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Cell penetrating recombinant Foxp3 protein enhances Treg function and ameliorates arthritis

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ABSTRACT

Foxp3 is the master transcription factor for T regulatory (Treg) cell differentiation and function. This study aimed to test the therapeutic potential of cell penetrating recombinant Foxp3 protein in arthritis. Recombinant Foxp3 protein was fused to a cell penetrating polyarginine (Foxp3-11R) tag to facilitate intracellular transduction. *In vitro* Foxp3-11R treated $CD4^+$ T cells showed a 50% increase in suppressive function compared with control protein treated cells. Severity of arthritis in Foxp3-11R treated mice was significantly reduced compared with those treated with a control protein. $CD4^+$ T cells of lymph nodes and spleen from Foxp3-11R treated mice showed increased levels of Foxp3 expression compared with those of a control protein treated. These results demonstrated that Foxp3-11R can enhance T cell suppressive function and ameliorate experimental arthritis and suggest that cell penetrating recombinant Foxp3 is a potentially useful agent in therapy of arthritis.

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1. Introduction

Immune homeostasis is mediated by a complex system of central and peripheral mechanisms. T regulatory (Treg) cells play a crucial role in maintenance of self-tolerance in periphery. Treg cells are a subset of $CD4^+$ helper T cells and Foxp3 is the master transcription factor for Treg function [1]. The exact mechanisms how Foxp3 regulates Treg function is not fully elucidated yet, but it is considered to be achieved through its interaction with other transcription factors such as NFAT, NF-κB and RORC, and regulation of multiple other genes [2]. Mutations in Foxp3 gene result in dysfunction of Treg cells and severe autoimmune inflammation [3]. Decreased number of and impaired function of Treg cells have been demonstrated in autoimmune disorders including systemic lupus erythematosus, rheumatoid arthritis (RA), multiple sclerosis and type 1 diabetes [4]. Therefore, restoration of Treg cell function and administration of *in vitro* generated Treg cells are a potential therapeutic strategy to those autoimmune diseases [4]. Previous studies using Treg cells as therapy have focused on cell based treatment. Although some positive effects have been reported, cell based therapy have suffered from intrinsic disadvantages with requirement of long term *in vitro* expansion and maintenance of Treg cells. Injected Treg cells proved to be instable and have the potential to change phenotype and loss of regulatory function. Moreover, injected Treg cells may produce detrimental rather than therapeutic effects since Treg cells can transform *in vivo* to pathogenic Th17 or Th1 like effector cells [5–7]. To overcome the problems associated with cell based Treg cell therapy, a novel approach to promotion of Treg cell function *in vivo* has been attempted. Since induction of Foxp3 in naïve T cells converts naive T cells into Treg-like cells [1,8], several studies have tried genetically induction and modification, or direct delivery of Foxp3 for clinical use [9–11]. However, the application is still limited due to its potential risk of delivery method or lower transfection efficiency.

Several short peptides, such as HIV tat and polyarginine, can cross cellular membrane. Previous reports have demonstrated stable delivery of recombinant proteins into cells using these short peptides [12,13]. In order to facilitate Foxp3 protein delivery, we created recombinant Foxp3 protein fused with polyarginine (11R). In this report, we demonstrated that Foxp3-11R converted mouse T cells into $Foxp3^{high}$ Treg-like cells and these Treg-like cells suppress other T cell proliferation *in vitro*. Additionally, systemic administration of Foxp3-11R alleviated arthritis of SKG mice. These results propose a stable delivery method of Foxp3 proteins into T cells and its potential use for therapy of autoimmune diseases.

2. Materials and methods

2.1. Gene construction and protein production of recombinant Foxp3-11R and ASCL-1-11R

Our target protein construct is a human Foxp3 protein (NP_054728.2) fused with a C-terminal polyarginine tag (11R) via

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a linker (ESGGGGSPG). The cDNA sequence of this construct was first optimized for high level protein expression in *Escherichia coli* and then synthesized by GenScript (Piscataway, NJ). Next, the gene was sub-cloned into vector pET-15b via NdeI and BamHI restriction enzyme sites. As a result, the expressed protein also has an N-terminal polyhistidine (6H) tag (Fig. 1A). The gene of ASCL1-11R was designed and constructed in the same way. The above protein expression plasmids were transformed into BL21 (DE3) competent cells, and protein production, refolding, and purification were carried out with procedures as previously described [13].

2.2. Flow cytometry

The mAbs used for flow cytometric analysis were: FITC- or APC anti-CD3 (2C11; BD bioscience), FITC- or PE-anti-CD4 (G.K1.5; BioLegend), PE-anti-CD25 (3C7; BD Bioscience), APC-Foxp3 (FJK16s; eBioscience). For analysis of Foxp3 transduction, cells were fixed and permeabilized using Fixation/Permeabilization buffer (eBioscience) followed by incubation with the specific Abs. FITC-Annexin V (BioLegend) staining was performed by following a standard protocol as described [14].

2.3. Foxp3-11R transduction assay

SKG mouse splenocytes were cultured with 10 μg/ml of Foxp3-11R or ASCL1-11R in anti-CD3/28 mAb (Bioxcel) pre-coated round bottomed 96-well plate for 48 h. After incubation, intracellular Foxp3 and CD25 on cell surface were stained.

2.4. Treg-like cell suppression assay

As suppressor cells, SKG mouse splenocytes were incubated with 10 μg/ml of Foxp3-11R or ASCL1-11R for 1 h and washed twice with culture medium. As responder cells, SKG mouse splenocytes were stained with 1 μg/ml of CFSE for 10 min, and washed twice with warmed PBS and culture medium. After creating suppressor and responder cells, both cells were cultured at various ratio (res:sup = 2:1, 1:5) and were stimulated with anti-CD3/CD28 coated 96 well plate for 72 h. Cell proliferation of responder cells were measured by flow cytometry.

2.5. SKG mice, arthritis induction and disease monitoring

Arthritis was induced by intraperitoneal injection of 2 mg of zymosan in 6 weeks old female SKG mice. The day of zymosan injection was defined as day 1. From day 8 to day 14, 40 μg of Foxp3-11R or ASCL1-11R were injected intraperitoneally and severity of arthritis was graded. Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.1, mild swelling of one finger joint; 0.2; severe swelling of one finger joint; 0.5, mild swelling of wrist or ankle; 1.0, moderate swelling of write or ankle; 1.5, severe swelling of wrist or ankle. Scores for all fingers

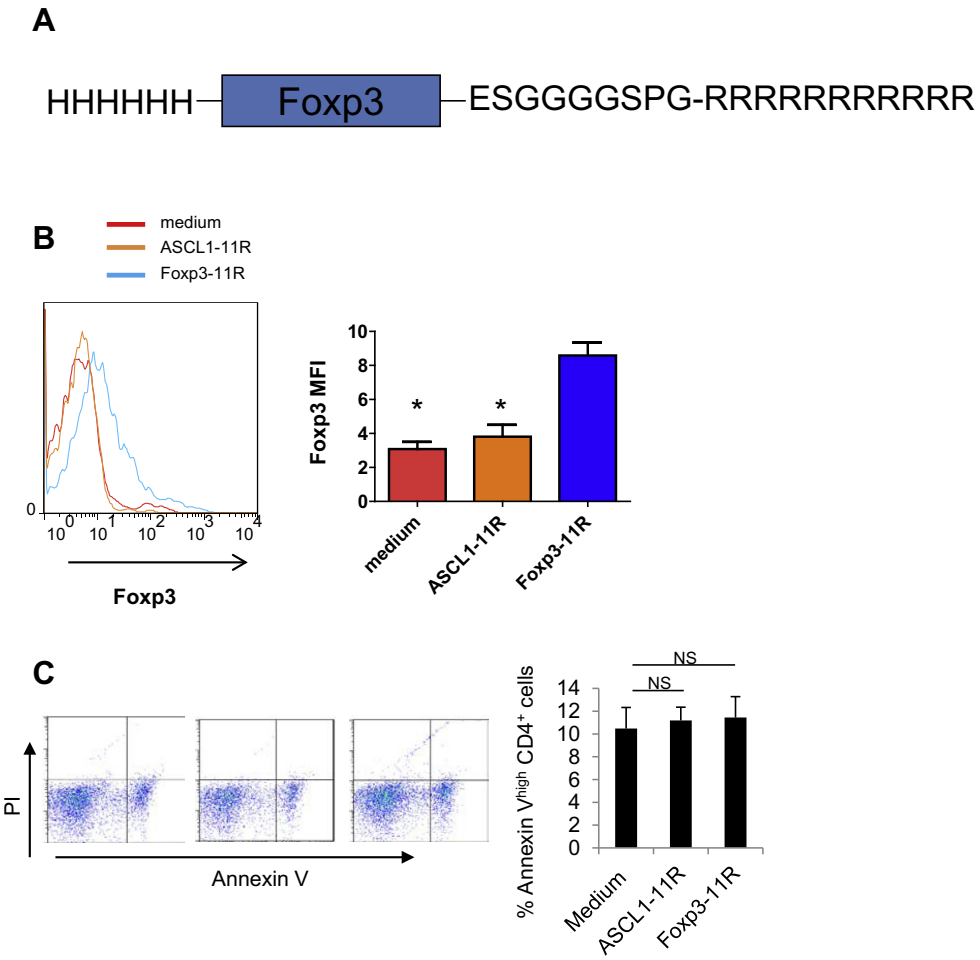

Fig. 1. Generation of cell-permeable Foxp3-11R. (A) A schematic presentation of the Foxp3 fusion protein. (B) SKG mouse splenocytes were incubated with 10 μg/ml of Foxp3-11R, ASCL1-11R or culture medium in anti-CD3/CD28 coated plates for 120 h, and Foxp3 was stained intracellularly. Mean fluorescence of Foxp3 was compared between the three treatment groups. Data are representative of three independent experiments and mean ± SD ($n = 3$), $^{*}p < 0.05$. (C) Splenocytes were treated as in (B) and stained with Annexin V and propidium iodide (PI). Figure shows $CD4^{+}$ T cells stained by Annexin V (pooled data of three experiments, $n = 6$ in each group).

of fore paws and hind paws, wrists and ankles were summed for each mouse. The maximum of score for each mouse is 9.6. The study was approved by the Institutional Animal Care and Use Committee of Portland VA Medical Center.

2.6. Statistics

Two-tailed Student *t* test and Mann–Whitney *U* test were performed for statistical analysis.

3. Results and discussion

3.1. Foxp3-11R penetrate cell membrane

To create cell-penetrating Foxp3 protein, we designed and fused a polyarginine (i.e. 11R) protein transduction domain to the C-terminus of the human Foxp3 protein (Fig. 1A). We also added a polyhistidine (6H) tag to the N-terminus for purification and detection. The protein was expressed in *E. coli* in inclusion body form, which was then solubilized, refolded, and further purified. The protein identity was confirmed by SDS–PAGE and Western blot analysis. Similarly, we constructed and prepared the ASCL1-11R protein as a control protein. To examine the transduction efficiency, splenocytes from SKG mouse were incubated with Foxp3-11R or irrelevant control protein ASCL1-11R, and intracellular Foxp3 level was assessed. Higher levels of Foxp3 were detected in Foxp3-11R treated group compared to control groups, and this confirmed transduction of Foxp3-11R (Fig. 1B). Since polyarginine is cell membrane permeant cationic and potentially cytotoxic, we assessed the viability of the spleen cells by Annexin V staining after exposure to Foxp3-11R or ASCL1-11R. As shown in Fig. 1C, there is no significant increase in Annexin V positive cells in either Foxp3-11R or ASCL1-11R treated spleen cells as compared with those cultured in medium alone. These data indicate that polyarginine tagged Foxp3 (Foxp3-11R) is not toxic to the cells and is potentially safe to be administered *in vivo* as a therapeutic agent.

3.2. Foxp3-11R converted T cells to Treg-like cells

Next, we investigated T cell suppressive function of Foxp3-11R. Treg cells were initially characterized as $CD4^{+}CD25^{high}$ T cells with immunosuppressive function [15]. We examined CD25 expression after Foxp3-11R treatment. Surprisingly, CD25 was not altered by Foxp3-11R treatment (Fig. 2A). In order to assess its role in T cells, we performed Treg-like cell suppression assay. In this assay, suppressor cells were prepared by Foxp3-11R treatment, and responder cells were stained with CFSE. Subsequently, suppressor cells and responder cells were mixed at various ratios and function of Treg-like cell was evaluated by proliferation of responder cells. Foxp3-11R treated T cells significantly inhibited proliferation of responder cells (Fig. 2B). This indicated that Foxp3-11R treated T cells gained suppressive function and may have been converted to Treg-like cells.

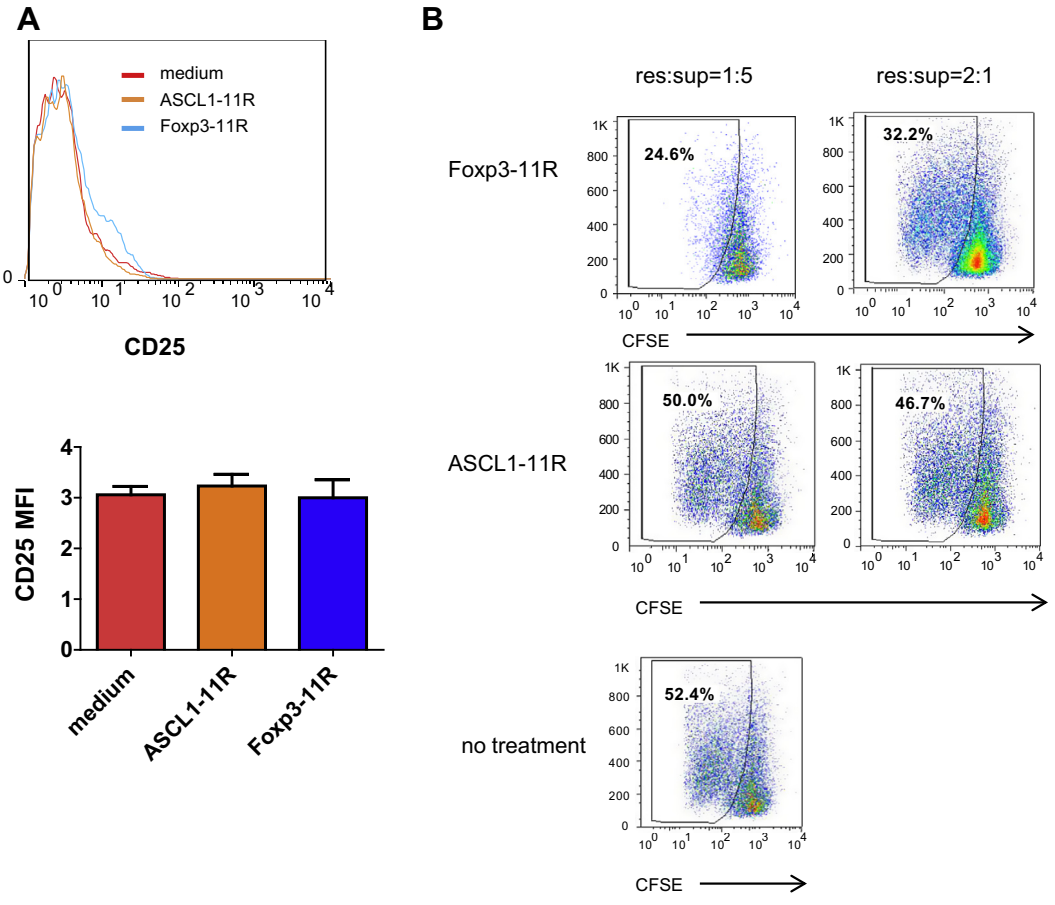

Fig. 2. Foxp3-11R treatment did not upregulate CD25, but induced Treg-like cells. (A) SKG mouse splenocytes were incubated with 10 μg/ml of Foxp3-11R, ASCL1-11R or culture medium in anti-CD3/CD28 coated plates for 120 h, and CD25 on cell surface was stained. (B) Suppressor cells (sup) were prepared by incubating SKG mouse splenocytes with 40 μg/ml of Foxp3-11R, ASCL-11R, or culture medium for 1 h and washed twice. As responder cells (res), SKG mouse splenocytes were stained with CFSE. Suppressor cells and responder cells were mixed at various ratios. Data are representative of three independent experiments.

3.3. Foxp3-11R ameliorated arthritis in SKG mice

We next investigated the clinical application of Foxp3-11R in arthritis model. SKG mice have a mutation in gene encoding ZAP-70 along with a defective Treg function and develop chronic arthritis which most closely resembles human rheumatoid arthritis [16]. The impaired Treg cell function might be a contribution factor for SKG mice developing arthritis. Therefore, SKG mouse arthritis serves as a right model to test the capacity of Foxp3-11R in treating arthritis. First, we assessed *in vivo* delivery of Foxp3-11R. Seven days after induction of arthritis by zymosan, Foxp3-11R or ASCL1-11R was given systemically for 7 days. Fourteen days after final treatment, lymph nodes and splenocytes were harvested and intracellular Foxp3 level was measured. As shown in Fig. 3A, increased level of Foxp3 was detected from Foxp3-11R treated group and this indicated that Foxp3-11R was delivered intracellularly even by systemic injection.

Development of clinical arthritis in SKG mice became visible around 2 weeks after zymosan injection. Mice in ASCL1-11R treated group develop more severe arthritis and the disease progressed (Fig. 3B). In contrast, mice in Foxp3-11R treated group had significantly reduced severity of arthritis. This effect lasted for 7 weeks. These results showed that Foxp3-11R treatment ameliorated arthritis in SKG mice.

Impaired Treg function is associated with multiple autoimmune diseases. Intensive research has been focused on therapeutic interventions to enhance Treg cell numbers and Treg cell function in autoimmune diseases [17]. Those approaches include *ex vivo* expansion of Treg by cytokines and viral mediated Foxp3 transfection [9,18–20]. However, stability of Treg cells created *ex vivo* is still questionable and viral mediated transfection has concern in its safety for human application. Moreover, *in vitro* generated Treg cells can potentially differentiate into effector cells *in vivo* [5–7]. In this report, we demonstrated that recombinant Foxp3-11R was delivered into cells stably both *in vitro* and *in vivo* and converted T cells to Treg-like cells which inhibited proliferation of responder T cells and ameliorated arthritis.

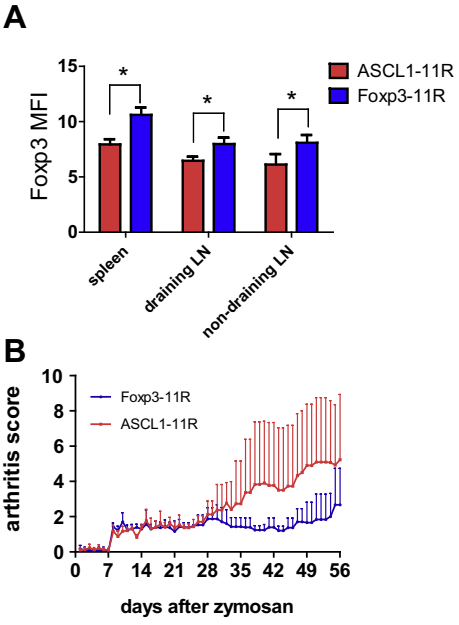

Fig. 3. Foxp3-11R prevented progression of arthritis in SKG mice. (A) Seven days after arthritis induction, SKG mice were treated with Foxp3-11R or ASCL1-11R for 7 days. Fourteen days after final treatment, splenocytes were harvested and Foxp3 was stained intracellularly. Data are CD4 T cell gated and mean ± SD ($n = 4$), $^{*}p < 0.05$. (B) Severity of arthritis was monitored daily after the above treatment ($n = 10$ in each group), ($p = 0.78$ at week 4, $p = 0.02$ at week 6 and $p = 0.033$ at week 8, pooled data of two experiments).

It is interesting that Foxp3-11R treatment enhanced Treg cell function both *in vitro* and *in vivo* by enhancing Foxp3 expression but did not seem to affect expression of CD25. CD25 is the α-chain of high affinity IL-2R complex. IL-2R receptor signaling is critical for functional maturation of natural Treg cells during thymic development [21] and consumption of IL-2 by means of high CD25 expression is considered to be an important mechanism in maintaining Foxp3 expression in periphery [22]. Indeed, CD25 deficient mice have poorly functional Treg due to lower Foxp3 [23]. All these data indicate the role of CD25 in Treg development and function is mediating Foxp3 expression. However, the ability of the $Foxp3^{+}$ T cells to act as suppressors is not dependent on expression of CD25. For instance, infection of $CD4^{+}/CD25^{-}$ T cells with a retrovirus expression *foxp3* gene converted those cells to a regulatory phenotype [1,24]. Therefore, induction of Foxp3 expression is necessary and sufficient for regulatory cell function but induction of CD25 expression is not absolutely required.

In summary, we demonstrated that recombinant Foxp3 protein with cell penetration capacity could enhance Treg cell function and of potentially therapeutic in arthritis with impaired Treg cell function.

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