cells (IL-4, IL-5, IL-13) are involved in allergic reactions. In our study, when the c-kit gene was silenced, H-2K DCs significantly enhanced allogeneic lymphocyte proliferation. In addition, H-2K DCs polarized a TH1 response which was confirmed by high IFN-γ production and much lower IL-4 production from allogeneic lymphocytes.

To further assess the function of c-kit silenced DCs, an initial observation was conducted on the antitumor effect of these cells in vitro. Interestingly, much stronger cytotoxic activity was detected when the gastric cancer cell line MFC was co-cultured with CTL induced by c-kit siRNA and MFC RNA cotransfected DCs. It indicated that silencing the c-kit gene in DCs may enhance their antitumor ability in vitro. The reason for low cytotoxic activity was detected in the control cell lines CT26 and B16 is that these tumor cell lines may share some antigens encoded by the MFC RNA to which the cytotoxic response is directed.

In conclusion, we showed that silencing the c-kit gene did not affect the viability and maturation of DCs derived from peripheral blood. Furthermore, c-kit siRNA-treated DCs significantly increased allogeneic lymphocyte proliferation and polarized allogeneic lymphocytes toward the TH1 response. Our findings have ramifications for DC-based vaccines in cancer therapy, where inhibition of c-Kit may promote the efficacy of the vaccine by augmenting a TH1 response.

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Disclosure of conflict of interest

None.

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