Development of a Human IFN-β Expression System using Chinese Hamster Ovarian Cells

by

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A Senior Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

by

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May, 2019

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Abstract

Two human IFN-β expression systems were derived based on the pIRES2-AcGFP1 plasmid backbone. One expression plasmid encoded human IFN-β fused to a C-terminal linker and an 8-histidine affinity chromatography tag. A second expression plasmid encoded human IFN-β without the C-terminal additions to determine if the addition of the 8-his tag alters IFN-β function. Both expression vectors encoded the native signal sequence to direct secretion of IFN-β as a glycosylated soluble protein. These plasmids were then transfected into Chinese Hamster Ovary (CHO) cells. Stable transfected CHO cells were selected based on plasmid-encoded resistance to the antibiotic Geneticin. IFN-β-producing cells were selected by Fluorescence-Activated Cell Sorting of the brightest 10% fraction of GFP^+ cells. Expression supernatants from each cell line exhibited similar amounts of cytotoxic activity in the IFN-β reactive TF-1 erythroleukemia cell line. These results provided suggestive evidence that the C-terminal affinity tag did not adversely affect the activity of the N-terminal IFN-β cytokine domain. This IFN-β-8his recombinant protein was purified by Ni-NTA affinity chromatography and was shown to exhibit potent activity in the *in vitro* TF-1 cytotoxicity assay. Human peripheral blood mononuclear cells (PBMCs) were activated with Con-A, IL-2, and either IFN-β, TGF-β, IFN-β + TGF-β, or no additional cytokine. Cell numbers were counted at each passage. The main finding was that IFN-β caused the induction of T cell anergy. Human T cells (90% $CD8^+$) were activated with RS4 (11) cells (acute lymphoblastic leukemia cell line), Con-A, and IL-2 in the presence or absence of IFN-β, TGF-β, IFN-β + TGF-β. T cells were cultured for eight days, and then reactivated. Supernatants were collected from reactivation cultures to measure IL-2 production as a measure of T cell responsiveness. Human T cells activated in the presence of IFN-β and TGF-β produced less IL-2 compared to T cells activated in the presence of TGF-β

alone. This expression system will be used to reveal whether IFN-β elicits differentiation of human FOXP3$^+$ Tregs.

Introduction

Multiple Sclerosis (MS) is an inflammatory, demyelinating autoimmune disorder of the central nervous system (CNS) that afflicts more than 2 million people globally (1). MS is a prominent cause of neurological disability in young adult populations (2). MS is characterized by a loss of either sensory or motor function due to permanent axonal damage (2). MS is demonstrated clinically through a variety of nervous system complications. The common presentations of MS include optic neuritis, cognitive impairment, partial transverse myelitis, or diplopia (3). Cerebral hemisphere lesions are also presented with symptoms including aphasia and encephalopathy (3). Most frequently, individuals with MS experience periods of relapsing symptoms followed by periods of remission, which is termed relapsing-remitting multiple sclerosis (1). Despite the prevalence of MS, the underlying mechanism causing the disease remains unknown. Current evidence suggests peripheral Th1 and Th17 cells are activated and migrate into the CNS by inducing a proinflammatory response via cytokine production (4). Due to molecular mimicry, myelin reactive T cells are reactivated in the CNS to release inflammatory cytokines and chemokines, permitting migration of CD8$^+$ T cells and neutrophils into the CNS (4). As a result, the myelin sheath is damaged by the inflammatory response (4).

Currently there is no cure for MS, though IFN-β has historically been used as the primary FDA-approved treatment for the disease. Recombinant IFN-β produced in E. coli was the first approved form of MS treatment in 1993 (5). By 1996, recombinant IFN-β drugs were produced by use of CHO cells to express IFN-β (5). These medications demonstrated a reduced rate of

annual relapse of MS presentation (5). IFN-β also elicits a reduction in brain lesion formation as observed in clinical trials (5). Evidence suggests IFN-β has the capability to lessen the severity of MS presentation, though the mechanism of IFN-β function in the body is not fully understood. It is proposed that IFN-β increases anti-inflammatory cytokine production such as IL-10 and IL-4 (6). IFN-β is also suggested to decrease the production of proinflammatory cytokines such as IL-17 and osteopontin (6). There is no substantial evidence currently to affirm how IFN-β is able to increase or decrease specific cytokine production.

Recent research regarding IFN-β has focused on T regulatory (Treg) cells. Tregs are a subclass of T cells that are derived from naïve T cells in the thymus or the periphery (7). Tregs suppress effector T cells, inhibiting autoimmune responses from occurring in the body (7). $CD4^+CD25^+$ Treg development and function are regulated by the transcription factor Foxp3 (7). Foxp3 is crucial for T cell differentiation into Tregs, and high levels of Foxp3 is necessary for immunological suppression of effector T cells by Tregs (7). Past studies have demonstrated how TGF-β is required for T cell differentiation during immune responses, including the upregulation of Tregs (8). However, in regards to IFN-β, recent preclinical studies in mouse models of MS showed that IFN-β elicits an immunosuppressive subset of $FOXP3^+$ Tregs, which in turn suppress CNS demyelinating disease in mice (9). Given that mouse IFN-β elicits Tregs in mice, a central question is whether human IFN-β elicits differentiation of human $FOXP3^+$ Tregs in primary T cell cultures. The purpose of this project is to derive new mammalian IFN-β expression systems to support studies assessing whether human IFN-β elicits or stabilizes human Tregs.

Materials and Methods

Preparation of IFN-β and IFN-β-8his plasmids

Human IFN-β untagged clone plasmid was obtained from Origene. After reconstitution, the IFN-β plasmid was transformed into Top 10 E. coli by use of electroporation. An initial screen was performed via PCR to identify bacterial colonies with the IFN-β plasmid. The IFN-β plasmid from positive E. coli was amplified and prepared for the extension PCR reaction by PCR with forward and reverse primers. A different reverse primer was used to encode an 8-histidine tag at the C terminal end of the IFN-β gene on a select number of IFN-β plasmids, generating two different IFN-β plasmids.

Generation of pIRES2-AcGFP1 with IFN-β or IFN-β-8his

Previously prepared Top 10 E. coli containing the pIRES2-AcGFP1 (Clontech) plasmid were cultured on Luria Broth (LB) with kanamycin. pIRES2-AcGFP1 was isolated from the Top 10 E. coli and amplified. The IFN-β or IFN-β-8his PCR product was inserted into the pIRES2-AcGFP1 by an extension PCR reaction using 5 ul of the previous PCR product and 2 ug of pIRES2-AcGFP1. Extension PCR reaction products were digested with restriction endonucleases (SmaI, SalI-HF, EcoRI-HF, Eco53KI, AfeI, DpnI) to isolate the newly formed pIRES2-AcGFP1 IFN-β-8his plasmid. The pIRES2-AcGFP1 IFN-β and pIRES2-AcGFP1 IFN-β-8his plasmids were transformed into Top 10 E. coli. PCR screening of the Top 10 E. coli was conducted to verify the creation of the plasmids.

Transfection of pIRES2-AcGFP1 IFN-β and pIRES2-AcGFP1 IFN-β-8his into CHO Cells

CHO cells cultures were established and used for pIRES2-AcGFP1 transfection. 5 ug of either pIRES2-AcGFP1 IFN-β or pIRES2-AcGFP1 IFN-β-8his were prepared in serum free DMEM and Turbofect Transfection Reagent (ThermoFisher) before being added dropwise to CHO cell cultures in complete DMEM (CDMEM). Expression of Green Fluorescent Protein (GFP) by the CHO cells was examined by flow cytometry, indicating successful transfection. CHO cells were cultured in CDMEM with Geneticin (G418). The brightest fraction of GFP-expressing CHO cells were separated by flow cytometric sorting.

Purification of IFN-β-8his

Supernatant from culture media was periodically collected from the CHO cells that were stably transfected with the pIRES2-AcGFP1 IFN-β-8his plasmid. IFN-β-8his expressed in the CHO supernatant was purified on Ni-NTA affinity columns. Protein was collected by use of wash buffer with varying concentrations of imidazole. Protein electrophoresis was performed on the samples of wash buffer containing protein supernatant to determine which elution fractions contained the purified IFN-β-8his. The purified IFN-β-8his was concentrated by use of Amicon Ultrafiltration Devices for a final concentration of 0.875mg/ml.

Results

Purified Human IFN-β Cytotoxicity Assay

To determine whether IFN-β was successfully produced by the pIRES2-AcGFP1 IFN-β expression system, TF-1 cells were cultured with the purified IFN-β-8his collected by Ni-NTA affinity chromatography. IFN-β has a cytotoxic effect on TF-1 cells, allowing decreased counts per minute of cells do be indicative of IFN-β presence. After 3 days, IFN-β concentrations from 3.2 nM to 0.032 nM produced significantly less TF-1 cells (Figure 1). Results for the purified IFN-β-8his were comparable to an IFN-β stock previously purchased, indicating function of the IFN-β-8his was not altered by the 8 histidine tag attached. Based on the cytotoxicity present by the TF-1 cells, the Origene human IFN-β plasmid was successfully incorporated in the pIRES2-AcGFP1 plasmid and transformed into CHO cells.

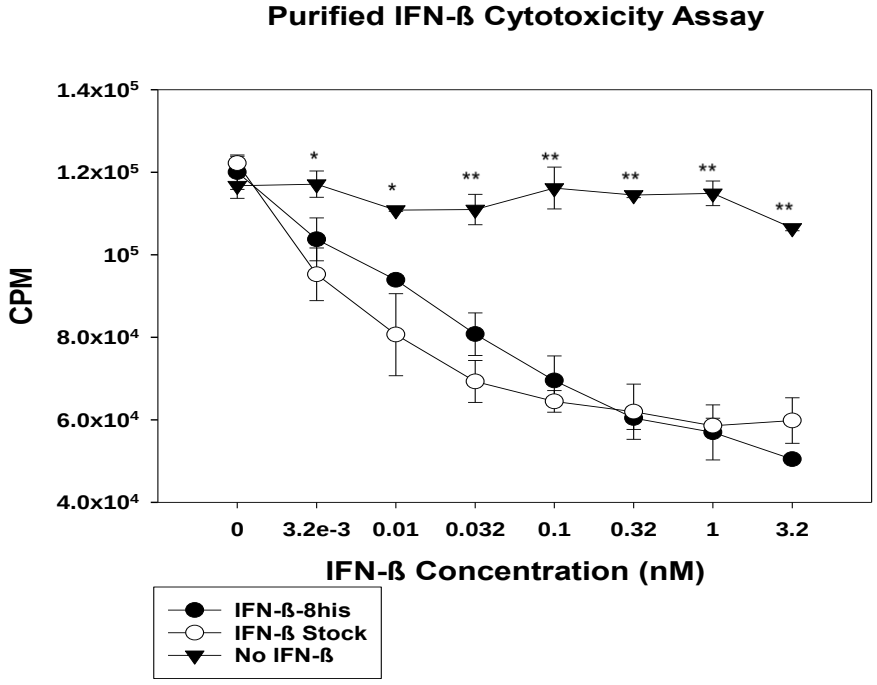

Figure 1. Purified human IFN-β-8his cytotoxicity assay. On day 0, 10,000 TF-1 cells were cultured with designated concentrations of IFN-β or IFN-β-8his. 2 ng/mL GMCSF were added per well to stimulate cell growth in all wells. On day 2, the plate was pulsed with 1 μCi/well [^{3}H] thymidine. On day 3, the plate was harvested and counts per minute (CPM) were analyzed. ** indicates $P<0.001$. * indicates $P<0.05$.

Induction of T Cell Anergy by IFN-β

In a preliminary study to investigate the ability of IFN-β to create a T regulatory cell line required for MS studies, PBMCs were isolated from patient blood samples and activated for 3 days with IFN-β and/or TGF-β. The cells were then washed and propagated in IL-2 without IFN-β or TGF-β. The growth rate was determined on days 3, 6, 11, and 14. On day 6, the prior exposure to TGF-β had no effect on the fold change in growth relative to the cell population that was activated without cytokine (Figure 2). In contrast, PBMC populations that were activated in the presence of IFN-β had a relative decrease in growth compared to control populations. This decrement in growth was also observed in the PBMC population activated with both IFN-β and TGF-β. The data present provided suggestive evidence that IFN-β dampens mitogenic T cell responses, in a primary T cell activation, by inducing T cell anergy.

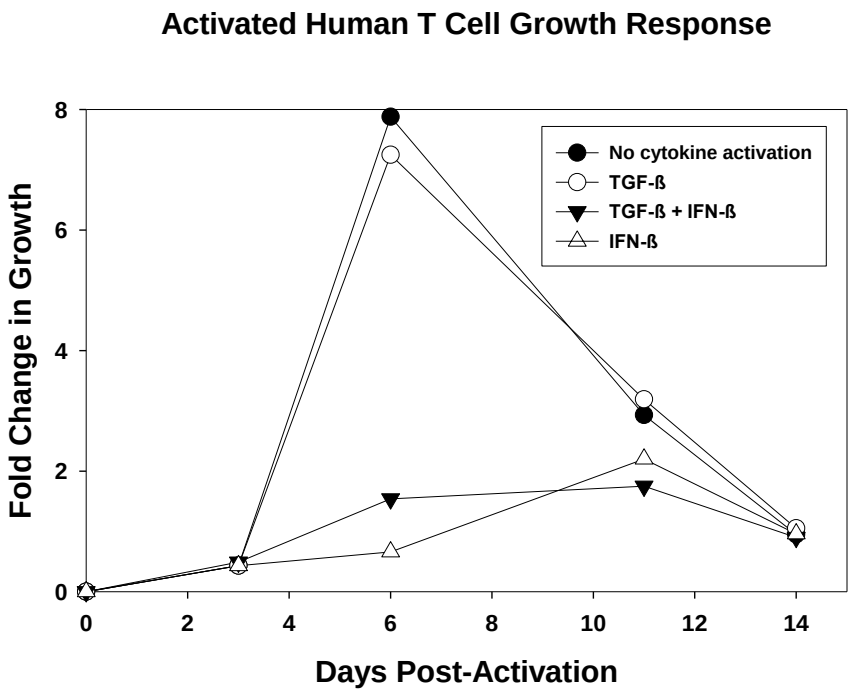

Figure 2. IFN-β induced T cell anergy. On day 0, human PBMCs were isolated from whole blood by use of a density centrifugation gradient protocol. PBMCs were activated for 3 days in presence of 2.5 μg/mL Con-A, 1% IL-2, and either 10 nM TGF-β, IFN-β, TGF-β + IFN-β, or no cytokine. Cells were counted and passaged every three to five days in fresh media and 1% IL-2 (without IFN-β and/ or TGF-β). Fold change in growth was determined relative to the previous passage.

IFN-β Induced Inhibition of IL-2 Production by Human T Cells

In order to evaluate the ability of IFN-β to inhibit T cell proliferation, a T cell line comprised of 90% CD8+ human T cells was activated with either IFN-β and/or TGF-β with irradiated RS4 (11) cells and IL-2. Once removed from activation media, the cells were cultured for seven days in IL-2 in the absence of IFN-β and/ or TGF-β. The cell populations were then reactivated in a recall assay (without additional IL-2) to measure mitogen-stimulated IL-2

production. Supernatants from these cultures were used to culture SJL-PLP T cells, mouse IL-2 indicator T cell line, in a proliferation assay to assess SJL-PLP cell growth. Based on the SJL-PLP proliferation assay, IFN-β alone did not have a distinguishable effect on IL-2 production by the human T cells compared to the control group (Figure 3). TGF-β enabled robust IL-2 production by the human T cells, as measured by SJL-PLP expansion. However, in human T cell cultures that were initially activated with TGF-β versus TGF-β + IFN-β, the reactivation showed that inclusion of IFN-β in the original culture resulted in significantly less IL-2 production in the recall assay. The results suggest that IFN-β dampens human T cell responsiveness to reactivation, inhibiting IL-2 production and thus preventing T cell expansion.

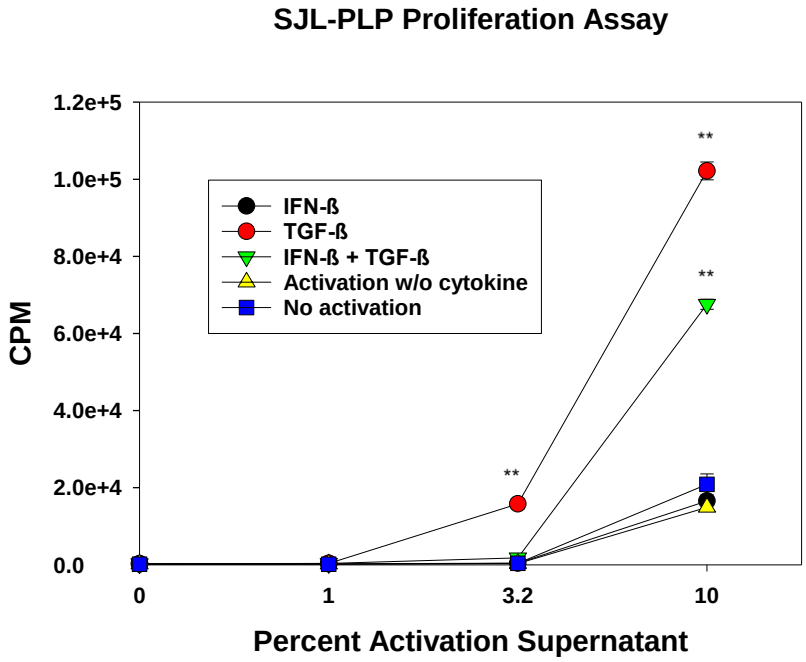

Figure 3. **SJL-PLP proliferation assay.** On day 0, human T cells were activated with either 10 nM IFN-β, TGF-β, IFN-β + TGF- β, no cytokine, or no activation. In a 96 well plate, triplicates of each group contained a 1:1 ratio of irradiated RS4 (11) cells and human T cells (90% $CD8^+$ T cells), 2.5 μg/mL Con-A, and 1% IL-2, with the control group containing only T cells and IL-2.

On day 1, T cells were removed from activation cultures and passaged every three days in new media and 1% IL-2 (without TGF-β and/ or IFN-β). On day 7, each group of human T cells were reactivated in the presence of a 1:1 ratio of irradiated RS4 (11) cells to T cells and 2.5 μg/mL Con-A. As a measure of T cell responsiveness, IL-2 production was measured in an IL-2 indicator cell proliferation assay. On day 8, supernatants were collected and were used to culture 10,000 IL-2 indicator cells per well at designated titrations (x-axis). On day 10, the plate was pulsed with 1 μCi/well [^{3}H]thymidine. On day 11, the plate was harvested and counts per minute (CPM) were analyzed. ** indicates $P<0.001$.

Discussion

The pIRES2-AcGFP1 IFN-β-8his plasmid provides a reliable method for mass producing and purifying IFN-β for future experiments in the laboratory. In particular, IFN-β produced by this expression system can be used to activate human T cells to observe whether IFN-β results in the differentiation of $Foxp3^+$ Tregs. As observed in the study, IFN-β induced T cell anergy in a primary activation of PBMCs and inhibited IL-2 production by T cells after an initial activation with IFN-β. These findings are the foundation of creating a consistent method of producing a pure Treg cell line required for future research and treatments. One of the newer treatments for autoimmune disorders being investigated is adoptive cellular immunotherapy (10). Adoptive immunotherapy involves the reintroduction of Foxp3+ Tregs into an individual in order to suppress the autoimmune disease (10). This therapy, unlike previous treatments using only IFN-β, aims to be a curative treatment for autoimmune diseases. Use of Tregs has already been performed in clinical trials involving kidney transplant patients. Results from clinical trials suggested that laboratory-expanded Tregs increased circulating Treg populations in patients and prevented transplant rejection for two years (11). In order for cellular adoptive immunotherapy to be used in MS clinical trials, methods for robust pure Treg cell expansion need to be devised.

The first step for producing a pure Treg cell line would be testing human T cell activation in the presence of human IFN-β, anti-human CD25 monoclonal antibodies, and Rapamycin. As previously stated, IFN-β has been found to cause the differentiation of $Foxp3^+$ Treg cells in mice, thus promoting Treg cell expansion (9). Anti-human CD25 monoclonal antibodies would be used due to the disparity of CD25 expression on Tregs compared to effector T cells (10). Effector T cell populations have decreased amounts of CD25 expression, allowing for complete inhibition of IL-2 uptake by CD25 in effector T cells while only partially inhibiting IL-2 uptake in Tregs

(10). Due to the inhibition of CD25, effector T cell proliferation would be inhibited. This can be further induced by use of Rapamycin, a drug capable of blocking PI3K-mediated signaling in effector T cells (12). Effector T cells use a PI3K-mediated and a JAK/STAT-IL-2 signaling pathway for cell growth, whereas Tregs only require the JAK/STAT-IL-2 signaling pathway (12). Therefore, by inhibiting only the PI3K-mediated signaling pathway, pure Treg expansion may be able to occur (12). A pure Treg cell line will be beneficial for future research regarding adoptive immunotherapy, MS treatment, including the potential effect IFN-β has on the differentiation of $Foxp3^+$ Tregs.

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