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The Role of TGF-β in CTB-Insulin Regulated Human Dendritic Cell Tolerance

Grace Edosewe Esebanmen

LOMA LINDA UNIVERSITY
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The Role of TGF-β in CTB-Insulin Regulated Human Dendritic Cell Tolerance

by

Grace Edosewe Esebanmen

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

_____, Chairperson
William H.R. Langridge, Professor of Biochemistry

_____
Leonard Brand, Professor of Earth and Biological Sciences

_____
Anthony Firek, Assistant Professor of Medicine

_____
Salma Khan, Assistant Research Professor of Biochemistry

_____
Kevin Nick, Associate Professor of Earth and Biological Sciences

_____
Suzanne Phillips, Assistant Professor of Earth and Biological Sciences

_____
Ubaldo Soto, Assistant Research Professor of Microbiology and Molecular Genetics

_____
Juli Unternaehrer, Assistant Professor of Biochemistry

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To all I say, “Let your light shine” – Matthew 5:16

CONTENT

FIGURES

Figures Page

TABLES

Tables Page

ABBREVIATIONS

Ab	Antibody
Ahr	Aryl hydrocarbon receptor
ALK	Activin Receptor-like Kinase
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BAFF	B cell-activating Factor
CCR7	C-C motif Chemokine Receptor type 7
CD	Cluster of Differentiation
ChIP	Chromatin Immunoprecipitation
CLR	C-type Lectin Receptors
CTB	Cholera Toxin B subunit
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
DAMP	Damaged Associated Molecular Pattern
DC	Dendritic Cell
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraaceticacid
ELISA	Enzyme-linked Imunosorbent Assay
ENTPD-1	Ectonucleoside Triphosphate Diphosphohydrolase-1
ERK	Extracellular signal-regulated Kinases
FcγRIIB	Fragment, crystallizable gamma receptor IIB

FBS	Fetal Bovine Serum
FSC	Forward Scatter
GC	Glucocorticoids
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HCL	Hydrochloric Acid
HO-1	Heme oxygenase-1
IDO	Indoleamine 2, 3-dioxygenase
IL	Interleukin
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motifs
iPSC	Induced Pluripotent Stem Cell
IVIg	Intravenous Immunoglobulins
KCL	Potassium chloride
LN	Lymph node
LAP	Latency-associated Peptide
MHC	Major Histocompatibility Complex
moDC	Monocyte-derived Dendritic Cell
MS	Multiple Sclerosis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB Inducing Kinase
NLT	Non-lymphoid tissues
NOD	Non-obese Diabetic Mouse
PAMP	Pathogen Associated Molecular Pattern

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid Dendritic Cells
PVDF	Polyvinylidene difluoride
RANK	Receptor Activator of Nuclear κ B
RGD	Arginine-Glycine-Aspartic amino acid sequence
SLC	Small latent complex
SSC	Side Scatter
T1D	Type 1 Diabetes
TCR	T cell Receptor
TβRI	TGF-β Receptor type I
TGF-β	Transforming Growth Factor Beta
TGFβRII	TGF-β Receptor type II
TGFβRI	TGF-β Receptor type I
Th	T helper cell
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factor
Treg	Regulatory T cell

ABSTRACT OF THE DISSERTATION

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Grace Edosewe Esebanmen

Doctor of Philosophy, Graduate Program in Biology
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Dr. William H. R Langridge

Cholera toxin B subunit fusion to autoantigen vaccines such as proinsulin (CTB-INS) down regulates dendritic cell (DC) activation and induces the synthesis of DC immunosuppressive cytokines. Recent studies of CTB-INS induction of immune tolerance in human DCs indicate that increased IDO1 biosynthesis may play an important role in CTB-INS vaccine inhibition of DC activation. Previous studies suggest transforming growth factor beta (TGF-β) may play a role in the stimulation of IDO1 biosynthesis for induction of immunological tolerance in murine DCs. To elucidate the mechanisms by which CTB-INS may stimulate IDO1 biosynthesis and mediate tolerance in human DCs, we investigated the contribution of TGF-β superfamily ligand proteins to CTB-INS induction of IDO biosynthesis in human monocyte-derived DCs (moDCs). Our previous studies demonstrated that CTB-INS activates the non-canonical NF-κB pathway to induce IDO1 biosynthesis in human DCs. However, experiments presented in this dissertation demonstrate that the CTB-INS fusion protein also stimulates biosynthesis of the immunoregulatory molecules TGF-β1, activin-A and integrin αvβ8 a molecule known to activate TGF-β1. In addition, CTB-INS stimulates increased levels of Smad2/3 phosphorylation in the vaccinated DCs. Further, we confirmed that CTB-INS upregulation of the TGF-β superfamily is unrelated to IDO1 biosynthesis in human

moDCs. In conclusion, our experimental findings identified novel immunoregulatory functions of CTB-INS fusion protein and suggest the fusion protein may utilize previously unidentified mechanisms for the regulation of human DC activation to mediate tolerance, a significant finding for development of clinical applications in the therapy of tissue specific autoimmunity.

CHAPTER ONE

INTRODUCTION: AUTOIMMUNITY, STRATEGIES FOR THERAPY, TOLEROGENIC DENDRITIC CELLS

Autoimmune Diseases and Type 1 Diabetes

Autoimmunity is the break-down of immunological mechanisms that control the function of the immune system. Autoimmunity is characterized by immune cell attack on self-tissues that lead to the development of a chronic inflammatory diseased state (Freitag et al., 2016). Autoimmune diseases affect greater than 5 percent of the worldwide population and in recent years, the incidences of autoimmune diseases have increased dramatically. The conditions of autoimmune disease are profoundly debilitating, limiting, cause severe discomfort and result in a significant reduction in longevity, and can lead to severe socio-economic deprivation (Freitag et al., 2016; Kamradt and Mitchison 2001).

The classical mechanism for initiation of autoimmunity involves the presentation of peptides from self-antigens on MHC-II molecules by activated antigen presenting cells (APCs) such as dendritic cells (DCs) to self-reactive T cells leading to the activation and proliferation of T cells. Further expression of adhesion molecules and chemokine factors by activated T cells enable homing and infiltration of target tissues. Subsequently, the autoreactive effector T cells induce inflammation and immune-mediated destruction of the target tissues (Benson et al., 2014; Benson et al., 2010; Roncarolo and Battaglia, 2007).

Autoimmune conditions can be generally classified into two categories: autoimmune disorders that lead to the destruction of several tissues or organs are described as systemic autoimmune diseases and examples of such are rheumatoid

arthritis, systemic lupus erythematosus and scleroderma; the other class of autoimmune process directly damages specific tissues or organ and are labeled tissue-specific autoimmune diseases. They include Type 1 diabetes (T1D), Hashimoto's thyroiditis and multiple sclerosis (MS) (Van Brussel et al., 2014). T1D is a chronic autoimmune disease that develops from the destruction of the islet β cells of the pancreas as mediated by effector T cells, leading to total insulin deficiency. A trio of genetic predisposition, epigenetic, and environmental factors have been implicated as causes for the condition (Stankov et al., 2013). Epidemiological studies reveal a general increase in autoimmune disease incidence of about 3%, with approximately 65,000 new cases of T1D documented every year in children less than 15 years of age (Borchers et al., 2010; Stankov et al., 2013). The destructive processes that lead to loss of the insulin-producing β cells of the pancreas are largely mediated by CD4+ and CD8+ classes of T cells that are activated by antigen presenting cells (APCs) such as DCs and macrophages. APCs have been shown to present β cell antigens to the effector T cells to stimulate an autoimmune response (Morran et al., 2008; Wang et al., 2016).

Dendritic cells are the principal APCs of the human immune system and are crucial for priming T cells during the immune response. These cells also prevent autoimmunity by maintaining central and peripheral tolerance under steady-state conditions (Benson et al., 2014; Ohnmacht et al., 2009). DCs are a heterogeneous group of antigen presenting cells and are widely distributed within body tissues, mediating and shaping immune signals between innate immunity and the adaptive immune response, thereby playing a central role in the break-down of self-tolerance during the pathogenesis of autoimmune diseases (Benson et al., 2014).

Strategies for Therapy

Numerous biologic therapeutic options have been approved for many autoimmune diseases such as psoriasis and multiple sclerosis. However, the limiting factors of cost and determination of the therapeutic choice best suited for individual patients continue to persist (Steinman et al., 2012). Despite the progress in the development of therapy for autoimmune conditions, it is known now that one therapeutic strategy may not be applicable for treatment across all autoimmune disease spectrum, as the underlying biological mechanism for each autoimmune disease differs(Steinman et al., 2012). For example, although TNF-α has been implicated in the pathology of multiple sclerosis (MS) and therapeutic strategy involving the blockade of TNF has been developed, its role in the regeneration and proliferation of oligodendrocytes has been established thereby confounding the benefits of any therapy for MS that is based on antagonizing TNF-α (Arnett et al., 2001; Group and Group, 1999). An understanding of the basic mechanism of the autoimmune disease is therefore imperative for the development of effective therapeutic strategies.

Even with these apparent successes of therapy, there are still some autoimmune conditions that lack approved therapies such as type 1 diabetes (T1D) and myasthenia gravis (Steinman et al., 2012). Available treatments are broad sweeping, targeting normal immune cells as well as the pathogenic cells; therefore re-directing the antigen-specificity of the autoreactive immune cells present in these conditions towards self- tolerance, may be key to therapeutic management of these autoimmune conditions (Peakman and von Herrath, 2010; Steinman et al., 2012).

Current strategies for the treatment of autoimmune diseases include biologic and non-biologic agents that modulate the immune response by interacting with either specific effector cells, secreted pro-inflammatory factors or molecular pathways (Coutinho and Chapman, 2011; Her and Kavanaugh, 2016; Kivity et al., 2010; Lewis and Allen, 2016; Rosman et al., 2013; Sharma and Scott, 2015)(Table 1.1).

Table 1.1 Approved therapies for autoimmune diseases.

Category	Agents	Mechanisms
Biologic	Golimumab, Adalimumab, Infliximab (chimeric)	Monoclonal antibodies directed against TNF-α.
	Etanercept	TNF receptor IgG Fc fusion protein that binds to TNF.
	Sifalimumab	A monoclonal antibody acting on IFN-α.
	Tociluzumab	Anti-IL-6- receptor monoclonal antibody.
	Secukinumab	A monoclonal antibody directed against IL-17A.
	Anakinra, Canakinumab	Blocks IL-1
	Rituximab (chimeric), Ofatumumab	Monoclonal antibodies directed against CD20 protein expressed on B cells.
	Epratuzumab	Anti-CD22 monoclonal antibody acting on CD22 molecule expressed on B cells.
	Intravenous immunoglobulins (IVIg)	Diverse mechanisms are proposed; IVIg are purified immunoglobulin gamma obtained from plasma, pooled from thousands of healthy donors.
	Abatacept	A CTLA-4: IgG Fc fusion protein which binds to costimulatory molecules on antigen presenting cells (APC), CD80/CD86, to inhibit CD28 activation on T cells.
Non-biologic	Methotrexate	Exact mechanism unclear; A folate analogue, it probably inhibits actively dividing cells by blocking folate-dependent enzymes.
	Glucocorticoids (GC)	Immunosuppressive; Binds to GC receptors to inhibit NFκB signaling.
	Mycophenolate mofetil	An antibiotic derived from *Penicillium sp.* that targets GMP synthesis to inhibit T and B cell proliferation and cytokine expression of immune cells.

Abbreviations: *TNF*, Tumor Necrosis Factor; *IL*, interleukin; *IFN*, interferon.

With advancements in medical science and biotechnology, immunotherapy (the use of immunological materials for treatment purposes (Feldmann and Steinman, 2005)) is not restricted to the biologic agents listed in Table 1.1, and these biologic agents may also result in general immunosuppression. Other forms of immunotherapy have been developed to mitigate the condition of autoimmunity; these include the application of immune cells and vaccines (Feldmann and Steinman, 2005). Cell-based therapy which includes adoptive transfer of regulatory T cells (Tregs), proffers an antigen-specific strategy to inhibit the activity and proliferation of auto-inflammatory immune cells (Arellano et al., 2016). Therapies applying the regulating potential of T cells are more advantageous than nonspecific immune regulating strategies, since nonspecific approaches may result in generalized blocking of the immune response, initiating immune-compromise and susceptibility of the immune system to infections (Arellano et al., 2016). Preclinical animal studies and a Phase I clinical trial have demonstrated the prevention of autoimmune diseases by adoptive transfer of regulatory T cells (Marek-Trzonkowska et al., 2014; Roncarolo and Battaglia, 2007).

Stem cells are another group of cell-based therapy currently under investigation and development for the treatment of autoimmune conditions. Mesenchymal stem cells (MSC) possess immune regulating properties (Munir and McGettrick, 2015) that can be harnessed for autoimmune disease therapy and induced pluripotent stem cells (iPSCs) can be developed into antigen-specific Tregs for adoptive transfer therapy in autoimmune disease conditions(Haque et al., 2016).

The use of vaccines to induce “inverse vaccination” is currently under development. Inverse vaccination implies the antigenic stimulation of the immune

response towards specific inhibition or elimination of specific antibody and T cell immunological responses (Steinman, 2010). The aim of inverse vaccination is to specifically tolerize the pathological response of the adaptive immune system of the autoimmune diseases (Steinman, 2010). For example, DNA vaccination strategy delivering plasmids encoding self-antigens to immune cells, have demonstrated efficacy in preventing and reversing autoimmune conditions in experimental autoimmune encephalomyelitis EAE mouse models (Garren et al., 2001; Ho et al., 2005). Human trials of DNA vaccines have been initiated for multiple sclerosis (MS) and efforts to develop such DNA vaccines for T1D are in progress (Garren et al., 2008; Steinman, 2010). Other vaccine strategies for the induction of tolerance involve the use fusion proteins consisting of epitopes of self-antigens and cytokines (Mannie et al., 2012; Mannie et al., 2007), or self-antigens and immunogenic subunits (Carter et al., 2006b; Sadeghi et al., 2002).

Most of these vaccine strategies target the feature of antigen presenting cells (APC) especially DCs, in processing and presenting antigens, to deliver the potency of the vaccine in inducing tolerance. With the growing necessity for treatment options for autoimmune diseases that are potent and safe, DC-based vaccines are arising as potential tools for therapy of autoimmune diseases. This form of cell-based therapy combines the principles of inverse vaccination with adoptive transfer of antigen-specific immunological materials, which in this case are DCs, for the induction and restoration of immune regulation (Sabado et al., 2017; Ten Brinke et al., 2015). In the following sub-section, a brief discussion of an immunosuppressive vaccine strategy is provided.

The Immunosuppressive Fusion Protein Vaccine CTB-PROINSULIN (CTB-INS)

Cholera toxin B subunit-proinsulin fusion protein (CTB-INS) is a chimeric fusion protein vaccine comprised of the cholera toxin B subunit conjugated to the diabetes autoantigen, proinsulin. The chimeric protein is translated from a fusion gene encoding the cholera toxin B sub-unit linked by a c-terminal fusion to proinsulin, in the *Escherichia coli* expression vector, pRSET A (Carter et al., 2006a; Odumosu et al., 2010) (Fig. 1.1). The rationale for applying CTB-INS as strategy for the therapy of autoimmune diabetes, emanates from the observation that administration of autoantigens reduce the development of autoimmune conditions in several studies and the induced tolerance could be prolonged, sustained and amplified by utilizing the potential of CTB as an efficient trans-mucosal carrier delivery molecule (Sadeghi et al., 2002; Sun et al., 1994; Zhang et al., 1991) to increase the efficiency of delivery and enhance presentation of the autoantigen to effector T cells (Sadeghi et al., 2002). Murine studies have shown that oral and peritoneal delivery of a conjugate of the non-toxic B subunit of the cholera toxin (CTB) to specific autoantigens, was able to induce tolerance, in several autoimmune conditions in mice, such as uveitis, autoimmune encephalomyelitis and autoimmune chondritis (Kim et al., 2001; Phipps et al., 2003; Sun et al., 2000). Specific linkage of the CTB subunit to the diabetes autoantigen, insulin, has been shown to suppress development of autoimmune diabetes in the non-obese diabetic (NOD) mice (Arakawa et al., 1998; Aspord and Thivolet, 2002; Dénes et al., 2006). Recent experiments in our laboratory have revealed the vaccine can suppress pro-inflammatory activation of human dendritic cells (Odumosu et al., 2011b), induce upregulation of IDO biosynthesis (Mbongue et al., 2015), and upregulate expression of immunoregulatory proteins and

cytokines, such as integrins and TGF-β1 (Chapter 3). The mechanisms of CTB-INS immuno-modulatory effects in human moDCs is under investigation (Chapter 2; Chapter 3) and is essential for evaluation of vaccine efficacy and safety as a therapeutic agent for T1D.

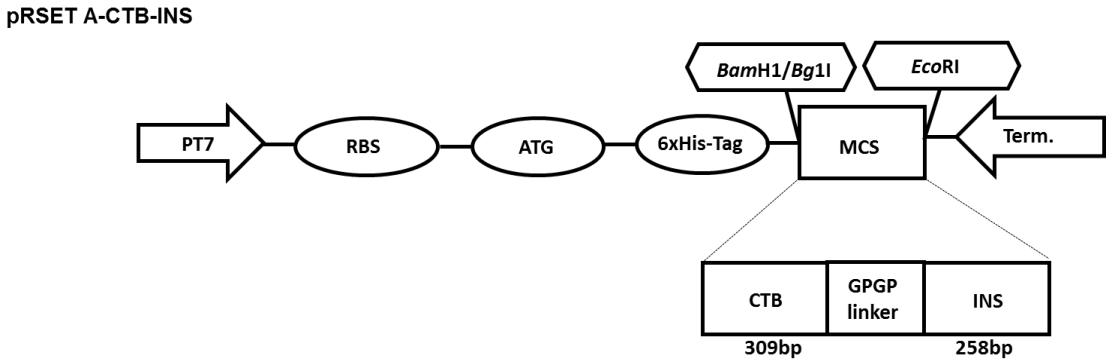

Figure 1.1: Plasmid map of the *E. coli* expression vector, pRSET A (Invitrogen, Carlsbad, CA), carrying the CTB-INS fusion gene. The expression vector is under the control of the bacteriophage T7 promoter and contains an oligonucleotide region that encodes 6 histidine amino acid residues immediately 5' upstream of the CTB gene sequence.

Dendritic Cells and Their Role in the Induction of Immune Tolerance

Dendritic cells (DC) are a heterogeneous population of APCs that develop from bone marrow precursors that migrate to the lymphoid and non-lymphoid tissues (NLT) for further differentiation (Boltjes and van Wijk, 2014). Three circulating DC subsets, in peripheral human blood have been described: The $CD11c^+$ myeloid/conventional DCs expressing the $CD1c^+$ surface marker, the $CD11c^+$ myeloid/conventional DCs expressing $CD141^+$, and $CD123^+$ $BDCA\text{-}2^+$ plasmacytoid DCs (Boltjes and van Wijk, 2014). Plasmacytoid DCs circulate in the blood and lymphoid tissues and produce copious amount of Type 1 interferons (IFN) in response to viral pathogens. These cells appear tolerogenic when inactive, but attain immunogenic phenotypic expression upon activation (Boltjes and van Wijk, 2014; Mathan et al., 2013). Conventional DCs are found in the blood, lymph node (LN), spleen, and NLT. Both $CD11c^+$ myeloid/conventional DC types show differences in cytokine/chemokine repertoire and TLR patterns during an immunological response (Boltjes and van Wijk, 2014). A distinct population of DCs has also been recently identified to be associated with inflammatory cellular environments and appear to be the *in vivo* equivalents of *in vitro* monocyte-derived dendritic cells (moDCs) as confirmed by transcriptome analyses (Segura et al., 2013). The discovery of the potential for human peripheral blood monocytes to differentiate into DCs is a valuable tool and has enabled research in DC biology and development of DC therapeutic applications which were otherwise a challenge due to the low level of circulating DCs (León et al., 2005; Sallusto and Lanzavecchia, 1994).

DCs are the most potent antigen presenting cells and are crucial in initiating and modulating adaptive immune cells. The flexibility of DCs' features and function in

response to cellular environmental cues, enables them to direct the outcome of the adaptive immune response (van Duivenvoorde et al., 2006). As sentinels of the immune system DCs constantly patrol for danger signals of antigens from pathogens, self-tissues, and cancerous cells called pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) that are detected using pattern recognition receptors (PRR) such as Toll-like receptors (TLR) and C-type lectin receptors (CLRs) expressed on the DC cell surface (Schinnerling et al., 2015; Van Brussel et al., 2014). Recognition of any of these 'red flag' molecular signatures activates and induces a DC differentiation process that involves DC maturation and homing to T lymphocyte zones (Randolph et al., 2008). Activating signals may arise from direct DC contact with antigens, by indirect stimulation via nonspecific tissue response induced by the pathogen or by cytokine expression from the cellular environment (Kaliński et al., 1999; Schinnerling et al., 2015). Upon DC uptake of the antigens, simultaneous processing, presentation of the peptide antigen on major histocompatibility molecules (MHC), and upregulation of costimulatory molecules (CD80 and CD86) follows and DCs migrate to the lymph nodes. DC engagement of peptide-MHC complex with T cell receptor (TCR) followed by delivery of costimulatory molecule signals, and DC priming of T cells with specific cytokine profile, initiates naïve T cell activation and polarization to antigen specific T helper type 1 (Th1) or Th2, or stimulation of effector and memory T cell, depending on the signal delivered (García-González et al., 2016; Kaliński et al., 1999; Randolph et al., 2008; Schinnerling et al., 2015).

Tolerogenic Dendritic Cells

Tolerogenic DCs are generally characterized by an immature or semi-immature phenotype demonstrated by expression of low costimulatory molecules, high expression of anti-inflammatory cytokines, decreased expression of pro-inflammatory cytokines, and induction of hyporesponsive CD4+ T cells (Hilkens et al., 2010; Schinnerling et al., 2015; Ten Brinke et al., 2015). Tolerogenic DCs can be generated *in vitro* for further use as cellular vaccines, by genetically engineering DC expression of immunoregulatory factors, and by applying pharmacological agents such as dexamethasone, vitamin D_3, and rapamycin to DCs. Otherwise, conditioning of DCs with anti-inflammatory cytokines such as IL-10 and TGF-β may biologically mediate the induction of tolerogenic DCs (Hilkens et al., 2010). DCs may also be targeted *in vivo* by utilizing an auto-antigen delivery system that specifically primes the DCs to induce tolerance of adaptive immune cells involved in an autoimmune response (Hilkens et al., 2010; Mukhopadhaya et al., 2008). Tolerogenic DCs mediate tolerance by inducing anergy (inactivation of T cells), or, apoptosis of antigen specific autoreactive T cells (Mahnke et al., 2002), and skew effector T cell immune response into the anti-inflammatory Th2-like phenotype or induce the generation of Treg cells (Van Brussel et al., 2014; van Duivenvoorde et al., 2006). Successful experimentation in various animal models have led to application of tolerogenic DC therapy in several clinical trials (Benham et al., 2015; Giannoukakis et al., 2011; Jauregui-Amezaga et al., 2015).

Although a specific and unique molecular biomarker has not been identified to indicate DC tolerogenicity, several molecules and pathways have been shown to be involved with DC modulatory properties (Schinnerling et al., 2015). These include (1)

signaling enzymes, (2) transcription factors and adapter molecules, (3) DC membrane inhibitory receptors as described below.

Signaling Enzymes

Heme oxygenase-1 (HO-1) regulates DC activation, induces antigen specific Tregs in a murine model (Wong et al., 2016), inhibits the expression of proinflammatory cytokines, and is mainly expressed by immature DCs (Blancou et al., 2011; Schinnerling et al., 2015). Extracellular signal-regulated kinases (ERK) activation in DCs induces expansion of Treg population and generation of regulatory DCs (Arce et al., 2011; Schinnerling et al., 2015). DCs upregulate ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1), also known as CD39, in response to IL-27 signaling, for the induction of Treg cells and to suppress the progression of experimental autoimmune encephalomyelitis (EAE), the mice model of MS (Mascanfroni et al., 2013). ENTPD1 is a cell surface enzyme predominantly expressed by conventional DCs (cDCs) that limits levels of extracellular ATP (Mascanfroni et al., 2013). ENTPD1 deficiency resulted in increased IL-1β release and DCs conditioned with IL-27 utilized ENTPD1 to suppress the differentiation of pathogenic effector T cells (Mascanfroni et al., 2013).

Transcription Factors and Adaptor Molecules

The nuclear factor-κB1 (NF-κB1) p50 homodimer promotes the anti-inflammatory response of DC by acting as a transcriptional repressor of DC activation, and regulating DC expression of effector T cell activating factors (Dissanayake et al., 2011; Schinnerling et al., 2015). Others include, Peroxisome-proliferator activated

receptor (PPAR)-γ which is highly expressed on immature human monocyte-derived DCs (Gosset et al., 2001) and has been shown to reduce the capacity for T cells to induce lymphocyte proliferation in these cells (Nencioni et al., 2002). Suppressor of cytokine signaling (SOCS) proteins, a group which has numerous immunoregulatory functions including the modulation DC phenotype into a tolerogenic profile and the suppression of DC activation (Hanada et al., 2003; Li et al., 2006c). The duo of aryl hydrocarbon receptor (Ahr) and indoleamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan into immunosuppressive metabolites such as kynurenines, modulates DC mediated induction of Tregs and inhibition of T cell proliferation (Nguyen et al., 2010).

Dendritic Cell Membrane Inhibitory Receptors

Surface molecules such as Fc gamma receptor IIB (FcγRIIB), expressed on DCs, modulates peripheral tolerance by suppressing DC costimulatory molecule expression and directing T cell differentiation to a tolerogenic phenotype (Samsom et al., 2005). Also, overexpression of FcγRIIB enables immature DCs to resist toll-like receptor (TLR) induced maturation signals (Zhang et al., 2011). Cell membrane Ig-like transcripts (ILTs) are represented by members such as ILT3 and ILT4 that transmit suppressive signals via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM), resulting in increase of circulating Treg cells (Cella et al., 1997; Stallone et al.). Targeting autoantigens to DEC-205, a dendritic cell C-type lectin receptor (CLR), has been demonstrated to induce the conversion of autoreactive effector T cells to Foxp3(+) Treg cells (Petzold et al., 2010).

Further research to elucidate the key regulators of DC tolerogenicity remain highly imperative so as to monitor the induction and therapeutic efficacy of tolerogenic DCs (Schinnerling et al., 2015). In the following section, specific immunomodulatory factors implicated in CTB-INS-mediated immune tolerance of human moDCs are further discussed.

Potential Molecules and Pathways Involved in CTB-INS Immunomodulation of DCs

Indoleamine 2, 3- dioxygenase (IDO)

Indoleamine 2, 3- dioxygenase is an enzyme that catalyzes the breakdown of tryptophan, an essential amino acid, to degradation products called kynurenines. The activity of the enzyme has been shown to impact immune suppression and peripheral tolerance (Fallarino et al., 2012; Harden and Egilmez, 2012). Immune cells express IDO for immune regulation. Recently, B cells have been shown to induce regulatory T-cells by a TGF-β/IDO axis requiring the production of TGF-β1 and IDO upon stimulation of the B cells (Nouël et al., 2015). A combined inhibition of TGF-β1 and IDO resulted in the loss of B cell regulatory capacity (Nouël et al., 2015). Dendritic cells expressing IDO generate regulatory T cells in an arthritis mouse model (Park et al., 2008). The role of IDO in conferring tolerance to immune cells, specifically dendritic cells, has also been highlighted in other experimental models of autoimmune diseases such as autoimmune haemolytic anaemia, asthma, and autoimmune diabetes (Dahal et al., 2013; Grohmann et al., 2003; Hayashi et al., 2004). These studies indicate that IDO may be a marker for tolerogenicity. Further, recent studies in our lab show that CTB-INS induces IDO biosynthesis in human moDCs (Mbongue et al., 2015).

Integrin alpha v beta 8 (αvβ8)

Integrins are heterodimeric glycoprotein molecules that function as cell adhesion receptors to modulate the cellular response to the extracellular environment by interacting with ligands, cytoskeletal and cytoplasmic-signaling molecules (Nishimura et al., 1994). These molecules consist of an α and a β subunit, each with a large extracellular domain, a transmembrane and a cytoplasmic domain (Evans et al., 2009; Nishimura et al., 1994). Members of the integrin αv family, which include αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, are able to bind to a tri-amino acid motif, Arg-Gly-Asp (RGD), present on the latency associated peptide (LAP) region of the latent TGF-β1 protein complex, and some αv integrins can further activate latent TGF-β1, following binding to the RGD sequence. Knock-in mice with a point mutation in the TGF-β1 integrin binding site exhibit a phenotype similar to TGF-β1 null mice and die from multi-organ inflammatory disease early in their development indicating the relevance of the integrin binding site to normal functioning of TGF-beta (Worthington et al., 2012; Yang et al., 2007). All αv or RGD-binding integrins have been implicated in the activation of TGF-β1 (Henderson et al., 2013; Hinz, 2013; Leask and Hutchenreuther, 2014; Reed et al., 2015; Song et al., 2016; Wipff and Hinz, 2008). Integrin αvβ8 has been well characterized to be critical for TGF-β1 activation and in maintaining immune homeostasis. Mice whose leucocytes are deficient in integrin αvβ8 develop a wasting inflammatory disorder (Aluwihare et al., 2009b; Mu et al., 2002; Travis et al., 2007b). This abnormality is matched by mice specifically lacking the expression of integrin αvβ8 integrin on DCs and the integrin-deficient DCs fail to induce regulatory T cell differentiation *in vitro* (Aluwihare et al., 2009b; Travis et al., 2007a). Alpha (v) integrins are also considered as apoptotic cell receptors associated with

clearance of apoptotic cells; These molecules appear to enable apoptotic cell recognition for TGF-β activation and the initiation of immunosuppressive signaling (Païdassi et al., 2010). In murine immune cells, expression of integrin αvβ8 is mostly restricted to $CD4^+$T cells and DCs (Travis et al., 2007b; Travis and Sheppard, 2014). Also, integrin αvβ8 appears to be the only αv integrin these cells express (Edwards et al., 2014; Travis et al., 2007a; Worthington et al., 2012). Integrin αvβ8 is highly expressed in intestinal DC subsets of mice and is required for generation and function of regulatory T cells. (Païdassi et al., 2011; Worthington et al., 2012; Worthington et al., 2015). Recent investigations in our laboratory reveal that CTB-INS induces upregulation of integrin αvβ8 expression in human moDCs (Chapter 3).

Transforming Growth Factor Beta (TGF-β)

The pleiotropic cytokine TGF-β belongs to the TGF-β superfamily of growth factors and functions in numerous physiological and pathological processes including the immune response. The cytokine plays a central role in T cell development, homeostasis, tolerance and differentiation (Guerder et al., 2013). While TGFβ exists in three isoforms in mammals as TGF-β1, TGF-β2 and TGF-β3 (Mu et al., 2002), TGF-β1 is the most expressed isoform in immune cells (Li et al., 2006a; Melton et al., 2010). TGF-β1 is critical for the regulation of DC immune responses and in the maintenance of immune cell homeostasis (Worthington et al., 2012). Increase in TGF-β1 production has been shown to be associated with immune defense and/or recovery from autoimmune diseases (Li et al., 2006b; Prud'homme and Piccirillo, 2000). In mice, gene deletion of TGF-β1 or TGF-β1 signaling proteins results in multifocal autoimmune disorders and inflammatory

states that mimick several autoimmune diseases (Aoki et al., 2005; Kriegel et al., 2006; Prud'homme and Piccirillo, 2000). Studies have shown that TGF-β1 induced IDO expression and signaling in murine plasmacytoid DCs (pDCs) and splenic DCs in a manner that resulted in sustained tolerance in the DCs (Belladonna et al., 2008; Pallotta et al., 2011). However, our studies reveal that TGF-β1 may not induce IDO expression in human moDCs, but may be involved in CTB-INS tolerance induction of human moDCs by alternative mechanisms that require further clarification (Chapter 3).

TGF-β1 is synthesized and expressed as a latent form that requires activation to exert its biological functions. After furin cleavage in the Golgi, the inactive complex consists of an N-terminal portion that is a disulfide-linked homodimer called the latency associated peptide (LAP), and a C-terminal fragment that is also a disulfide-linked homodimer of the mature cytokine. Both peptides are non-covalently associated and form the small latent complex (SLC) also called latent TGF-β1. The crystal structure of the SLC has revealed that the LAP encircles the active TGF-β1 fragment thereby prohibiting its contact sites from interacting with TGF-β receptors and thus prevents induction of a signaling response (Li et al., 2006b; Travis and Sheppard, 2014). TGF-β1 activation can be mediated by extreme pH and heat, thrombospondins, proteases and integrins (Li and Flavell, 2008; Li et al., 2006b). The active cytokine signals by binding to the TGF-β receptor type II dimer (TGFβRII) that associates with the TGF-β receptor type I dimer (TGFβRI) to form a tetrameric receptor complex, inducing phosphorylation of the cytoplasmic domain of TGFβRI, and subsequent phosphorylation of the intracellular signal transducers, Smad2 and Smad3. Upon activation, the Smad2/3 proteins bind to Smad4 to form a complex and translocate to the nucleus where the complex initiates

transcription of target genes (Massagué, 2012; Travis and Sheppard, 2014; Wipff and Hinz, 2008). TGF-β1 also signals through Smad-independent routes and the mechanisms for these pathways remain to be fully elucidated (Li et al., 2006b; Travis and Sheppard, 2014). Our studies show that CTB-INS stimulates TGF-β1 expression and Smad2/3 signaling in human moDCs (Chapter 3).

Summary and Conclusion

The need to develop effective therapy for autoimmune conditions that is antigen-specific, efficient and safe remains paramount, and DCs are promising tools for therapy based on their unique functions in the immune system. CTB-INS has proven to be an effective agent for inducing tolerance in human DCs and may hold promise for application as a therapeutic tool in the treatment of type 1 diabetes. However, the mechanism of action of CTB-INS requires further validation.

In this dissertation, we document our findings on the mechanism of CTB-INS induction of tolerance in human DCs. Data presented here reflect our efforts to test the hypotheses presented in Fig. 1.2. We also attempt to provide clarification on the relationship between the immunosuppressive cytokine TGF-β1 and immunomodulatory enzyme, IDO, as studied in human moDCs. We anticipate that these novel findings will improve the understanding of human moDC biology and the mechanisms and molecular interactions that occur when human moDCs acquire tolerogenicity.

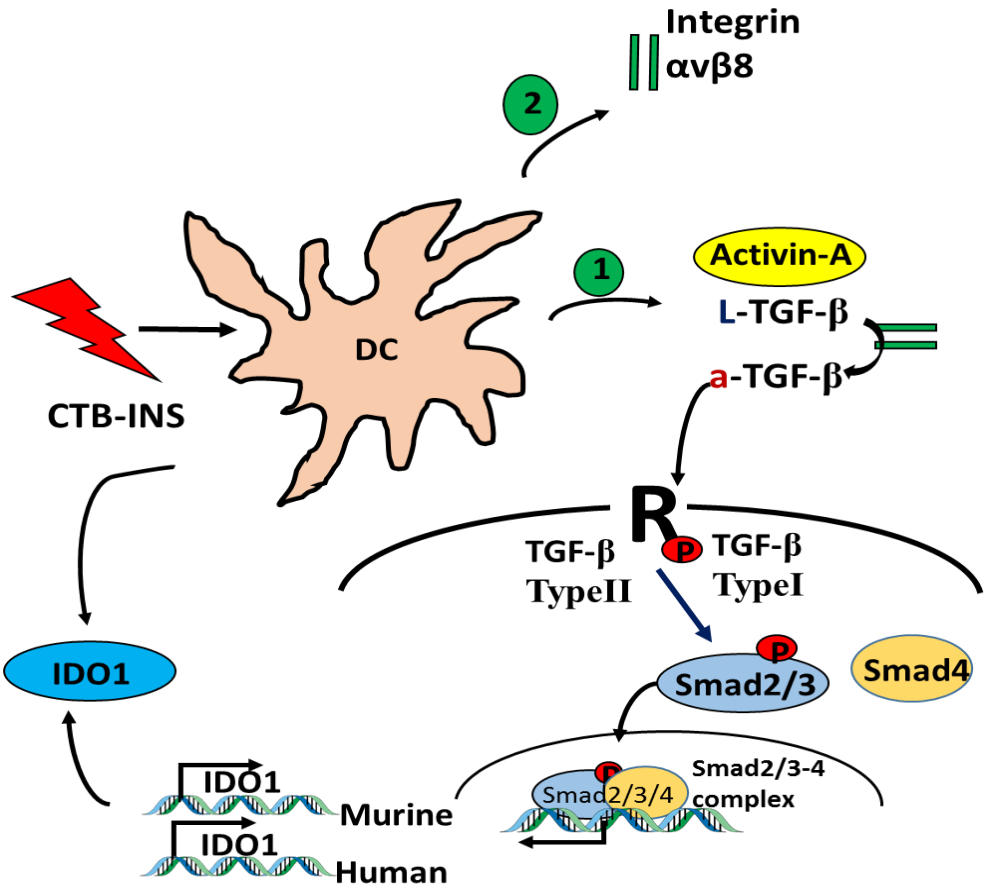

Figure 1.2. Schematic of the hypotheses tested in this dissertation. Hypothesis 1: CTB-INS stimulates biosynthesis of TGF-β superfamily members in human moDCs for the induction of intracellular signaling of gene activation that leads to IDO1 transcription and protein synthesis; 2: CTB-INS induces integrin αvβ8 production to mediate TGF-β1 activation for tolerogenic functions such as induction of IDO1 biosynthesis. a-TGF-β, active TGF-β; L- TGF-β, Latent TGF-β.

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CHAPTER TWO

CHIMERIC VACCINE STIMULATION OF HUMAN DENDRITIC CELL INDOLEAMINE 2, 3-DIOXYGENASE OCCURS VIA THE NON-CANONICAL NF-κB PATHWAY

Nan-Sun Kim[1, 2], Jacques C. Mbongue[1, 3], Dequina A. Nicholas[1,5], Grace Esebanmen [1,4,] Juli J. Unternaehrer[5], Anthony F. Firek[6] and William H. R. Langridge[1,5*]

[1] Center for Health Disparities and Molecular Medicine, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA, USA

[2] Department of Molecular Biology, Chonbuk National University, Jeon-Ju, Republic of Korea.

[3] Loma Linda University School of Medicine, Department of Basic Sciences, Division of Physiology, Loma Linda, CA, USA

[4] Loma Linda University School of Medicine, Department of Earth and Biological Sciences, Loma Linda, CA, USA

[5] Loma Linda University School of Medicine, Department of Basic Sciences, Division of Biochemistry, Loma Linda, CA, USA

[6] Endocrinology Section, JL Pettis Memorial VA Medical Center, Loma Linda, CA, USA

*Corresponding author:

Email: *blangridge@llu.edu* (WHRL)

Abstract

A chimeric protein vaccine composed of the cholera toxin B subunit fused to proinsulin (CTB-INS) was shown to suppress type 1 diabetes onset in NOD mice and upregulate biosynthesis of the tryptophan catabolic enzyme indoleamine 2, 3-dioxygenase (IDO1) in human dendritic cells (DCs). Here we demonstrate siRNA inhibition of the NF-κB-inducing kinase (NIK) suppresses vaccine-induced IDO1 biosynthesis as well as IKKα phosphorylation. Chromatin immunoprecipitation (ChIP) analysis of CTB-INS–inoculated DCs showed that RelB bound to NF-κB consensus sequences in the IDO1 promoter, suggesting vaccine stimulation of the non-canonical NF-κB pathway activates IDO1 expression *in vivo*. The addition of Tumor Necrosis Factor Associated Factors (TRAF) TRAF 2, 3 and TRAF6 blocking peptides to vaccine inoculated DCs was shown to inhibit IDO1 biosynthesis. This experimental outcome suggests vaccine activation of the TNFR super-family receptor pathway leads to upregulation of IDO1 biosynthesis in CTB-INS inoculated dendritic cells. Together, our experimental data suggest the CTB-INS vaccine uses a TNFR-dependent signaling pathway of the non-canonical NF-κB signaling pathway resulting in suppression of dendritic cell mediated type 1 diabetes autoimmunity.

Introduction

Type 1 diabetes (T1D) is a well-studied prototypic tissue specific autoimmune disease resulting from auto-reactive lymphocyte destruction of the pancreatic islet insulin-producing β-cells (Eisenbarth, 1986; Tisch & McDevitt, 1996). The progressive

loss of islet β-cell function leads to insulin deficiency and high blood glucose levels (hyperglycemia). Increased levels of cellular oxidative stress and chronic inflammation generated by hyperglycemia leads to neural and circulatory complications that result in an early mortality from amputation, loss of kidney function, blindness, heart attack, and stroke (Forbes & Cooper, 2013; Melendez-Ramirez, Richards, & Cefalu, 2010).

Due to the high cost and extended duration of palliative patient care, there is an urgent need for therapeutics that can safely deliver specific effective therapy that protects against the onset and reverses the progression of tissue specific autoimmunity. Dendritic cells, which are considered the most prominent subset of professional antigen presenting cells (APC), have been implicated in the initiation of diabetes-related islet β-cell destruction (Allen et al., 2009; Ganguly, Haak, Sisirak, & Reizis, 2013; J. Mbongue, Nicholas, Firek, & Langridge, 2014). Effective immunological suppression strategies include chimeric vaccines that link immuno-stimulatory molecules (adjuvants) with autoantigens to enhance vaccine efficacy (Aspord & Thivolet, 2002; Bergerot et al., 1997; Ploix et al., 1999). Prominent among the adjuvants used is the cholera toxin B subunit (CTB) (Odumosu, Nicholas, Yano, & Langridge, 2010). C-terminal linkage of CTB to the diabetes autoantigen proinsulin (CTB-INS), generated a fusion protein shown to protect against T1D onset (Ploix et al., 1999). Oral immunization experiments showed that feeding small amounts (2–20μg) of CTB-INS vaccine protein alone or in recombinant plant tissues effectively suppressed β-cell destruction and clinical diabetes in adult non-obese diabetic (NOD) mice (Aspord & Thivolet, 2002; Bergerot et al., 1997; Denes et al., 2005).

Proteomic analysis of human dendritic cells inoculated with CTB-INS revealed strong up-regulation of the tryptophan catabolic enzyme indoleamine 2, 3-dioxygenase (IDO1) (J. C. Mbongue et al., 2015). Previous observations showed that CD40 ligand (CD40L) induced IDO1 biosynthesis in human DCs through activation of the non-canonical NF-κB signaling pathway (Tas et al., 2007). Thus, we assessed the requirement for NF-κB activation in vaccine up-regulation of IDO1 using ACHP and DHMEQ, pharmacological inhibitors of NF-κB (J. C. Mbongue et al., 2015). While these experiments revealed vaccine stimulation of NF-κB was essential to activate IDO1 biosynthesis, the relative contributions of canonical and non-canonical NF-κB pathways required for CTB-INS induction of IDO1 biosynthesis remained undetermined.

The NF-κB family in mammals is composed of five members, including c-Rel, RelA also known as p65, NF-κB1 known as p50, RelB, and NF-κB2 known as p52. These NF-κB family members form a variety of dimeric complexes capable of transactivating numerous target genes through binding to the κB enhancer (Sun, 2011). The NF-κB subunit proteins are normally found inactive in the cell cytoplasm due to binding by a family of inhibitors that include IκBa and several additional related ankyrin repeat containing proteins (Sun, 2011; Sun & Ley, 2008).

Due to the diversity of NF-κB functions, its activity is under tight control at multiple levels by positive and negative regulatory elements. Under resting conditions, NF-κB dimers are bound to inhibitory IκB proteins that retain NF-κB complexes in the cytoplasm. In the canonical signaling pathway, the degradation of IκB inhibitor proteins is initiated through stimulus-induced phosphorylation by IκB kinase (IKK), a molecular complex consisting of two catalytically active kinases, IKKα and IKKβ, and their

regulatory subunit IKKγ (NEMO). Phosphorylation of IκB proteins target them for ubiquitination and proteasome degradation, releasing the NF-κB RelA (p65) and p50 protein dimers for translocation into the nucleus.

The non-canonical NF-κB signaling pathway was discovered during analysis of non-canonical p100 subunit processing (Sun, 2011; Xiao, Fong, & Sun, 2004). In addition to serving as a precursor of the functional p52 subunit, p100 was shown to function like an IκB inhibitor molecule, preferentially inhibiting RelB nuclear translocation (Solan, Miyoshi, Carmona, Bren, & Paya, 2002; Sun, 2011). Partial proteasome processing of p100 serves to generate p52 and to induce nuclear translocation of the RelB/p52 heterodimer, as RelB binds to DNA with p52. The p52 subunit is actively generated predominantly in specific immune cell types including B-cells and dendritic cells, leading to the idea that p100 processing might be a signal-regulated event. Indeed, the NF-κB-inducing kinase (NIK) is required for p100 processing and is required for *in vivo* p100 processing in splenocytes (Xiao et al., 2004; Xiao, Harhaj, & Sun, 2001). The first component of the non-canonical signaling pathway to be identified was NIK, a MAP-kinase kinase kinase (MAP3K) member originally implicated in NF-κB activation by the TNF receptor (TNFR) pathway (Malinin, Boldin, Kovalenko, & Wallach, 1997). To date, all of the non-canonical NF-κB inducers identified are known to signal through NIK (Coope et al., 2002; Manches, Fernandez, Plumas, Chaperot, & Bhardwaj, 2012; Tas et al., 2007).

Here we focus on identification of non-canonical NF-κB signaling pathway contributions to CTB-INS vaccine induction of IDO1 in human dendritic cells as a

prerequisite for application of chimeric vaccine immune suppression strategies in the clinic.

Materials and Methods

Construction of a Bacterial Expression Vector Containing the Cholera Toxin B Subunit – Proinsulin Gene

A DNA sequence encoding 258bp of the human proinsulin gene (INS M12913.1) was linked to the carboxyl-terminus of a DNA fragment (309bp) encoding the cholera toxin B subunit gene (CTB U25679.1) to generate the fusion gene CTB-INS according to a previously used protocol (J. C. Mbongue et al., 2015) (Figure 2.1).

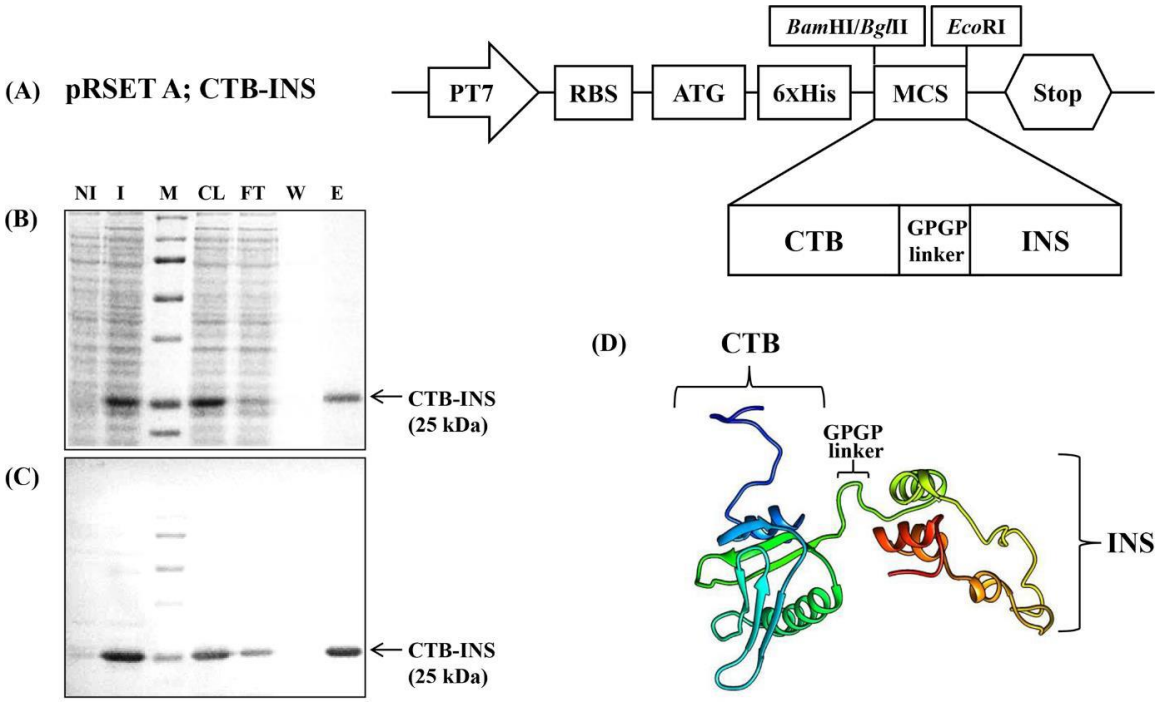

Figure 2.1. CTB-INS fusion protein was expressed from the E.coli pRSET A expression vector and purified using Ni-NTA agarose with the indicated imidazole concentration in the wash and elution steps. Panel (A) is a plasmid map of the *E. coli* expression vector pRSET A (Invitrogen, Carlsbad, CA), carrying the CTB-INS fusion gene. Panel (B) shows the SDS-PAGE. Proteins were visualized by Coomassie staining. Lane NI: non-induced *E. coli* (BL21) cell; I: induced E. coli cell; M: protein size marker; CL: cell lysate; FT: flow-through; W: wash; E: elution. Panel (C) Western blot detection of recombinant CTB-INS fusion protein identified with anti-CTB primary antibody. The arrow indicates the purified CTB-INS proteins. Panel (D) shows the predicted CTB-INS protein structure using its protein sequence generated by the RaptorX server.

Expression and Purification of CTB-INS Fusion Protein in E. coli

The *E. coli* strain BL21 was transformed with pRSET-CTB-INS as previously described (J. C. Mbongue et al., 2015).

Ethics

Ex vivo experiments on monocyte-derived DCs were performed, with aphaeresis blood provided by the Life Stream Blood Bank (San Bernardino, CA). These experiments were approved by the Loma Linda University Adventist Health Sciences Center Institutional Review Board and blood donor written consent. Blood donor information was anonymized and de-identified prior to the study.

Isolation and Culture of Monocyte - derived Dendritic Cells from Human Peripheral Blood

Monocyte-derived dendritic cells (MoDCs) were prepared from freshly collected human peripheral blood cells isolated from aphaeresis filter cones obtained from the LifeStream blood bank (San Bernardino, CA). The blood was incubated with a red blood cell lysis buffer (3.0 mL Lysis Buffer/ mL of blood) containing 8.3g/L NH_4Cl, 1g/L $KHCO_3$, and 1.8 mL 5% EDTA (Boston Bioproducts), and centrifuged for 5 minutes at 1,500 rpm at 4°C in a Beckman Coulter Allegra X-15R centrifuge, equipped with a SX4750 rotor. After a total of 3 washes in PBS to remove cellular debris and hemoglobin CD14+ monocytes were obtained from the total lymphocyte fraction by incubation with anti-CD14 antibodies bound to magnetic beads for 15 minutes at 4°C (Miltenyi Biotech, Auburn, CA). The monocytes were separated from other immune cells by binding to a

magnetic MACS column followed by elution of all other leucocytes (Miltenyi Biotech, Auburn, CA). The monocytes were eluted from the column and cultured at a concentration of 2-9 x 10^6 cells/well in 6-well non-pyrogenic polystyrene culture plates in RPMI 1640 culture medium (Mediatech Inc. Manassas, VA, USA), supplemented with 10% FBS, 1mM glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, 50ng/ml human recombinant GMCSF, and 10 ng/ml human recombinant IL-4 (ProSpec-Tany), at 37°C in a humidified atmosphere of 5% CO_2 (Preprotech, Rocky Hill, NJ). The monocyte cell culture was fed at 2-day intervals by gentle replacement of 50% of the medium with fresh pre-warmed culture medium. The cells were cultured for a total of 6 days to allow monocyte differentiation into DCs prior to vaccine treatment. The cells were monitored by phase contrast microscopy to assess dendrite formation, a marker indicating DC differentiation.

IDO1 Protein Synthesis in Vaccinated Dendritic Cells

Approximately 2-9 x 106 monocyte-derived DCs generated from each of several subjects were inoculated with CTB-INS (0.1, 0.5, 1.0, 2.5, 5.0 and 10µg/ml), 500ng/ml of CD40L (Immunex, Seattle, WA), 500ng/ml of TRAF 2, 3 binding peptide (Proteintech Group, San Diego, CA) and 500 ng/ml of TRAF 6 binding peptide (Proteintech Group). The vaccinated DCs were incubated for 6, 12, 24, 48 or 96 hours and lysed in buffer C (20mM HEPES, 0.42 M KCl, 26% Glycerol, 0.1mM EDTA, 5mM MgCl2, 0.2% NP40, 37ºC) containing a tablet of complete protease inhibitor (Roche, Basel, Switzerland) according to the manufacturer instructions. At least 50µg of protein isolated from the total DC lysate was separated by electrophoresis on a 12% polyacrylamide gel (SDS-

PAGE). After transfer of the separated proteins to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA), the presence of IDO1 protein (NP_002155.1) was detected by incubation of the blot for 12 hours at 4^{o}C with an anti-IDO1 rabbit monoclonal primary antibody (Cat. 04-1056, clone EPR1230Y) (Millipore, Temecula, CA). For signal detection, the blot was washed 3 times with PBST (1X PBS, 0.02% tween 20, pH 7.4) and incubated for 2 hours at room temperature in the presence of a monoclonal anti-rabbit IgG γ-chain specific alkaline phosphatase conjugated secondary antibody (Cat. A-2556, clone RG-96) (Sigma-Aldrich). The immunoblots were washed 3 times in PBST and incubated in 200μL of Novex® AP chemiluminescent substrate (Invitrogen™) for 5 minutes prior to exposure to x-ray film (Kodak X-Omat) for 3 minutes. The IDO signal intensity was quantified via Image J software v. 1.48h. (Image J, NIH).

Small Interfering RNA (siRNA) Transfection

No pharmacological inhibitors for IKKα exist that selectively block the noncanonical pathway of NF-κB activation (Karin, Yamamoto, & Wang, 2004; Tas et al. 2007). Here we used siRNA to specifically target this pathway. To define the regulatory effect of the NF-κB pathway on CTB-INS-induced IDO expression, human NIK-small interfering RNA (NIK siRNA, sc-36065) and non-targeting siRNA (Control siRNA Fluorescein conjugate, sc-36869) were purchased from Santa Cruz Biotechnology (Santa Cruz, Delaware, CA, USA). Monocytes were cultured in six-well plates for 4 days with 50 ng/ml of hGM-CSF and 10 ng/ml of IL-4 to induce differentiation of DCs. siRNAs were transfected into DC cells using Lipofectamine® RNAiMAX reagent (Invitrogen,

Carlsbad, CA, USA) according to the manufacturer's protocol. Three microliters of 10μM siRNA was mixture in 150μl of Opti-MEM (Gibco-Life Technologies, Paisley, UK), while 9μl of Lipofectamine® RNAiMAX reagent was incubated in 150μl of Opti-MEM at room temperature for 5 min. Then the diluted siRNA and Lipofectamine® RNAiMAX reagent were incubated for a further 20 min at room temperature for complex formation. The complexes were added to wells. The final siRNA concentration was 25pmol. DC continued to be incubated at 37°C in 5% humidified CO2 for 48 h which was sufficient to significantly knock down the target protein levels. Expression of IDO1 was induced by 5μg of CTB-INS for 24h after siRNA transfection. To evaluate transfection efficiency, FITC-labeled control RNA was substituted for siRNA. After 24 hours, incubation, the transfected DCs were analyzed by fluorescence microscopy for intracellular FITC content. To confirm IKKα phosphorylation, DCs were treated with 10μg/ml of CTB-INS for 6hrs after transfection and lysed in 100μL buffer C/well containing phosphatase inhibitors (50mM Sodium-beta-glycerophosphate, 1mM Sodium fluoride, 1mM Sodium-ortho-vanadate). Western blot analysis with Anti-IKK alpha (phospho S176+S180) (Abcam 1:1000) and anti-rabbit IgG whole molecule conjugated AP (Sigma-Aldrich 1:1000) was performed prior to band detection on x-ray film (Kodak XOmat) of IKKα.expression.

Total RNA Preparation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from 1 X 106 DC was prepared using Trizol (Invitrogen, Carlsbad, CA, USA) and complementary DNA was synthesized from 2μg total RNA with oligo

(dT) primer in a 20µl reaction volume according to the manufacturer's recommendations (Thermo Fisher Scientific Inc, Waltham, MA, USA). Polymerase chain reaction (PCR) amplification was performed at 95°C for 1m, 58 °C for 1m, 72°C for 30s, and PCR was done for 35 cycles. The primers used in this study were NIK (h)-PR (sc-36065-PR, Santa Cruze), product size 537bp; and β-actin forward, 5'-GCA TTG CTT TCG TGT AAA TTA TGT-3' β-actin reverse, 5'-ACC AAA AGC CTT CAT ACA TCT CA-3', product size 211bps. The PCR products were size-separated on 1.5% agarose gels and visualized by Et-Br DNA gel staining.

ChIP Analysis of CTB-INS Induction of NF-κB Activation in Vivo

A chromatin immunoprecipitation (ChIP) assay was performed to identify the specific binding sequences in the IDO1 promoter region for the non-canonical NF-κB subunits RelB protein using a MAGnify™ Chromatin Immunoprecipitation System (Invitrogen) according to the manufacturer's instructions. 3-8 x 10^6 Human dendritic cells were left unstimulated or were stimulated with CTB-INS for 3h, after which DC cells were harvested and washed with 1X PBS and were fixed in formaldehyde (Sigma, St Louis, MO, USA) to a final concentration of 1%. After 10 min, 1.25M glycine was added to stop crosslinking reaction. After centrifugation, cells were lysed for 5 min in Lysis buffer supplemented with protease inhibitors. Chromatin was sheared by sonication (5 X 12s at one-fifth of the maximum potency) with a Sonic 60 Dismembrator (Fisher Scientific, Sunnyvale, CA, USA), centrifuged to pellet debris, and diluted in Dilution Buffer which was recommend in manufacturer's instructions. Fragmented chromatin was immunoprecipitated with a ChIP-grade antibody against RelB (GeneTex, Irvine, CA,

USA) which was coupled with Dynabeads ®, at 4°C overnight. Immune complexes were washed with IP Buffer 1 and 2 in the DynaMagTM-PCR Magnet. For reversing the crosslinking, Reverse Crosslinking Buffer with proteinase K was added to both input control (fragmented chromatin without immunoprecipitation) and immune complexes and incubated at 55°C for 15 min. The DNA was purified with DNA Purification Magnetic Beads and buffers provided in the kit according to the manufacturer's instructions. The immunoprecipitated DNA was used in each real-time PCR assay using primers specific for indicated regions of the DNA. The primers were designed using Primer Express 2.0 software (PE Applied Biosystems, USA) under default parameters. The primers that were used are as follows: 5'-CGT TAA TGG TGA ATT CAG TGA TG-3' (2732 F1) and: 5'-TGC AGA GGG ACC TTC ATT CAA G-3' (2732 R1), 5'-GGT AGA GAT GTT CCT CAG GCA G-3' (2961 F2) and 5'-CTC TAT GGC CTC CTA CAT CTG-3' (2961 R2), 5'-TGA GTT CTG GCT TTC AGG AG-3' (3072 F3) and 5'-GAT CTT GTC TTC ATT CAC CTT G-3' (3072 R3). Real-time PCR amplification reactions were performed using SYBR Green detection chemistry and run as triplicate samples on 96-well plates using the CFX 96TM Real-Time PCR Detection System (Bio-Rad). The PCR reactions were prepared in a total volume of 25µl containing: 5µl of Chip or input template DNA, 2µl of each amplification primer (final concentration 50nM), and 12.5µl of 2X iQ SYBR Green Supermix (Bio-Rad). The cycling conditions were set as follows: an initial denaturation step of 95 °C for 10 min to activate the iTaq DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min. The amplification process was followed by a melting curve analysis, ranging from 65 °C to 95 °C, with temperature increasing at steps of 2°C every 1 min. Baseline and threshold cycles (Ct),

were automatically determined using the Bio-Rad CFX Manager 2.1. The samples were electrophoresed on a 1.5% (w/v) agarose gel, and the banding pattern observed under UV light. Two biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate.

Blocking TNFR Activation of IDO1 Biosynthesis

Peptides containing the CD40 receptor TRAF2, 3 and TRAF6 binding sites were linked to the TAT47–57 cell penetrating peptide. The sequences for the CD40–TRAF2, 3 and the CD40–TRAF6 blocking peptides were NH2-NTAAPVQETLHGYGRKKRRQRRR-OH and NH2- KQEPQEI*D*FPDD YGRKKRRQRRR-OH respectively. The TAT47–57 sequence is underlined. Control peptides consisted of either TAT47–57 alone or TAT47–57 linked to a scrambled peptide. The peptides were manufactured by Proteintech Group (San Diego, CA) and were low in endotoxin and > 98% pure as measured by HPLC (Portillo, Greene, Schwartz, Subauste, & Subauste, 2015; J. A. Portillo et al., 2014).

CTB-INS Amino Acid Sequence Alignment with TNF Receptor Family Members

The protein amino acid sequence of CTB-INS (FASTA sequence-MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIYTLNDKIFSYTESLAGKR EMAIITFKNGAIFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNN KTPHAIAAISMANGPGPFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDL QVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCNSEKDE), was aligned with the following tumor necrosis factor (TNF) superfamily member ligands:

CD40L (Accession: NP_000065.1), TNFR14 (Accession: NP_003798.2), RANKL (Accession: NP_003692.1), and BAFF (Accession: NP_006564.1) using the T-Coffee server (http://tcoffee.crg.cat) (Notredame, Higgins, & Heringa, 2000). The protein alignment graphics were constructed using Jalview software v1.6 (Waterhouse, Procter, Martin, Clamp, & Barton, 2009).

Results

IDO1 Expression Following CTB-INS Incubation

Concentrations of 0.1-10μg/ml CTB-INS were used to assess the minimal concentration of CTB-INS needed to allow detection of IDO1 in monocyte-derived DCs. Expression of IDO1 induced by CTB-INS occurred at concentrations as low as 0.5μg/ml of CTB-INS (Figure 2.2A). Monocyte-derived DCs were incubated with CTB-INS for 6 to 96 hours with the medium replaced at 2 day intervals. The levels of IDO1 in vaccine inoculated DCs increased continuously for 96 hours following vaccination (Figure 2.2B).

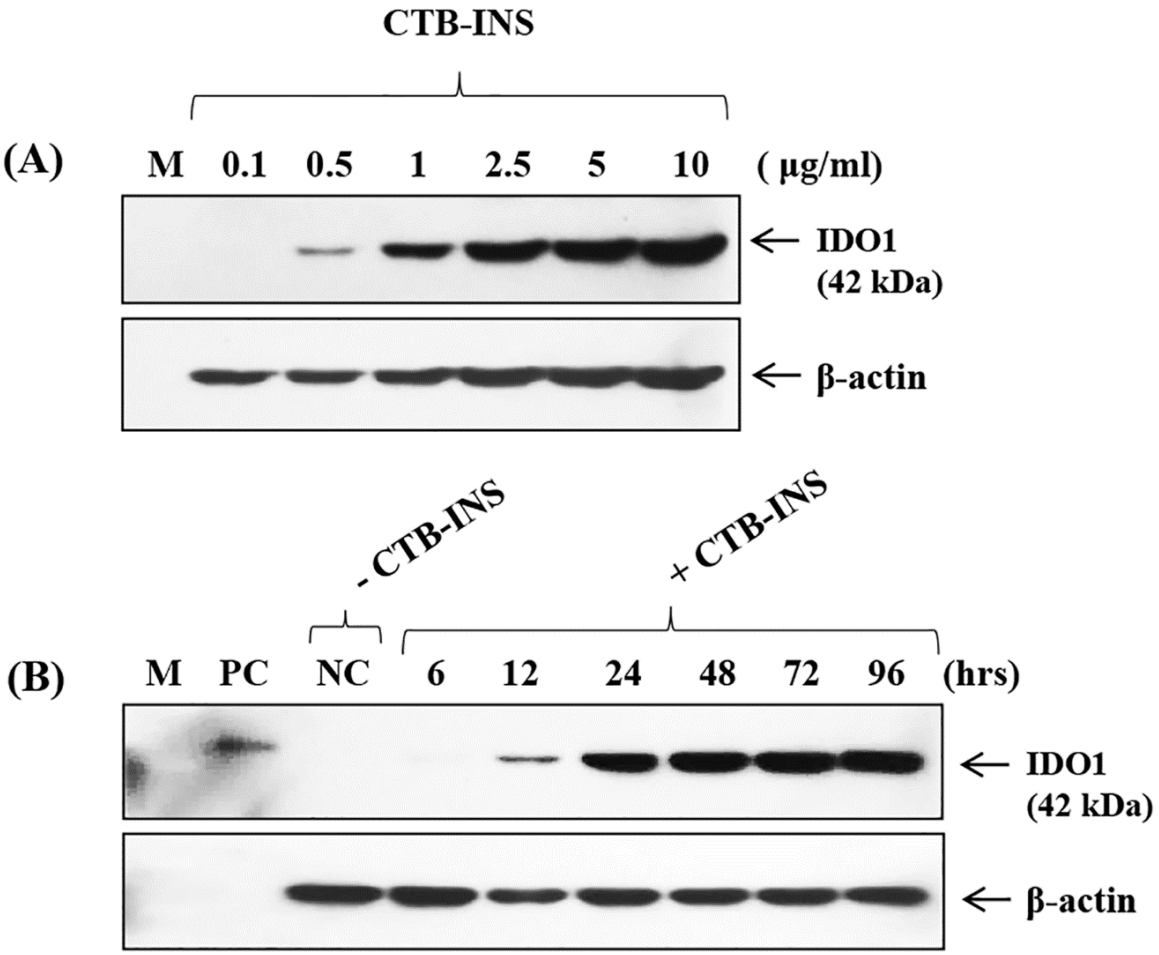

Figure 2.2. IDO1 Expression with varying concentrations of CTB-INS and incubation times and conditions. In panel (A) varying concentrations of CTB-INS from 0.1 to 10µg/ml were used to assess the minimal concentration of CTB-INS needed to cause detection of IDO1 in monocyte-derived DCs. In Panel (B) monocyte-derived DCs were incubated with CTB-INS for 6 hours to 96 hours with the medium changed every 2 days. IDO1 expression went increasingly unabated for 96 hours. M: Marker. PC: Positive Control (IDO1 recombinant protein) NC: Negative Control (Untreated).

CTB-INS Stimulation of the Non-canonical NF-κB Pathway Induces IDO1 Synthesis in DCs

To assess non-canonical NF-κB pathway contributions to CTB-INS-induced IDO1 expression we used siRNA technology to knock down the non-canonical pathway dependent kinase NIK and measured the level of IDO1 expression in treated DCs. The knockdown of NIK in CTB-INS stimulated DCs resulted in a significant reduction in NIK mRNA levels (Figure 2.3A) and IDO1 protein expression as well as a decrease in phosphorylated IKKα in comparison with non-specific siRNA-treated dendritic cells (Figure 2.3B). This experimental result demonstrated that CTB-INS-induced IDO expression in human DCs was dependent upon vaccine activation of the non-canonical NF-κB pathway.

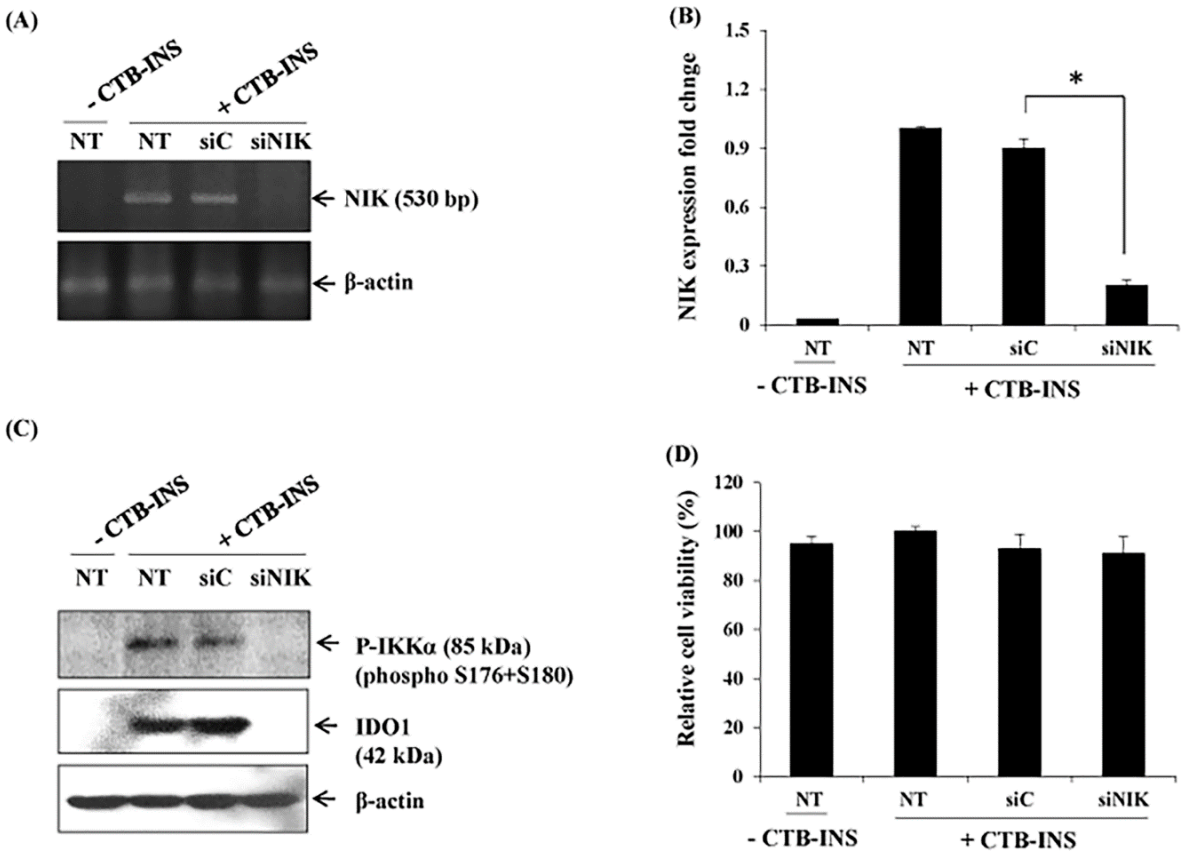

Figure 2.3. The non-canonical NF-κB pathway is required for CTB-INS-induced IDO1 expression in monocyte-derived DCs. Panel (A) shows NIK mRNA levels in monocyte-derived DCs transfected with NIK-specific siRNA (siNIK) or negative control (siC) examined by RT-PCR using NIK specific primers. The β-actin gene was used as an internal standard in RT-PCR. This image is representative of two independent experiments. Panel (B) graphic representation of NIK mRNA levels in CTB-INS vaccinated and unvaccinated DCs. Panel (C) shows the expression of IDO1 protein and phosphorylated IKKα in moDCs transfected with NIK-specific siRNA (siNIK) or negative control (siC) examined by Western blot analysis using anti-IDO1 as the primary antibody. This image is representative of three independent experiments. Panel (D) shows graphic representation of the cell viability of non-transfected or transfected with NIK-specific siRNA (siNIK) or negative control (siC) in mDCs. Dendritic cell viability was measured by determination of the percentage of vaccinated DCs negative for annexin V and propidium iodide. NT means non-siRNA CTB-INS transfected DCs, and – or + means without or with CTB-INS. Samples were assayed in triplicates and the results represent the mean ± SD of three independent experiments $p < 0.01$. Statistical analysis was performed using one way analysis of variance (ANOVA).

CTB-INS Leads to Non-Canonical NF-κB RelB Translocation to Drive IDO1 Expression in DCs in vivo

The IDO1 promoter region was shown to contain three partial RelB/p52 binding sites (AGGAGACACA, GGGAGACAGA, and AGGAGAAAGA), with a consensus non-canonical binding sequence PuGGAGApyTTPu located close to position -2000 (Figure 2.4A), (Bonizzi & Karin, 2004; Manches et al., 2012; Puccetti & Grohmann, 2007). To demonstrate direct binding of RelB/p52 to the IDO1 promoter, we performed a ChIP analysis experiment using RelB binding to pull-down the IDO1 promoter (Figure 2.4B). Immunoprecipitation of RelB induced by CTB-INS showed binding to all three IDO1 non-canonical binding sequences with increased binding at the GGGAGACAGA promoter sequence (Figure 2.4B, C).

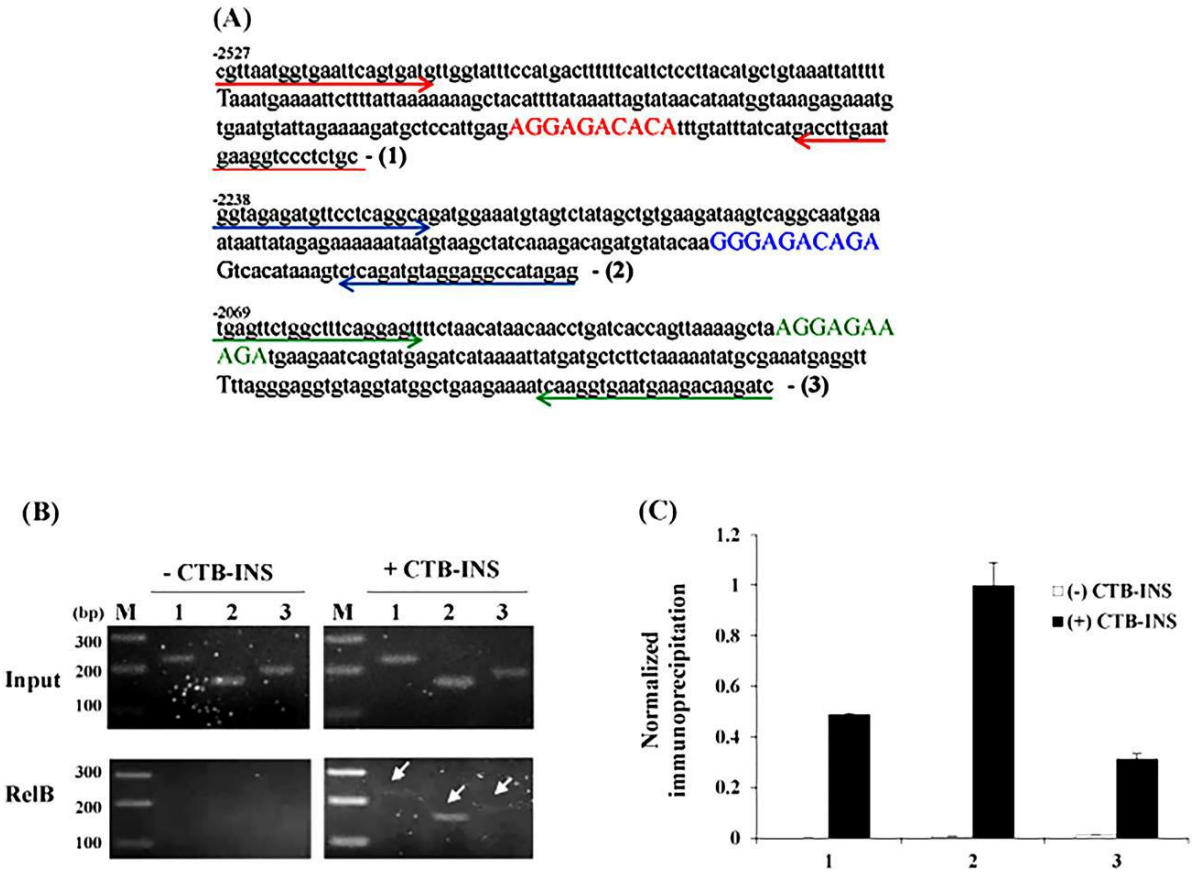

Figure 2.4. ChIP analysis showing vaccine stimulation of NF-κB RelB binding to the human dendritic cell IDO1 promoter region in vivo. Panel (A) shows the sequence of three partial non-canonical NF-κB RelB binding sites in the IDO1 promoter (capitalized nucleotides). The arrows indicate the primer sequences for detection of the three RelB binding sites. Panel (B) shows the PCR products after chromatin immunoprecipitation (ChIP). Immature human DCs were stimulated with the CTB-INS fusion protein vaccine for 0 (-) or 6 hr (+). Protein-DNA complexes were cross-linked, the DNA sheared and RelB genomic DNA complexes immunoprecipitated with RelB monoclonal antibody. After purification of the DNA, Real-time PCR was performed using primers flanking the three consensus NF-κB RelB binding sites in the human IDO1 promoter region shown in panel A. The Input control consists of PCR amplification of the IDO1 promoter obtained from total genomic DNA prior to immunoprecipitation. Lane M: DNA fragment size marker, Lanes 1, 2, 3: show the products of PCR amplification with primer sets detecting the three consensus RelB binding sites in the vaccinated human IDO1 promoter region (white arrows). Panel (C) Quantification of immunoprecipitation was performed for three experiments, by normalizing the intensity of each immunoprecipitated band to its input.

Vaccine Stimulation of the TNFR Signaling Pathway Induces IDO1 Biosynthesis in Human DCs

Control of NIK post-translational stability may be essential for non-canonical NF-κB signaling modulation. Therefore, the control of NIK stability is one of the prime questions for understanding regulation of the non-canonical NF-κB signaling pathway. Mounting evidence suggests that TNF receptor-associated factors, TRAF2, TRAF3 and TRAF 6, are critical molecules involved in negative regulation of NIK activity (Hostager & Bishop, 2013; Song & Kang, 2010; Vallabhapurapu et al., 2008). (Figure 2.5). Pharmacological inactivation of these proteins or their deletion also allows for basal NIK accumulation in the absence of ligand (Elgueta et al., 2009; Portillo et al., 2015; J. A. Portillo et al., 2014; Sun, 2010, 2011; Xiao et al., 2004; Xiao et al., 2001).

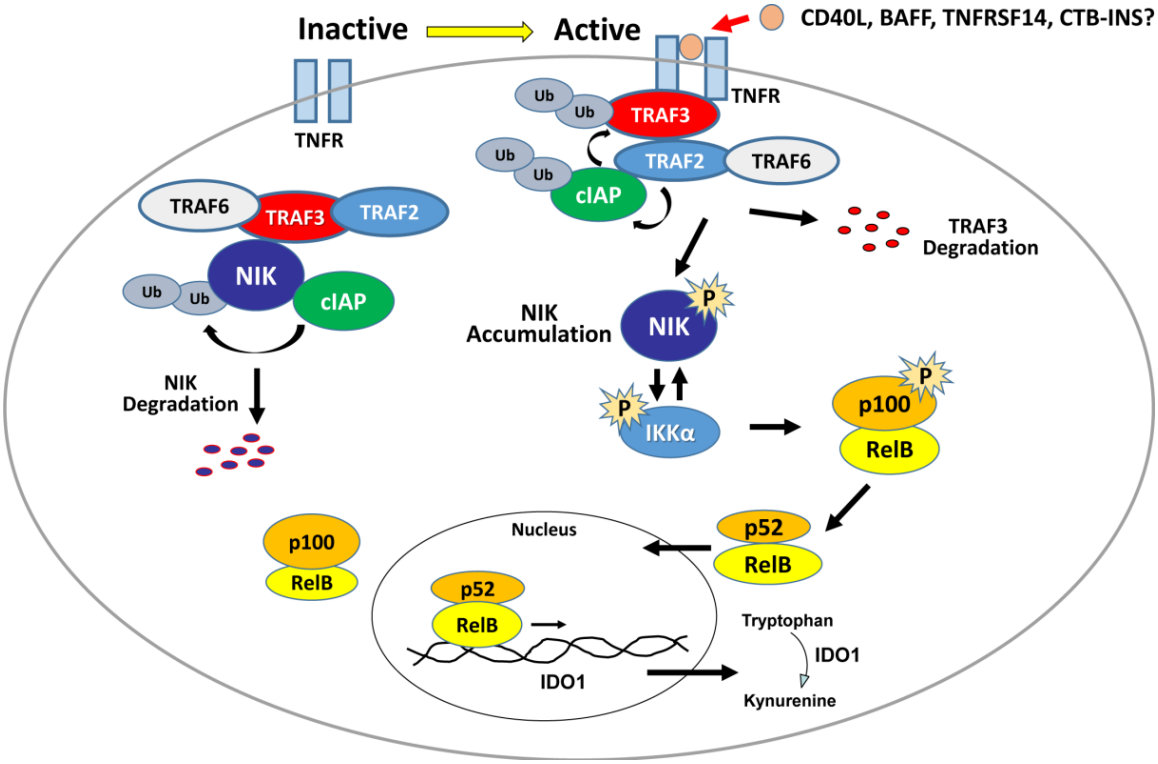

Figure 2.5. Activation of the non-canonical NF-κB pathway. In the basal inactive state (left), the TRAF-cIAP complex catalyzes ubiquitination of NIK, leading to constitutive NIK degradation in the proteasome leaving p100-containing RelB complexes isolated in the cytoplasm. During activation (right), the TRAF-cIAP complex is recruited to the CD40 receptor. Upon ligand binding, TRAF2-mediated, ubiquitination of cIAP1/2 switches its ubiquitin ligase activity from NIK to TRAF3. The resultant TRAF3 degradation destabilizes the TRAF-cIAP complex allowing accumulation of newly synthesized NIK. Phosphorylated NIK then transfers a phosphate to IKKα. Now activated, IKKα phosphorylates p100 leading to its partial proteosomal degradation releasing p52: RelB heterodimers that translocate into the nucleus. Further, activated IKKα phosphorylates NIK, destabilizing it thereby limiting downstream activation events.

The engagement of CD40 by CD40L promotes clustering of CD40 inducing the recruitment of adapter proteins known as TNFR-associated factors (TRAFs) to the cytoplasmic domain of CD40 (Elgueta et al., 2009). Previous reports showed that cell permeable peptides that include the TRAF2, 3 or TRAF6 binding site to CD40 are able to block the CD40-TRAF signaling pathway (Chatzigeorgiou et al., 2014; Chatzigeorgioua et al., 2014; Elgueta et al., 2009; Lutgens, 2012; Portillo et al., 2015; J. A. C. Portillo et al., 2014; Vallabhapurapu et al., 2008). Inhibition of the TNFR pathway permits examination of its role in induction of IDO1 in vaccinated dendritic cells. Therefore, monocyte-derived DCs were incubated with peptides containing the amino acid sequence of the TRAF2, 3 and the TRAF6 binding sites to CD40. The DCs were then stimulated with CD40 ligand (CD154), and CTB-INS. The CD40-TRAF2, 3 and CD40-TRAF6 blocking peptides were shown to impair upregulation of IDO1 in response to CD154, and CTB-INS treatment. The most impairment of IDO upregulation was detected when both TRAF 2, 3 and TRAF 6 inhibitors were used in combination. (Figure 2.6).

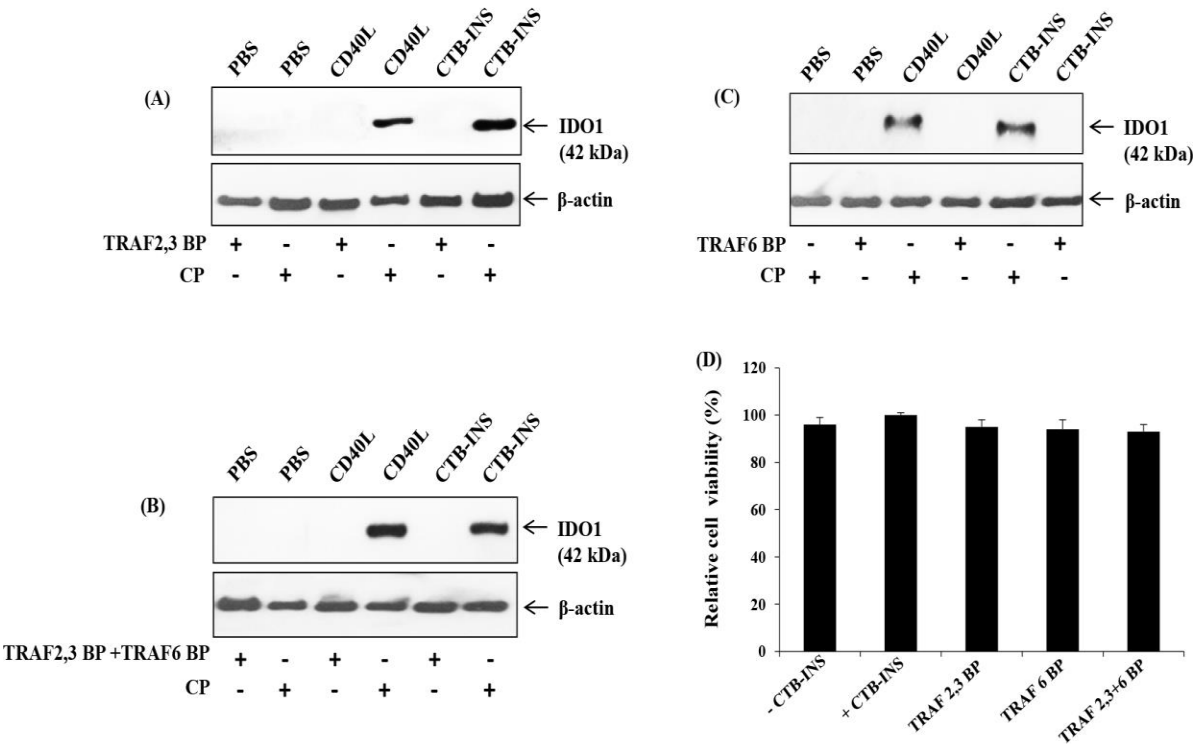

Figure 2.6. The TNFR-TRAF pathway is required for CTB-INS vaccine induction of IDO1 protein biosynthesis. In Panels (A,B,C) monocyte-derived DCs were inoculated with blocking peptides containing the amino acid sequence of TRAF2, 3 or TRAF6 binding sites of CD40 linked to the TAT47–57 membrane transport peptide. After blocking peptide binding, the DCs were stimulated with 500ng/ml of CD40L (CD154), Immunex, Seattle, WA), LPS (1μg/ml) and CTB-INS (10μg/ml). Both CD40-TRAF2, 3 and CD40-TRAF6 blocking peptides impaired upregulation of IDO1 in response to CD154, LPS and CTBINS. Each image is representative of two independent experiments. (D) Relative cell viability of cells treated with CTB-INS in combination with TRAF 6BP and TRAF 2, 3 BP.

CTB-INS as a Ligand for Members of the TNFR Superfamily

Members of the Tumor Necrosis Factor (TNF) receptor family have been shown to stimulate DC maturation or modulate peripheral tolerance in autoimmunity by upregulation of IDO1 (Coope et al., 2002; Ma & Clark, 2009; Tas et al., 2007). Both CTB and LTB enterotoxin protein binding subunits were shown to stimulate antigen presenting cell CD40 surface expression and DCs were found to upregulate IDO1 mediated immune suppression through activation of the NF-κB non-canonical signaling pathway (Tas et al., 2007). Protein functional homology analysis (PROPSEARCH^{TM}) identified the probability of functional homology between CTB-INS and the TNF subfamily of ligands to be >87%, (unpublished data). Based on the data in (Figure 2.6) we hypothesize that CTB-INS interacts with TNF receptors to stimulate IDO1 synthesis in vaccinated DCs. To test this hypothesis, we aligned CTB-INS protein amino acid sequence with the following tumor necrosis factor (TNF) superfamily member ligands: CD40L (Accession: NP_000065.1), TNFR14L (Accession: NP_003798.2), RANKL (Accession: NP_003692.1), and BAFF (Accession: NP_006564.1) using the T-Coffee server. We observed that the CTB-INS vaccine shares significant levels of amino sequence homology with ligands of the TNFR superfamily (Figure 2.7).

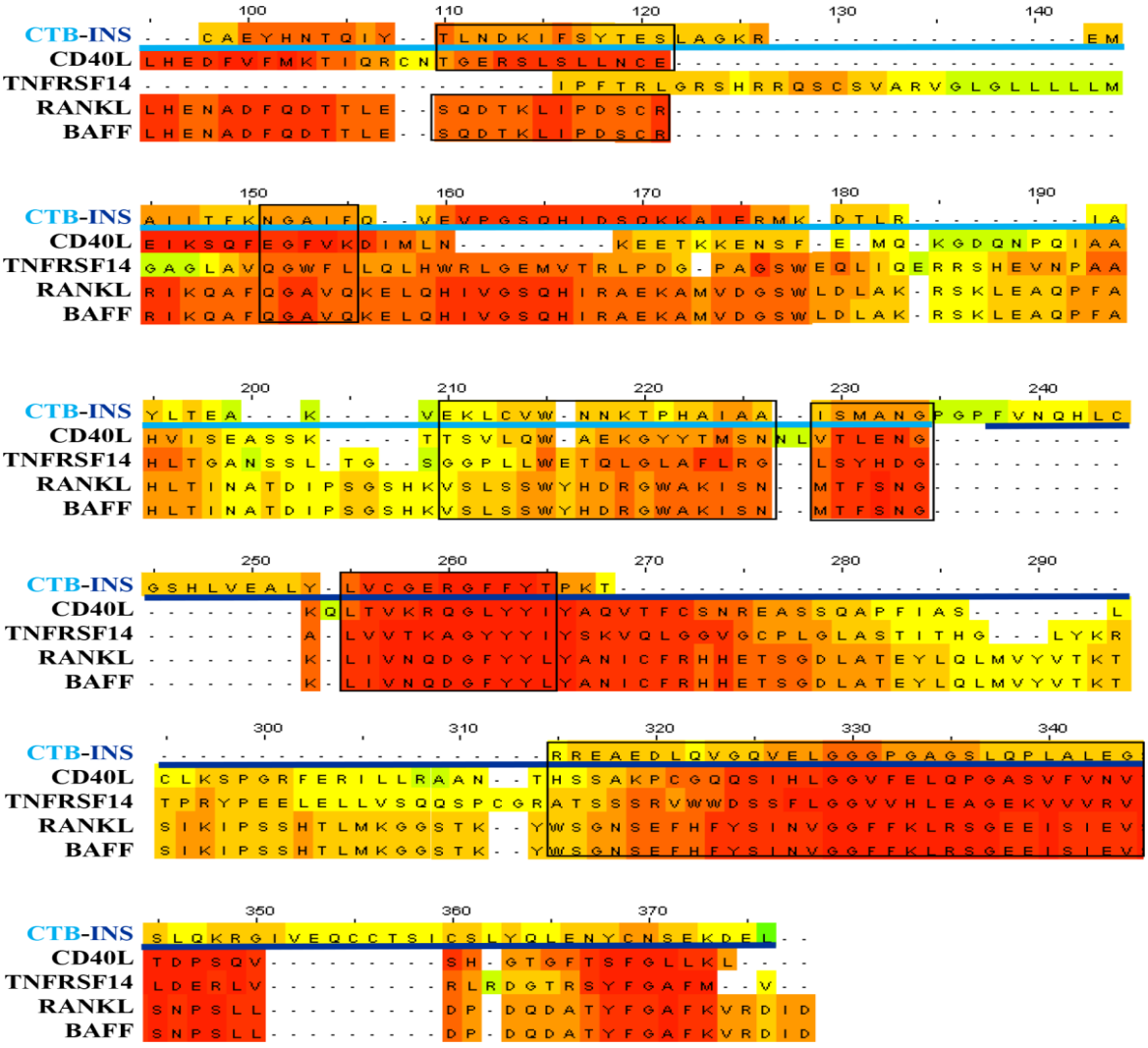

Figure 2.7. Comparison of the amino acid sequence of CTB-INS with ligands of the Tumor Necrosis Factor Receptor (TNFR) superfamily (CD40L, TNF14, RANKL and BAFF). The light-blue horizontal line corresponds to the amino acid sequence of CTB and the dark blue horizontal line denotes the amino acid sequence of proinsulin. The black boxes highlight areas of greater functional binding homology among TNFR family members, in which the red highlighted areas signify greater levels of amino acid homology and the green areas, low to no detectable homology.

Discussion

The mechanism of CTB-INS induction IDO1 biosynthesis was shown to be dependent on the NF-κB signaling pathway (J. C. Mbongue et al., 2015). However, the relative contributions of canonical and non-canonical branches of this pathway to IDO1 up regulation remain unknown. Earlier work by Tas and his colleagues showed that CD40L was responsible for stimulation of IDO1 via the non-canonical pathway (Tas et al., 2007), suggesting this pathway could play a significant role in CTB-INS induction of IDO1 in human DCs. Based on NIK-dependent activation experiments, our data suggest CTB-INS induces IDO1 in human monocyte-derived DCs via the non-canonical NF-κB pathway. In addition, ChIP analysis experiments showed that NF-κB RelB-p52 dimers bound to defined consensus sequences within the *IDO1* promoter *in vivo*, suggesting the non-canonical signaling pathway is active in vaccine induction of IDO1 in human DCs. Blocking TRAF adaptor molecule functions was shown to inhibit IDO1 biosynthesis in vaccinated DCs suggesting upregulation of IDO may occur through TNF receptor family stimulation of the NF-κB non-canonical signal transduction pathway (Song & Kang, 2010). In the TNF-activated signal transduction pathway, NIK is known to interact with TRAF2, and TRAF3 leading to non-canonical NF-κB activation (Vallabhapurapu et al., 2008).

Induction of IDO1 depends on fusion of CTB to proinsulin (J. C. Mbongue et al., 2015), suggesting that the vaccine may bind as a ligand to receptors responsible for NF-κB non-canonical pathway activation of IDO1 expression. Several ligands of the Tumor Necrosis Family Receptor (TNFR) superfamily were shown to activate the non-canonical NF-κB pathway (Bishop, Moore, Xie, Stunz, & Kraus, 2007; Hsu et al., 1997; Morrison,

Reiley, Zhang, & Sun, 2005; Novack et al., 2003; Tas et al., 2007; Theill, Boyle, & Penninger, 2002). For suggestive evidence of a link between CTB-INS with TNFR, we compared amino acid sequences of CTB-INS with those of four ligands of the TNFR family to assess any type of functional homology. We found that several areas where the vaccine could act as a TNFR ligand, represented by areas of greater amino acid homology (Figure 2.7).

The association between the ligand and its potential receptor suggests that CTBINS receptor binding may involve specificity of the autoantigen for its receptor rather than existing as a general mechanism for binding all CTB-autoantigen conjugates. Our experimental data suggests that CTB-INS induces non-canonical NF-κB signaling which is driven by TRAFs as TNFR signal mediators. Although, there is evidence that CTBINS elicits immunosuppressive effects through TNFRs, further experiments are needed to determine the probability of CTB-INS binding to individual members of the TNFR family.

The B-cell activating factor (BAFF) predominantly expressed in B cells, differs from many other TNFR superfamily members in that it generally activates the non-canonical NF-κB signaling pathway with only weak induction of canonical NF-κB pathway signaling (Claudio, Brown, Park, Wang, & Siebenlist, 2002; Morrison et al., 2005). This unique feature of the BAFF receptor (BAFFR) is due primarily to its possession of an atypical TRAF-binding sequence, which interacts with TRAF3 but not with TRAF2 (Morrison et al., 2005). The BAFFR-mediated induction of p100 processing to p52 contributes to the survival of transitional and mature B cells, likely through induction of anti-apoptotic genes like *bcl-2* and *bcl-x* (Claudio et al., 2002; Morrison et

al., 2005).

The CD40 molecule is a TNFR member expressed on a variety of cell types, including B cells, dendritic cells, monocytes, endothelial epithelial cells, and neurons (Bishop & Hostager, 2003; Ma & Clark, 2009). Activated T cells primarily express the ligand of CD40, alternatively referred to as CD40L or (CD154). In the immune system, a major function of CD40 signaling is to regulate B-cell activation and differentiation events, including proliferation and survival of activated B cells, germinal center formation, and antibody isotype switching. Another major function of CD40 is to mediate dendritic cell maturation and antigen presentation. Unlike BAFFR, CD40 elicits strong signals that target both the canonical and non-canonical NF-κB pathways (Ma & Clark, 2009; Tas et al., 2007). Upon ligation by CD40L, CD40 interacts via two different TRAF-binding motifs that include TRAF1, 2, 3, 5, and 6, and this interaction leads to proteolysis of both TRAF2 and TRAF3 (Bishop et al., 2007; Harnett, 2004). As indicated above, the degradation of TRAF2 and TRAF3 represent an important step in the activation of the non-canonical NF-κB signaling pathway (Sun, 2010, 2011; Sun & Ley, 2008).

The herpesvirus entry mediator (HVEM) or tumor necrosis factor receptor superfamily member 14 (TNFRSF-14) is a protein originally known as herpesvirus entry mediator A (HveA). Both HveB and HveC are structurally unrelated proteins of the immunoglobulin superfamily (Montgomery, Warner, Lum, & Spear, 1996). HvA is also known as CD270 (Hsu et al., 1997). Moreover it is also referred to as ATAR (another TRAF-associated receptor). Interactions between TNFRSF-

14 and TRAF2 were shown to activate the non-canonical NF-κB signaling pathway (Hsu et al., 1997).

The Receptor Activator of Nuclear Factor κ B (RANK) is best known for its role in osteoclastogenesis (Novack et al., 2003; Sun, 2011). However, it also regulates important immune functions that include dendritic cell survival and lymphoid organogenesis (Theill et al., 2002). RANK is expressed on osteoclast precursors, dendritic cells, and activated B cells, and in general, RANK signaling was shown to promote cell survival and differentiation. Analogous with CD40, the cytoplasmic domain of RANK was shown to bind TRAF1, 2, 3, 5, and 6 and mediates activation of both canonical and non-canonical NF-κB signaling pathways. Genetic evidence suggests an essential role for RANK-stimulated activation of non-canonical NF-κB activation during osteoclastogenesis and bone metabolism (Novack et al., 2003). The non-canonical NF-κB has been closely linked to immune suppression (J. C. Mbongue et al., 2015; Pallotta et al., 2011). Several ligands such as Glucocorticoid-induced tumor necrosis factor receptor (GITR) on T cells and its natural ligand, GITRL, on accessory cells contribute to the control of immune homeostasis. Grohmann et al. showed that reverse signaling through GITRL after engagement by soluble GITR initiates the immunoregulatory pathway of tryptophan catabolism in mouse plasmacytoid dendritic cells, by means of noncanonical NF-κB–dependent induction of IDO1 (Grohmann et al., 2007). Additionally, CpG-rich oligodeoxynucleotides activate the immune system, leading to innate and adaptive immune responses that have been shown to promote tolerogenic responses in mouse plasmacytoid dendritic cells in vivo and in an *in vitro* human DC model. Unveiling a previously undescribed role for TRIF and TRAF6 proteins in Toll-like receptor 9 (TLR9)

signaling, it was demonstrated that physical association of TLR9, TRIF and TRAF6 leads to activation of non-canonical NF-κB signaling and the induction of IRF3- and TGF-β-dependent immune-suppressive tryptophan catabolism (Volpi et al., 2013).

Understanding the link between vaccine activation of TNF receptor family members and the activation of non-canonical NF-κB signaling is an important step in elucidation of the mechanism underlying chimeric vaccine induction of immunological tolerance in dendritic cells. Understanding the mechanism of chimeric vaccine modulation of IDO1 induction and suppression of in human dendritic cell activation will facilitate development of chimeric vaccine strategies for effective and safe therapy for type 1 diabetes and a wide range of tissue specific autoimmune diseases.

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CHAPTER 3

CHIMERIC VACCINE STIMULATION OF TGF-B SIGNALING AND INDOLEAMINE 2, 3-DIOXYGENASE BIOSYNTHESIS IN HUMAN DENDRITIC CELLS ARE INDEPENDENT MECHANISMS

Grace E. Esebanmen [a, b] and William H.R. Langridge [b, c,*].

[a]Department of Earth and Biological Sciences, Loma Linda University School of Medicine, Loma Linda, CA, USA

[b]Center for Health Disparities and Molecular Medicine, Loma Linda University School of Medicine, Mortensen Hall, Loma Linda, CA, USA

[c]Division of Biochemistry, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA , USA

*Corresponding author: blangridge@llu.edu

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Abstract

Fusion of the cholera toxin B subunit fusion to autoantigens such as proinsulin (CTB-INS), down regulate dendritic cell (DC) activation and stimulate the synthesis of DC immunosuppressive cytokines. Recent studies of CTB-INS-induced immune tolerance in human DCs show that increased synthesis of tryptophan catabolic enzyme indoleamine 2, 3- dioxygenase (IDO1) may play an important role in CTB-INS vaccine suppression of DC activation. Studies in murine models of autoimmunity suggest transforming growth factor beta (TGF-β) may be involved in the stimulation of IDO1 biosynthesis, for induction of immunological tolerance in DCs. In this study, we investigated the contribution of TGF-β superfamily proteins to CTB-INS induction of IDO1 biosynthesis in human monocyte-derived DCs (moDCs). The fusion protein was shown to upregulate levels of TGF-β1, activin-A and the TGF-β activator, integrin αvβ8 in human DCs. The CTB-INS fusion protein was also shown to induce Smad2/3 phosphorylation; However, inhibition of endogenous TGF-β, activin-A or addition of biologically active TGF-β1, and activin-A, did not inhibit or stimulate IDO1 biosynthesis in human DCs treated with CTB-INS. While kinase inhibition with the inhibitor, RepSox, blocked Smad2/3 phosphorylation and diminished IDO1 biosynthesis in a concentration dependent manner, inhibition of TGF-β type 1 kinase receptor function with SB-431542 did not arrest IDO1 biosynthesis, suggesting involvement of a kinase pathway other than TGF-β type 1 kinase in CTB-INS induction of IDO1 in human moDCs. Together, our experimental findings identify alternative immunoregulatory proteins induced by CTB-INS fusion protein that suggest CTB-INS may utilize multiple mechanisms for induction of immune tolerance in human moDCs.

Introduction

The condition of autoimmunity is represented by a group of progressive chronic inflammatory diseases in which cells of the immune system induce a systemic or organ specific immune response against self-antigens. Autoimmunity generally attacks more women than men and affects 7-10% of the U.S. population (Agmon-Levin et al., 2011; Miller et al., 2012; Whitacre, 2001). Cells of the innate portion of the immune system are involved not only in the initiation of an immune response to pathogens but also in the initiation of autoimmune disease pathogenesis (Peng and Tian, 2014). Dendritic cells (DCs), considered to be the dominant antigen-processing and presenting cells of the body, are central to the regulation of innate and adaptive immune responses responsible for the maintenance of immune homeostasis (Raker et al., 2015). One major function of DCs is the processing and presentation of foreign and self-antigens to naive T cells resulting in the induction of T cell differentiation into either pro- or anti-inflammatory T cell populations. A break down in these normal DC functions can result in immunological impairment that includes autoimmunity and other chronic inflammatory diseases (Hammer and Ma, 2013; Thome et al., 2014). The tolerogenic functions of DCs have a therapeutic potential for prevention and treatment of autoimmune diseases, organ transplant rejection and other conditions of immune dysregulation that result in chronic inflammation(Osorio et al., 2015). Dendritic cells are known to induce immunological tolerance through the induction of T cell anergy, apoptosis or stimulation of regulatory T cell (Treg) proliferation. To alter the immune environment, the DC may express tolerogenic factors like IDO1 to regulate T cell differentiation and proliferation (Maldonado and von Andrian, 2010; Raker et al., 2015).

Type 1 diabetes (T1D) is a prototypic and economically important tissue specific autoimmune disease, in which loss of tolerance to islet-derived antigens, initiates autoreactive effector T cell (Teff) mediated destruction of the insulin-producing β cells located in the pancreatic islets of Langerhans (Morel, 2013). In the absence of insulin replacement therapy T1D leads to permanent insulin deficiency, diabetic coma and death. Considering the critical role dendritic cells play in maintenance of self-tolerance and in subverting autoimmunity, they have recently become the subjects of strategies designed to mediate therapy for autoimmune diseases. The application of DC mediated restoration of immunological tolerance holds particular promise for prevention of autoimmune disease onset and progression (Thomas, 2013).

A chimeric fusion protein vaccine composed of the cholera toxin B subunit linked at its c-terminus to the diabetes autoantigen proinsulin (CTB-INS), was shown to suppress autoimmune diabetes development in the non-obese diabetic (NOD) mouse (Arakawa et al., 1998; Aspord and Thivolet, 2002; Dénes et al., 2006). Further investigations using human monocyte-derived dendritic cells (moDCs) showed that vaccine efficacy depended on inhibition of dendritic cell activation through, suppression of DC costimulatory molecules, CD86 and CD80, suppression of pro-inflammatory cytokines and induction of the immunosuppressive tryptophan catabolic enzyme, indoleamine 2, 3-dioxygenase (IDO1) (Mbongue et al., 2015; Odumosu et al., 2011b). IDO1 catalyzes the breakdown of the essential amino acid tryptophan into degradation products called kynurenines and modulates immune suppression and peripheral tolerance (Fallarino et al., 2012; Harden and Egilmez, 2012). For example, in mice, T cell sensitivity to IDO1 reduction of tryptophan levels, inhibits their proliferation (Munn et

al., 2005). Further, the expression of IDO1 in human moDCs induces a state of immune tolerance through inhibition of pro-inflammatory T cell proliferation (Hwu et al., 2000; Planès and Bahraoui, 2013) and by the generation of regulatory T cell populations (Chung et al., 2009; Jürgens et al., 2009). The anti-inflammatory response of DCs to vaccine action may be significant in preventing the onset of type 1 diabetes (T1D). However, elucidation of the mechanism of immune suppression mediated by CTB-INS is required for validation of vaccine efficacy and safety prior to its use in therapy against T1D.

The Transforming Growth Factor-beta (TGF-β) superfamily is a group of pleiotropic cytokines that function in a variety of crucial biological activities that include cell growth and differentiation, cell death, early embryonic development, tumorigenesis, tissue homeostasis, immune responses and the regulation of inflammation (Isabel et al., 2014; Wakefield and Hill, 2013; Wijayarathna and de Kretser, 2016).The TGF-β superfamily includes the TGF-βs, activins, bone morphogenetic proteins (BMP), NODAL, growth and differentiation factors (GDF) and anti-Müllerian hormone (AMH), which make up the major subfamilies of up to 33 identified members encoded in the human genome (Morikawa et al., 2016; Moustakas and Heldin, 2009; Wakefield and Hill, 2013).

The TGF-β superfamily ligands transmit molecular signals by a mechanism conserved across all the members of the family. TGF-β signaling is initiated by binding of the ligand to its type II receptor which results in the recruitment of a type I receptor to form a type 2-type 1 receptor complex. The type I and type II cell surface receptors are transmembrane serine/threonine kinases, and upon receptor complex formation, the type I

receptor becomes phosphorylated and subsequently phosphorylates specific cytoplasmic transcription factor proteins called receptor-regulated Smads (R-Smads) (Huminiecki et al., 2009; Massague, 1998; Weiss and Attisano, 2013). The Smad proteins are the intracellular core machinery responsible for transducing external signals from TGF-β ligands through their receptors into the nucleus of the cell resulting in transcription of specific gene products. The R-Smads, Smad1, Smad5, and Smad8, are activated by the BMP/GDF pathway, while Smad2 and Smad3 are mainly activated by TGF-β, activin, and NODAL type I receptors (Huminiecki et al., 2009; Massagué, 2012; Moustakas and Heldin, 2009). Phosphorylation of the R-Smads results in their association with a common-mediator-Smad (Co-Smad) called Smad4. This association forms an R-Smad-Smad4 complex that translocates to the nucleus where it can associate with other DNA-binding transcription factors, to form a protein complex capable of binding to specific enhancer and promoter regions in a target gene to activate or repress transcription (Licona-Limón and Soldevila, 2007; Massague, 1998).

The TGF-β superfamily cytokines have been shown to modulate adaptive and innate immune responses. These cytokines critically regulate T cell differentiation and maturation, act synergistically to induce regulatory T cells (Tregs), mediate DC functions and natural killer cell-DC interactions, regulate macrophage polarization and attenuate pro-inflammatory cytokines (Licona-Limón and Soldevila, 2007; Lu et al., 2010; Robson et al., 2007; Seeger et al., 2014; Wang et al., 2014). The prototypic member of the TGF-β superfamily, TGF-β1, has been identified as a critical cytokine involved in the regulation of DC immune responses and in the maintenance of immune cell homeostasis via mechanisms of immune defense and/or recovery from autoimmune diseases (Li et al.,

2006b; Prud'homme and Piccirillo, 2000). Deficiency of the TGF-β1 gene, and gene deletions in the TGF-β1 signaling pathway in mice, results in multifocal inflammatory autoimmune disorders, or inflammatory states that mimic several autoimmune diseases (Aoki et al., 2005; Kriegel et al., 2006; Prud'homme and Piccirillo, 2000).

Despite the pleiotropic functions of TGF-β1, it is a tightly regulated cytokine: TGF-β1 is secreted and maintained as an inactive complex that requires activation to become functional (Hammer and Ma, 2013; Mu et al., 2002). The TGF-β1 cytokine is synthesized as a homodimeric pro-peptide consisting of the active TGF-β1 covalently linked to the latency associated peptide (LAP). Following enzymatic cleavage in the Golgi, the LAP remains attached to the mature TGF-β1 by non-covalent bonds in an association called the small latent complex (SLC) (Li et al., 2006b; Wipff and Hinz, 2008; Worthington et al., 2012). This interaction prevents the active TGF-β1 from binding to its receptors. Disassociation of the active peptide from LAP is termed latent TGF-β activation and can be mediated in biological systems by several proteases and cell transmembrane molecules called integrins (Travis and Sheppard, 2014). Members of the αv integrin family recognize specific arginine-glycine-aspartate (RGD) sequences present on LAP by which they bind to the latent TGF-β complex and liberate the active TGF-β to interact with its receptors (Song et al., 2016). The binding of the αv integrin family to the RGD is critical for TGF-β activation in maintaining immune regulation as mice with mutated binding sequences phenocopy TGF-β-null mice, dying from multi-organ inflammatory conditions (Yang et al., 2007). Integrin αvβ8 is the only αv member that has been detected on murine immune cells. The integrin is expressed only on murine DCs and CD4+ T cells but absent from other immune cells and has a critical role in

maintaining immune homeostasis by promoting TGF-β activation and signaling (Travis et al., 2007b; Worthington et al., 2015).

Studies on murine dendritic cells have revealed that IDO-dependent induction of tolerance in murine DCs requires TGF-β1, which both induces and maintains IDO synthesis in murine DCs by signaling that involves PI3/Akt phosphorylation and non-canonical NF-κB activation (Belladonna et al., 2008; Pallotta et al., 2011). Recent studies in our laboratory showed that CTB-INS induction of IDO1 biosynthesis in human moDCs involved non-canonical NF-κB activation (Kim et al., 2016). Therefore, in order to elucidate the mechanism by which CTB-INS induces immunological tolerance in human moDCs, we evaluated the role of TGF-β superfamily members in the induction of IDO1 biosynthesis in human moDCs following vaccination with CTB-INS. We analyzed IDO1 expression and Smad2/3 phosphorylation, following inhibition of Smad2/3 signaling in CTB-INS treated moDCs.

Materials and Methods

Preparation of Peripheral Blood Mononuclear Cells

This experimental study was approved by the Loma Linda University IRB and Research Ethics Committees. Experiments on peripheral blood mononuclear cells (PBMCs) were performed ex-vivo, with aphaeresis blood provided by the Life Stream Blood Bank (San Bernardino, CA) with blood donor consent. Blood donor information was anonymized before initiation of the study.

Synthesis and Isolation of CTB-INS Fusion Protein

The *Escherichia coli* producer strain, BL-21 (DE3) pLysS (Invitrogen, Carlsbad, CA), transformed with the CTB-INS fusion gene, was grown in 250 ml Luria Broth (LB) medium containing ampicillin (100mg/ml) with shaking at 37^{o}C for 7 to 8 hours. CTB-INS protein synthesis in the bacterial culture was stimulated with 2.0mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG), (Sigma Chemical Co. St Louis, Mo) at 3 hours of culture, and the CTB-INS protein extracted as previously described (Odumosu et al., 2011b) (Figure 1). Briefly, the bacterial cells were harvested at optimal culture density of up to 0.2-0.4 $O.D._{600}$, and CTB-INS protein was isolated from the bacterial cell homogenate using Maxwell Model 16 robotic protein purification system (Promega, Inc.) according to the manufacturer's instructions. The elution buffer was removed by dialysis at 4°C in phosphate buffered saline (PBS) and the purity of the isolated protein was determined by polyacrylamide gel electrophoretic mobility analysis followed by immunoblotting (Western) with anti-Cholera Toxin as the primary antibody (Sigma, Inc).

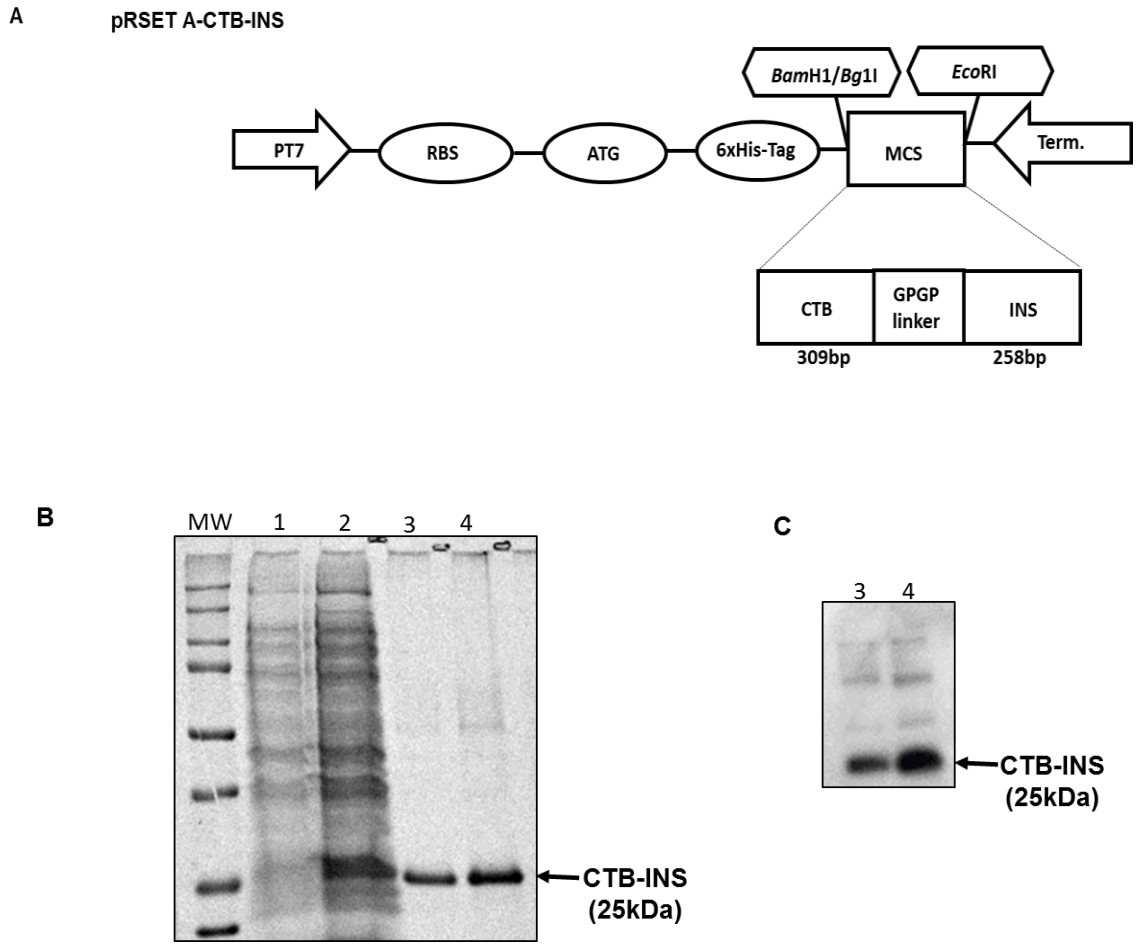

Figure 3.1. Gene construct and protein isolation of CTB-INS fusion protein. Panel (**A**) Plasmid map of the *E. coli* expression vector, pRSET A (Invitrogen, Carlsbad, CA), carrying the CTB-INS fusion gene. The expression vector is under the control of the bacteriophage T7 promoter and contains an oligonucleotide region that encodes 6 histidine amino acid residues immediately 5' upstream of the CTB gene sequence. The CTB-INS fusion protein was expressed using the *E. coli* pRSET A expression vector and purified by nickel binding isolation of the recombinant protein using a Maxwell 16 protein isolation robot (Promega Inc, Madison, WI, USA). (**B**) SDS-PAGE of CTB-INS protein visualized by Coomassie staining. Lane MW: protein size marker (BIO-RAD, Hercules, CA); 1: non-induced *E. coli* (BL-21) cell lysate; 2: IPTG induced *E. coli* lysate; 3: Eluted CTB-INS protein; 4: Eluted CTB-INS protein. (**C**) Immunoblot of recombinant CTB-INS fusion protein detected with anti-CTB primary antibody. Lane 3: Eluted CTB-INS protein; 4: Eluted CTB-INS protein.

Monocyte Isolation and Differentiation of DCs

Monocytes were isolated from peripheral blood using LS separation columns and anti-CD14 magnetic MicroBeads (Miltenyi Biotec) as previously described (Mbongue et al., 2015) and maintained in culture at a density of 5 x 10^5 to 1 x 10^6 cells/ml. Immature DCs were differentiated from CD14 positive monocytes by incubation for 6 days in RPMI 1640 medium (Hyclone, GE Healthcare Life Sciences) containing 10% FBS and supplemented with human GM-CSF (50ng/ml) and human IL-4 (10ng/ml) (Miltenyi Biotec). Half the medium was replaced with fresh medium every 2 days being careful not to dislodge the cells from the substrate.

Dendritic Cell Treatments

The differentiated immature moDCs (assessed by observation of dendrite formation using phase contrast microscopy) were incubated with 5µg/ml of CTB-INS protein or 20-100ng/ml recombinant bioactive human TGF-β1 (e-Bioscience), or 10-100ng/ml recombinant human activin-A (e-Bioscience) for 24hrs at 37^{o}C. To inhibit TGF-β signaling, the moDCs were treated with CTB-INS vaccine in the presence or absence of RepSox (12.5µM or 25µM; SIGMA), or the specific TβRI serine/threonine kinase inhibitor, SB-431542 (10µM; Reagents Direct) or DMSO as negative control, added 1hr before treatment with CTB-INS vaccine. To neutralize the presence of active TGF-β cytokine, the moDCs were pre-incubated for 15min to 1hr at 37°C with a pan-TGF-β (TGF-beta 1, 2, 3) neutralizing antibody clone 1D11 (20µg/ml; from R&D Systems, Minneapolis, MN, USA) or mouse IgG1 isotype control clone 11711 (R&D Systems, Minneapolis, MN, USA) (Belladonna et al., 2008) followed by treatment with CTB-INS

fusion protein (5µg/ml) for 24hrs. For neutralization of activin, the moDCs were incubated with recombinant human follistatin 288 (100-400ng/ml; R&D Systems, Minneapolis, MN, USA).

Real Time PCR Analysis

CTB-INS (5ug/ml) treated and untreated DCs were harvested at 1hr, 3hr, and 6hr time points. DCs incubated with the vaccine vehicle served as untreated controls. Total RNA was extracted from the DCs using RNA-STAT 60 isolation protocol (Tel-Test, Friendswood, TX). Total RNA concentration was measured using a NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific). Reverse transcription of RNA into cDNA was synthesized from 800ng- 1µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, CA, USA) according to the manufacturers' instructions. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was initiated by iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) according to the manufacturer's instructions. The PCR reactions (20µl) were performed in a CFX-96 Bio-Rad C-1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA). Analysis of the data was completed with Bio-Rad CFX manager software version 2.1 (Bio-Rad Laboratories). All the PCR measurements were performed in triplicate. Following amplification, the specificity of the reaction was confirmed by cDNA melting curve analysis. Relative quantitation of the gene products was determined using the comparative *C*T method with data normalized to β-actin mRNA and calibrated to the average Δ*C*T of untreated controls (Lai et al., 2009). RNA primers used for the PCR analysis were designed using Primer3 software and

purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The sequences are listed in Table 3.1.

Table 3.1. Oligonucleotide primers used for Real-Time PCR

Primers		Sequences
IDO1	Forward	5'-GCACCAGAGGAGCAGACTAC-3'
	Reverse	5'-GATTTGGCAGAGCAAAGCCC-3'
TGF-β1	Forward	5'-GAGCCTGAGGCCGACTACTA-3'
	Reverse	5'-GGGTTCAGGTACCGCTTCTC-3'
Activin-βA	Forward	5'-GGAGGGCAGAAATGAATGAA-3'
	Reverse	5'-CCTTGGAAATCTCGAAGTGC-3'
ITGB8	Forward	5-'CGAGGAGTTTGTGTTTGTGG-3'
	Reverse	5'-CATCTGCCTGCTTCACACTC-3'
Nodal	Forward	5'-AGACATCATCCGCAGCCTAC-3'
	Reverse	5'-CCATGCCAGATCCTCTTGTT-3'
β-Actin	Forward	5'-GCATTGCTTTCGTGTAAATTATGT-3'
	Reverse	5'-ACCAAAAGCCTTCATACATCTCA-3'

Immunoblotting

DCs were harvested by centrifugation (540g, 4^{o}C for 5mins) using an Allegra X-15R centrifuge (Beckman Coulter) equipped with a SX4750A rotor. The cells were washed with cold PBS and the pellets lysed with 150µl 1 X SDS (sodium dodecyl sulfate-polyacrylamide) sample buffer (Tris.Cl 50mM pH 6.8, 2% SDS, 10% glycerol). The cell membranes were further disrupted by sonication for 10 secs (3 x) with a Sonic 60 Dismembrator (Fisher Scientific, Sunnyvale, CA) at 10W. The cell extract was boiled at 99^{o}C for 5mins and centrifuged at 14,000 RPM for 30secs. The supernatants were transferred to fresh micro-centrifuge tubes and beta-mercaptoethanol (BME; 0.74M) was added after determining protein concentration using DC Protein Assay (Bio-Rad), before storage at -20^{o}C. Approximately 40µg of the total protein from the DC cell extracts was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel. The separated proteins were transferred, from the gel, to polyvinylidene difluoride (PVDF) (0.45µm; EMD Millipore) or nitrocellulose membranes (0.45µm; Thermo Scientific), and probed with antibodies against, phosphorylated Smad 2/3 (1:1000; Cell Signaling), Smad2/3 (1:1000; EMD Millipore) IDO1 (1:2500; Abcam), and beta-actin (dilution 1:1000; Cell Signaling) was used as a loading control. The primary antibodies were probed by horseradish peroxidase-conjugated anti-rabbit (1:1000; Cell Signaling) or anti-mouse IgG (1:2000-1:5000; Sigma). The membranes were exposed to X-ray film (CL-XPosure, Thermo-Scientific) and protein expression was detected between CTB-INS treated and untreated conditions and expression intensity was analyzed and quantified by densitometry, using Image J software v. 1.48h. (Image J, NIH), between CTB-INS treated and untreated conditions.

Statistical Analysis

The data was analyzed for statistical significance using the GraphPad prism 5 (GraphPad Software, San Diego, CA). Welch's *t*-Test (unpaired *t*-Test with Welch's correction) was used for each pairwise comparison and one-way analysis of variance (ANOVA) was used for multiple group comparisons.

Results

Vaccination of Human moDCs with CTB-INS Induces Integrin αvβ8, TGF-β1 and activin-A mRNA Synthesis

TGF-β1 was shown to induce IDO1 expression in murine DC subsets (Belladonna et al., 2008; Pallotta et al., 2014; Pallotta et al., 2011). We therefore assessed the potential for CTB-INS to activate TGF-β1 gene transcription and select members of the TGF-β1 superfamily of cytokines known to utilize similar signaling pattern, specifically activin-A and NODAL. Treatment of healthy subject moDCs with CTB-INS resulted in significantly increased TGF-β1 mRNA expression, although mRNA levels declined 6 hours after vaccine addition (Fig. 3.2A). Activin-A expression increased significantly reaching ~20-fold increase in expression at 6 hours after CTB-INS addition (Fig. 3.2B). IDO1 expression increased significantly from the first hour of culture and IDO1 expression was substantially amplified to more than 120-fold after 6hours (Fig. 3.2D), confirming earlier published data of CTB-INS induction of IDO biosynthesis(Mbongue et al., 2015). However, NODAL gene expression after CTB-INS treatment of moDCs did not increase significantly from basal levels (Fig. 3.2C). Integrin αvβ8 was shown to be critical for TGFβ1 activation in maintaining immune homeostasis (Aluwihare et al.,

2009a; Travis et al., 2007b). Interestingly, CTB-INS upregulated integrin αvβ8 expression by more than 30-fold at 6 hours of moDC treatment (Fig. 3.2E).

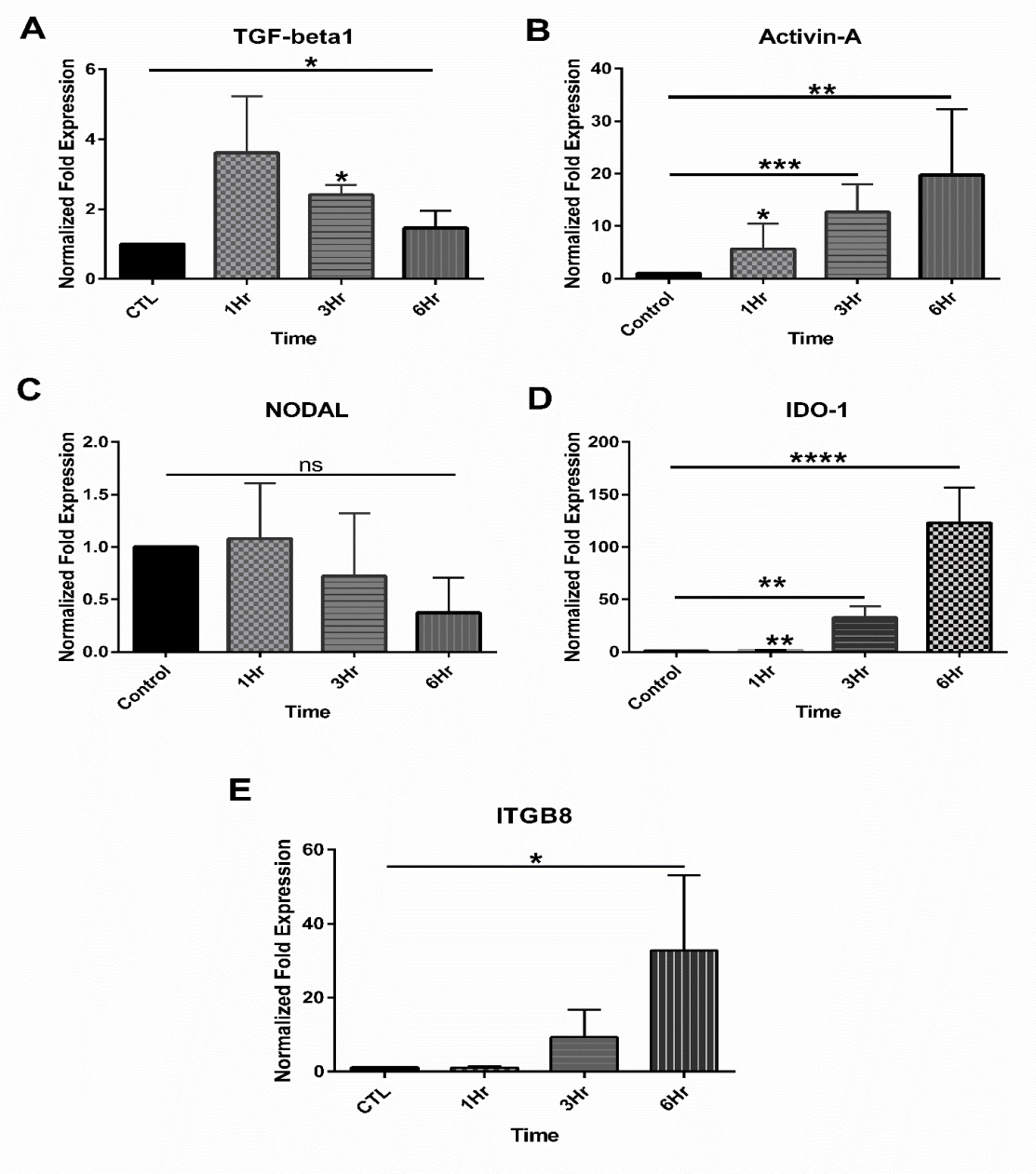

Figure 3.2. TGF-β1 superfamily and IDO1 mRNA synthesis in CTB-INS vaccinated moDCs. Healthy human moDCs were untreated (control), or treated with 5µg/ml of CTB-INS fusion protein. DC samples were harvested at 1hr, 3hr and 6hr after the addition of CTB-INS protein. The DCs were lysed and total RNA extracted for mRNA quantification normalized to β-Actin mRNA by real-time PCR. Fold change in the levels of: (**A**) TGF-β1 mRNA, (**B**) activin-βA subunit mRNA, (**C**) NODAL mRNA, (**D**) IDO1 and (**E**) Integrin β8 subunit mRNA, were normalized and presented relative to mRNA expression in untreated cells. Data for each gene represents the mean ± SD of four to six independent experiments. *p* values of treatment vs control was obtained using Welch's *t*-Test; ns, not significant; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001; *p* values on the bars were obtained by ordinary one-way ANOVA.

CTB-INS Induction of IDO1 Expression in Human moDCs is Independent of TGF-β1 or Activin-A Cytokine Expression

Next, we determined whether CTB-INS stimulated TGF-β cytokine production in order to induce IDO1 biosynthesis, through autocrine/paracrine signaling in human moDCs. To evaluate the effect of endogenous TGF-β induction by CTB-INS on IDO1 expression, we cultured DCs with pan-TGF-β neutralizing antibody in the presence of CTB-INS. IDO1 biosynthesis was not significantly reduced by antibody binding of endogenous TGF-β (Fig. 3.3A).

TGF-β propagates intracellular signals by inducing the phosphorylation of Smad2 and Smad3, which is the canonical signaling pathway for the ligand (Massagué, 2012). Assessment of the phosphorylation of Smad2/3 following TGF-β neutralization showed that deprivation of endogenous TGF-β did not inhibit phosphorylation of Smad2/3 in the CTB-INS treated DCs (Fig. 3.3B). This result suggests that CTB-INS stimulates Smad2/3 signaling independently of endogenous TGF-β1 levels. To confirm the validity of this experimental observation, the TGF-β neutralizing antibody was shown to effectively inhibit Smad2/3 phosphorylation in the presence of TGF-β1 stimulation (Fig. 3.3A, *Inset*).

Previously, TGF-β was shown to induce IDO expression in specific murine DC subsets (Belladonna et al., 2008; Pallotta et al., 2014; Pallotta et al., 2011). Based on these results, we evaluated the effect of exogenous TGF-β on human moDCs. Immature DC cultures were treated with increasing amounts of biologically active TGF-β1 and analyzed by Immunoblotting for detection of IDO1 expression. However, no IDO1 biosynthesis was detected in the TGF-β treated DCs (Fig. 3.3C), while, CTB-INS treated

DCs expressed high levels of IDO1 protein (Fig. 3.3C). The biological activity of the exogenous TGF-β1 was determined by measurement of Smad2/3 phosphorylation after TGF-β1 treatment. Phosphorylation of Smad2/3 increased in a dose dependent manner in response to exogenous TGF-β1 (Fig. 3.3D), showing that Smad2/3 signaling occurred in the presence of exogenous TGF-β1, although IDO1 expression was not induced.

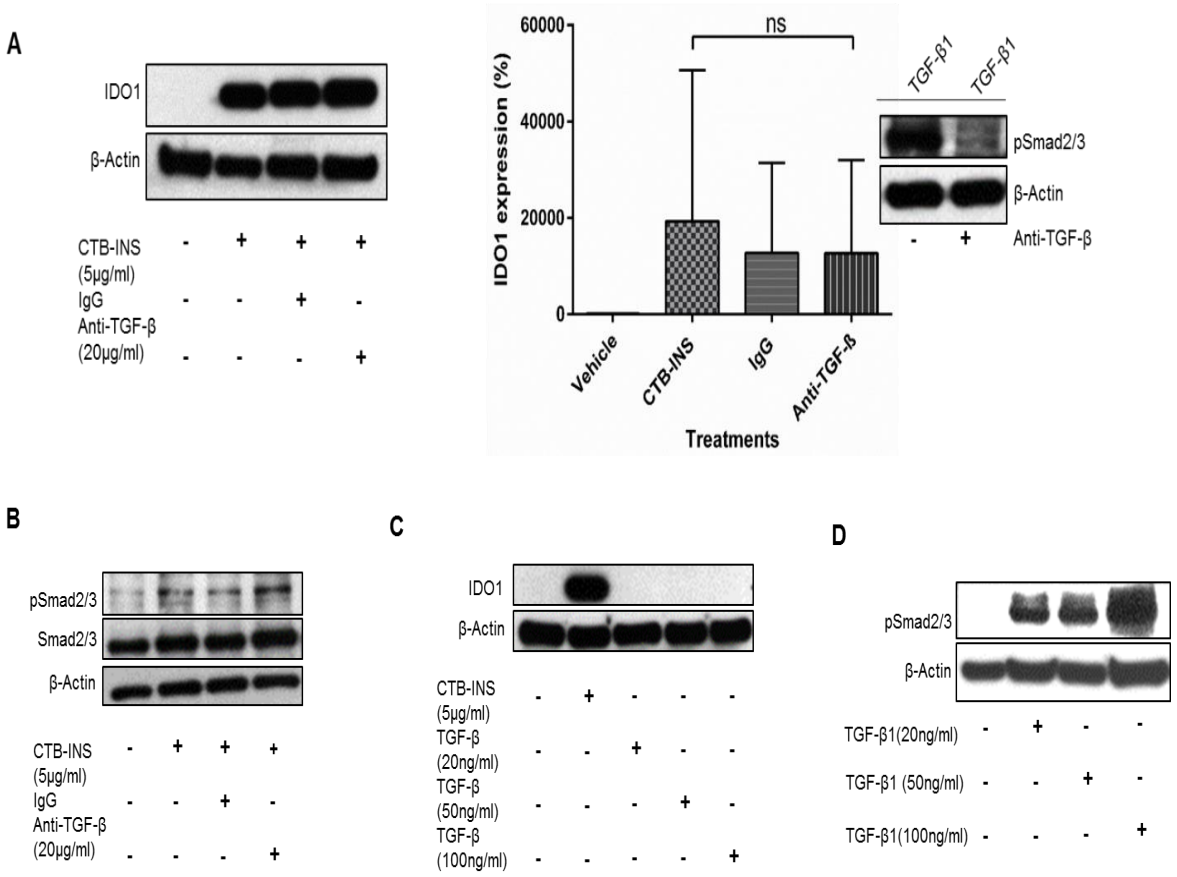

Figure 3.3. Effects of TGF-β on IDO1 biosynthesis in human moDCs. Panel (**A**) Immunoblot showing IDO1 expression of human moDCs, pre-incubated for 60 mins in the presence or absence of anti-TGF-β or isotype control prior to treatment with CTB-INS fusion protein or vehicle (PBS), for a period of 24 hours. β-actin was included as a control for sample loading. To the *right* of panel A is a graph showing the corresponding densitometry data (mean ± SD from three experiments; ns, not significant, obtained by Welch's *t*-Test for CTB-INS only vs CTB-INS+Anti-TGF-β) normalized to β-Actin, indicating IDO1 protein expression relative to normalized expression in the untreated cultures (i.e. cells exposed to vehicle only). Panel A graph *Inset*, confirms phospho-Smad2/3 biosynthesis in moDCs is stimulated with TGF-β (20ng/ml) and neutralized with anti-TGF-β antibody (20μg/ml) (**B**) A representative immunoblot of human moDCs pre-incubated with the anti-TGF-β neutralizing antibody and stimulated with CTB-INS for 30 mins prior to lysis and assessment of phosphorylated-Smad2/3 levels. Anti-Smad2/3 and anti-beta-actin antibody were used as loading controls. (**C**) Immunoblot of human peripheral blood moDCs treated with CTB-INS or increasing concentrations (20-100ng/ml) of TGF-β1 for 24 hours prior to assessment of IDO1 protein expression levels. (**D**) Immunoblot of phosphorylated Smad2/3 obtained from human moDCs treated for 30mins with increasing concentration of TGF-β for 30mins prior to total cell extraction. β-Actin was included as control for sample loading. Representative blots were taken from at least two independent experiments.

Activin-A is a member of the TGF-β superfamily that also signals via Smad2/3 proteins (Massagué, 2012; Xia and Schneyer, 2009). Considering that activin-A mRNA was upregulated (Fig. 3.2B) and phosphorylation of Smad 2/3 was increased in CTB-INS vaccinated human moDCs despite TGF-β neutralization (Fig. 3.3B), we investigated the possibility that CTB-INS stimulated endogenous production of activin-A resulting in the induction of IDO biosynthesis in human moDCs. Therefore, we repeated the experimental procedure by blocking endogenous activin-A biosynthesis with increasing concentrations of follistatin, a natural antagonist of activin-A that binds activin with high affinity (Hardy et al., 2015). Human moDC cultures were incubated +/- CTB-INS in the presence or absence of follistatin for 24 hours prior to assessment of IDO1 expression by immunoblot analysis. Follistatin neutralization of activin-A did not significantly suppress vaccine induction of IDO1 expression (Fig. 3.4A). In addition, cytokine neutralization of CTB-INS treated DCs with both TGF-β neutralizing antibody and follistatin, did not significantly decrease IDO1 protein expression (Fig 3.4A +Anti-TGF-β lane).

Based on earlier reports that, neutralization of activin by high concentrations (400ng/ml) of follistatin did not abrogate levels of activin-A stimulated in moDCs after 6 hours(Robson et al., 2007), we hypothesized that early biosynthesis of activin-A following CTB-INS stimulation of the moDCs might be responsible for induction of IDO1 expression. To test this hypothesis, we measured IDO1 mRNA by real-time PCR in vaccinated DC cultures incubated with or without follistatin, before and at 6 hours of culture. However, no significance difference in IDO1 expression was detected between cell cultures treated with or without follistatin (Fig. 3.4B), substantiating our previous observation that while activin-A is stimulated in CTB-INS treated DCs, the cytokine had

no effect on enhancement of vaccine stimulated IDO1 biosynthesis. To confirm these findings moDCs were treated with increasing concentrations of exogenous activin-A and IDO1 protein expression was assessed by immunoblot analysis. No IDO expression was detected (Fig. 3.4C). These novel findings suggest that TGF-β and activin-A cytokines exert no significant effect on the induction of IDO1 in human moDCs. Interestingly, as shown with TGF-β1, exogenous activin-A effected Smad2/3 signaling, as detected by increased phosphorylation of Smad2/3 after 30mins treatment with activin-A (Fig. 3.4D).

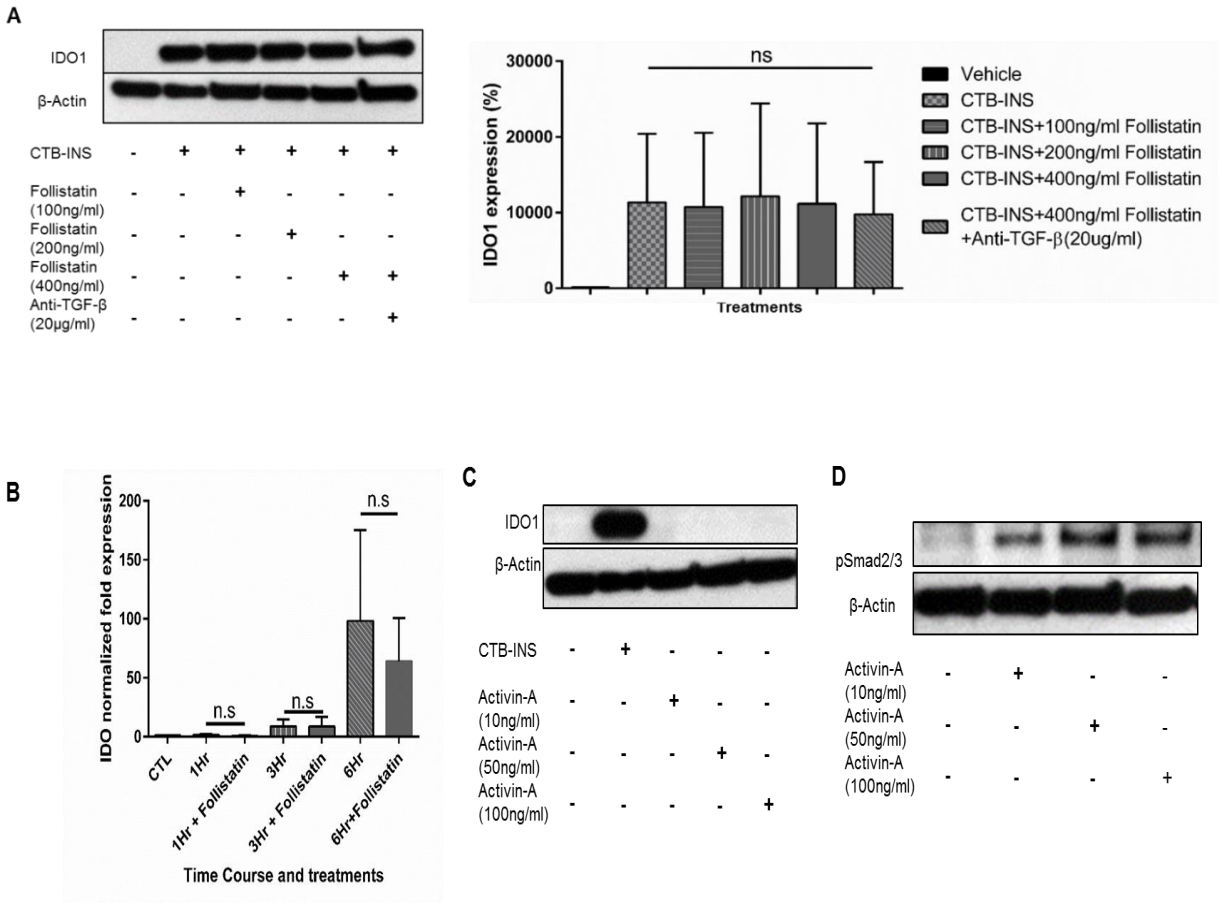

Figure 3.4. Effects of activin-A on the induction of IDO1 in moDCs. Panel (**A**) Immunoblot of IDO1 levels in human moDCs incubated with vehicle (PBS) or CTB-INS fusion protein incubated for 24hours in the presence of increasing concentrations (100-400ng/ml) of the activin-A inhibitor, follistatin, alone or follistatin (400ng/ml) plus pan-TGF-β neutralizing antibody (20µg/ml). β-actin was used as a loading control. Densitometry data (mean ± SD) indicating IDO1 protein expression in the samples from two independent experiments, normalized to β-Actin, is shown on the *right;* expression in the respective samples is relative to normalized expression in the untreated cultures (i.e. cells exposed to vehicle only). (**B**) Graphic representation of normalized fold change of IDO1 mRNA, relative to mRNA expression in control cells, of human moDCs treated with vehicle (control) or with CTB-INS (5µg/ml) at 1hr, 3hr and 6hr time points in the absence or presence of follistatin (400ng/ml). The moDCs were harvested and lysed and RNA was extracted for mRNA quantification by real-time PCR, relative to β-Actin mRNA. Data represents mean ± SD of three independent experiments. *p* values of paired comparison was obtained using Welch's t-Test; ns, not significant. (**C**) Immunoblot of IDO1 expression in moDCs treated with CTB-INS or increasing concentrations (10-100ng/ml) of activin-A for 24 hours. (**D**) Immunoblot of phosphorylated Smad2/3 in human moDCs untreated or treated with increasing concentration of activin-A for 30mins, after which total cell extracts were obtained for analysis. β-Actin was used as loading control. Representative blots are of at least two independent experiments.

CTB-INS Vaccine Activates Smad2/3-Independent Kinase Signaling to Stimulate IDO1 Biosynthesis in Human moDCs

Previous studies have demonstrated TGF-β-independent activation of Smad2/3 signaling pathway in rat vascular and renal cells (Rodríguez-Vita et al., 2005; Yang et al., 2009). Therefore, we investigated whether CTB-INS activated Smad2/3 signaling to induce IDO1 expression independently of TGF-β. To accomplish this goal, we examined the effect of a bioactive small molecule inhibitor of TGF-β signaling, RepSox, on CTB-INS induction of IDO1 expression in human moDCs. RepSox blocks TGF-β1 signaling by binding to and inhibiting phosphorylation of the TGF-β type I serine/threonine kinase receptor, ALK5/TβRI, thereby blocking phosphorylation of Smad2/3 (Gellibert et al., 2004; Ichida et al., 2009). Human moDC cultures were treated with CTB-INS in the presence or absence of the inhibitor. Western blot analysis of IDO1 protein expression showed that IDO1 biosynthesis was significantly decreased in the presence of RepSox (Fig. 3.5A). While RepSox (25μM) inhibited phosphorylation of Smad2/3 in the DCs (Fig. 3.5B), treatment of DCs with lower concentrations (12.5μM) of RepSox, did not decrease IDO1 protein expression (Fig. 3.5C), suggesting IDO1 suppression by RepSox is concentration dependent.

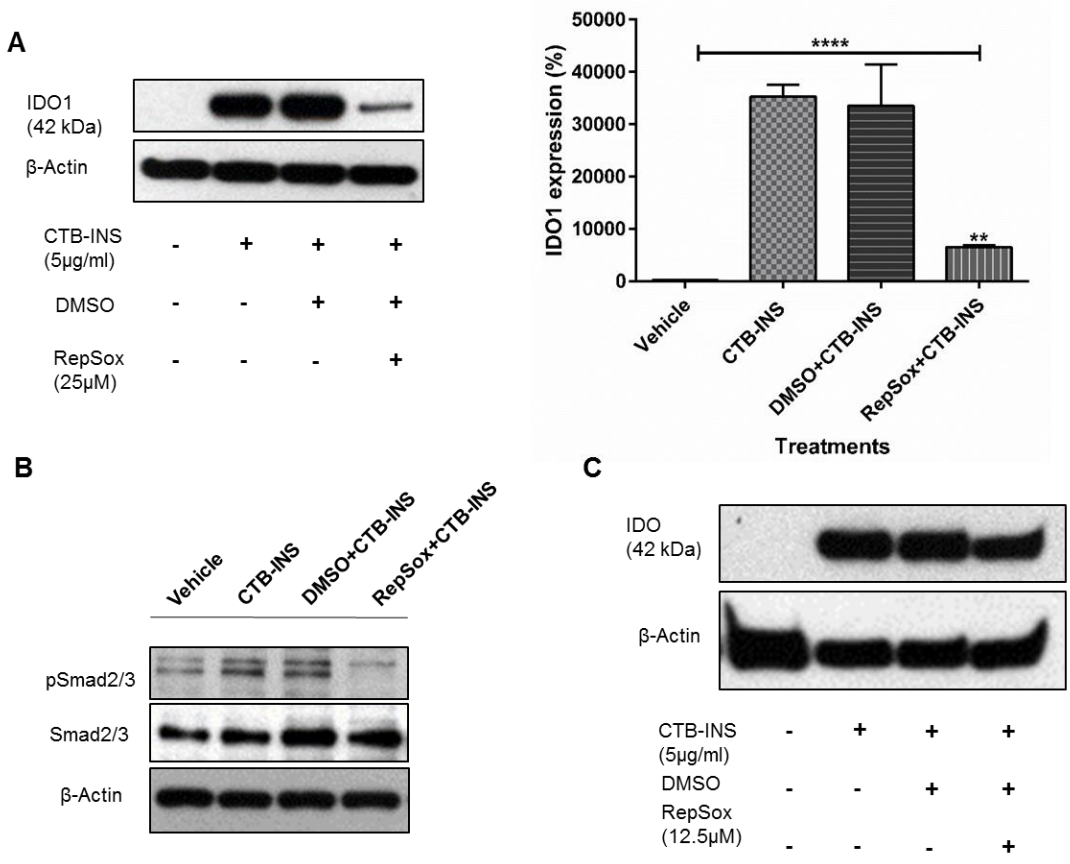

Figure 3.5. Arrest of IDO1 protein synthesis in CTB-INS vaccinated moDCs by the kinase inhibitor RepSox. Panel (**A**) A representative immunoblot analysis of IDO protein synthesis in moDCs incubated with RepSox (25μM) or DMSO (vehicle) for 1hr prior to 24hrs incubation with CTB-INS (5μg/ml). The graph at the *right* represents pooled densitometry data from 3 independent experiments (mean ± SD; $^{**}p < 0.01$ obtained by Welch's *t*-Test for CTB-INS only vs CTB-INS + RepSox DCs*;* $^{****}p < 0.0001$ obtained by ordinary one-way ANOVA comparing relative IDO expression between groups), normalized to beta-Actin. IDO1 expression in vaccinated moDCs is relative to normalized IDO1 expression in untreated cells. (**B**) Representative immunoblot of phospho-Smad2/3 biosynthesis in moDCs incubated with RepSox (25μM) or DMSO (vehicle) for 1hr followed by treatment with CTB-INS for 30mins, after which total cell extracts were subjected to immunoblot analysis. Anti-Smad2/3 and anti-beta-actin antibody were used as loading control. (**C**) Representative Immunoblot analysis of IDO1 expression in moDCs treated with RepSox (12.5μM) followed by treatment with CTB-INS (5μg/ml).

DCs were then treated with the small molecule serine/threonine kinase inhibitor, SB-431542 (10μM), known to selectively block the type I receptor kinases of TGF-β, activin and NODAL, resulting in the inhibition of Smad2/3 signaling (Inman et al., 2002). Although Smad2/3 signaling was inhibited (Fig. 3.6B), the application of SB-431542 did not suppress CTB-INS-stimulation of IDO1 protein synthesis (Fig. 3.6A). Interpretation of this data suggests that CTB-INS induces IDO1 protein expression in human moDCs independently of the Smad2/3 signaling pathway.

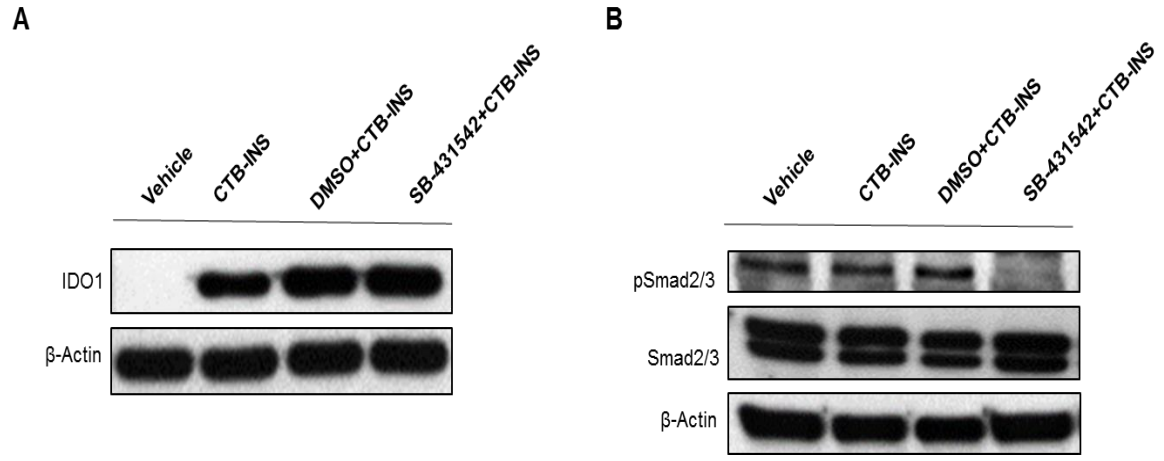

Figure 3.6. Effect of TβRI kinase inhibition on IDO1 protein synthesis and Smad2/3 phosphorylation in CTB-INS induced moDCs. Panel **(A)** Representative immunoblot of IDO1 expression in moDCs after pretreatment with vehicle (DMSO) or the serine/threonine kinase specific inhibitor SB-431542 (10μM) for 1 hour, followed by 24 hour incubation of the moDCs with CTB-INS (5μg/ml). Anti-β-Actin Ab was used as the loading control. (**B**) Immunoblot of phospho-Smad2/3 expression in moDCs pretreated for 1 hour with DMSO or SB-431542 followed by 30mins incubation with CTB-INS (5μg/ml). Immunoblot analysis was conducted for Smad2/3 expression and blots were stripped and re-probed with anti-β-Actin Ab as a control for equivalent sample loading. The data presented is representative of two independent experiments.

Discussion

Signaling pathways responsible for CTB-INS induction of IDO1 biosynthesis are poorly defined in human moDCs. Therefore, we examined the role of TGF-β superfamily members in CTB-INS vaccine upregulation of IDO1 biosynthesis in human DCs. In this study, we present experimental evidence that CTB-INS stimulation of IDO1 biosynthesis in human moDCs may occur through signaling pathways that are potentially dependent on the activation of kinases, but independent of members of TGF-β superfamily kinase receptors.

Our experimental data indicates that CTB-INS fusion protein vaccine upregulates TGF-β1 and activin-A mRNAs in human moDCs, and stimulates TGF-β signaling, further confirmed by detection of CTB-INS stimulation of Smad2/3 phosphorylation. Activin-A, a member of the TGF-β superfamily, regulates human moDC and modulates DC proinflammatory cytokine profile (Robson et al., 2007). Similarly, TGF-β signaling is a critical factor that contributes to maintaining immune cell homeostasis. Absence of TGF-β1 signaling in DCs leads to multi-organ autoimmune inflammation or autoimmune conditions (Boomershine et al., 2009; Ramalingam et al., 2012). In addition, TGF-β1 signaling is required for induction of tolerogenic DCs (Lan et al., 2012). It is plausible that the upregulation of these immunoregulatory factors are additional mechanisms by which CTB-INS modulates moDC towards a tolerogenic phenotype as previously demonstrated by our laboratory(Odumosu et al., 2011b).

Based on the observation that CTB-INS stimulated an increase in TGF-β or activin-A production, we speculated that autocrine/paracrine signaling of TGF-β or activin-A could be directly or indirectly involved in the upregulation of IDO1

biosynthesis, as previously suggested (Belladonna et al., 2008). However, our experimental data demonstrates that IDO1 biosynthesis in CTB-INS treated human moDCs is independent of endogenous TGF-β or activin-A. In experiments involving murine CD8-, plasmacytoid DCs (pDCs), and bone-marrow-derived DCs, the addition of biologically active TGF-β induced IDO biosynthesis and converted or maintained the DCs in a tolerogenic state (Belladonna et al., 2008; Pallotta et al., 2014; Pallotta et al., 2011; Song et al., 2014). However, in our experiments with human moDCs, the exogenous addition of bioactive TGF-β or activin-A cytokines did not induce IDO1 biosynthesis, thereby, further excluding potential, non-Smad TGF-β/activin-A signaling in the induction of IDO1 synthesis in human moDCs (Derynck and Zhang, 2003; Mu et al., 2012). While the molecular details are unknown, our data suggests TGF-β induction of IDO in immune cells may be species-specific, further confirming the findings of others that differences in immunological responses may occur between mammalian species (Cheng et al., 2015; Schmidt et al., 2010). Otherwise, the differences between our results and others (Belladonna et al., 2008; Pallotta et al., 2014; Pallotta et al., 2011; Song et al., 2014), may be a function of differences in DC ontogeny. To our knowledge this is the first time TGF-β family cytokine involvement in the induction of IDO1 biosynthesis in human moDCs has been documented and provides insight into signaling pathways involved in the induction of IDO1-expressing tolerogenic human moDCs, with the view of potential therapeutic applications. Further, we have previously demonstrated that CTB-INS activates tumor necrosis factor (TNF) receptor members in the modulation of IDO1 induction (Kim et al., 2016). However, additional research is required to clarify the mechanism of chimeric vaccine induction of IDO1 for immunological tolerance.

The serine/threonine kinase specific inhibitor, SB-431542 (Inman et al., 2002), did not block CTB-INS induction of IDO1 biosynthesis in moDCs (Fig. 3.6A), reinforcing the idea that CTB-INS induction of IDO1 biosynthesis in human moDCs may be independent of Smad2/3 signaling. Interestingly, data from the use of the kinase inhibitor, RepSox, is consistent with previous studies showing RepSox may inhibit the activity of serine/threonine kinases of other signaling pathways at concentrations greater than 16μM (Gellibert et al., 2004). Although RepSox blocked phosphorylation of the TGF-β type I kinase receptor, ALK5/TβRI, the application of RepSox at the concentration of 25μM may have inhibited kinase activity integral to other signaling mechanisms including the non-canonical NF-κB signaling pathway shown to be activated by CTB-INS in inducing IDO1 biosynthesis and activity in human moDCs (Kim et al., 2016). Thus, the mechanism underlying RepSox suppression of CTB-INS-mediated IDO1 biosynthesis may require further clarification.

CTB-INS stimulation of integrin αvβ8 expression reported in this study may represent a novel mechanism for CTB-INS induction of tolerance in human moDC via the activation of TGF-β1. The activation of latent TGF-β1 is critical for its immunoregulatory functions which include inhibition of DC-mediated immune responses (Hammer and Ma, 2013; Worthington et al., 2012). TGFβ controls and limits the differentiation of DCs at autoimmune-inflammatory sites and TGFβ–secreting DCs stimulate Treg proliferation (Ghiringhelli et al., 2005; Speck et al., 2014). In addition, TGFβ skews Th1/Th2 balance towards a Th2 profile (Becker et al., 2006; Maeda and Shiraishi, 1996). Studies have indicated a role for integrin αvβ8 in the suppression of autoimmunity by activation of TGFβ1. Mice with gene deletions of integrin αvβ8 or with

DCs deficient in integrin αvβ8 expression, developed immune dysfunction due to failure to activate TGFβ1 (Aluwihare et al., 2009a; Travis et al., 2007b). Further, specific intestinal subsets of murine DCs required the expression of integrin αvβ8 to activate TGF-β1 and generate regulatory T cells for the induction of tolerance to intestinal antigens (Païdassi et al., 2011; Worthington et al., 2011). It should be noted that in earlier reports of CTB-INS suppression of diabetes insulitis in NOD mice, the CTB-autoantigen fusion proteins were orally administered to the mice (Carter et al., 2006b). It is plausible that the mechanism of immune suppression in these early studies involved tolerance induction of intestinal DCs by integrin αvβ8 upregulation. The additional observation by others of integrin αvβ8 expression and requirement in immunosuppressive functions of Tregs, indicates the imperative for further investigation of the significance of integrin αvβ8 upregulation reported here for the first time in human moDCs (Worthington et al., 2015).

In conclusion, the present study identifies novel tolerogenic functions of CTB-INS fusion protein vaccine. CTB-INS stimulates both TGF-β and activin-A biosynthesis as well as Smad2/3 phosphorylation and induces integrin αvβ8 expression in human DCs. Although our data demonstrates that CTB-INS upregulation of TGF-β superfamily proteins is not related to the induction of IDO1 biosynthesis in human moDCs, it is likely that CTB-INS fusion protein activates multiple mechanisms for tolerizing DCs. Taken together, these observations suggest CTB-INS may employ TGF-β synthesis and activation in the induction of tolerance in human moDCs. Experiments to further validate the present experimental findings and to understand the mechanisms involved will help to

establish the vaccine function in moDCs and will provide a basis for determination of vaccine efficacy for induction of immunological tolerance in T1D.

Disclosures

The authors have no financial conflict of interest.

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CHAPTER 4

CHOLERA TOXIN B SUBUNIT-PROINSULIN VACCINE INDUCTION OF TGF-BETA IN HUMAN DENDRITIC CELLS

Introduction

Dendritic cells (DCs) are professional antigen presenting cells that coordinate the adaptive immune cells towards an immune response or to mediate immunological tolerance by mechanisms such as T cell deletion or anergy, Th2 skewing and induction of regulatory T cells (Tregs)(Danese et al., 2008) (Shortman and Naik, 2007). First described by Steinman in 1973, DCs are a widely distributed heterogeneous population of cells classified by ontogeny, phenotypic characteristics and functional attributes, with specialized functions (Liu and Cao, 2015; Steinman and Idoyaga, 2010). DCs modulate key immune processes including autoimmunity, which is an immune response characterized by failure of tolerance to self-antigens(Benson et al., 2010) . Tolerogenic DCs hold promise as potential therapy for autoimmune diseases and can be characterized by an immature or semi-immature phenotype demonstrated by expression of low costimulatory molecules, high expression of anti-inflammatory cytokines, and decreased expression of pro-inflammatory cytokines and induction of hyporesponsive CD4+ T cells (Hilkens et al., 2010; Schinnerling et al., 2015; Ten Brinke et al., 2015). Therefore, understanding the mechanisms that regulate DC homeostasis is crucial to harnessing their therapeutic potentials (Merad and Manz, 2009).

TGFβ is a pleiotropic cytokine affecting numerous cellular and immune processes, especially playing a central role in T cell development, homeostasis, tolerance and differentiation (Guerder et al., 2013). The immunosuppressive action of TGFβ on

dendritic cells can promote or inhibit DC-mediated immune responses (Worthington et al., 2012). TGFβ controls and limits the differentiation of DCs at autoimmune-inflammatory sites (Speck et al., 2014). TGF-β was shown to modulate homeostasis of DCs of the epidermis, also called Langerhans cells(LC) by suppression of maturation states of the LC (Kel et al., 2010). TβR1-deficient LCs significantly upregulated expression of CD86 and CCR7 compared to the wild-type, indicating switch in phenotype from immature to mature DCs and migratory potential of the DCs were also enhanced(Kel et al., 2010). Further, TGF-β-conditioned bone marrow-derived DCs (BMDCs) co-transplanted with islets, prolonged their survival and decreased T-cell infiltration of the graft islet (Thomas et al., 2013).The TGF-β-exposed DCs displayed low costimulatory molecule (CD80 and CD86) expression and reduced pro-inflammatory cytokine (IL-12 p70, IL-6, Il-1β, TNF-α) profile, maintaining IL-10 production, and poorly activating antigen-specific T cells cells (Thomas et al., 2013).

TGF-β is a tightly regulated cytokine that is secreted and maintained as an inactive precursor that requires activation to become functional (Hammer and Ma, 2013; Mu et al., 2002). TGF-β is synthesized as a homodimeric pro-protein consisting of the active TGF-β1 covalently linked to the latency associated peptide (LAP). After enzymatic cleavage in the Golgi, the LAP remains attached to the mature TGF-β1 by non-covalent bonds, and hinders the binding of the active TGF-β1 to its cognate receptors (Li et al., 2006b; Wipff and Hinz, 2008; Worthington et al., 2012). Integrin αv family recognize specific arginine-glycine-aspartate RGD sequences present on LAP by which they bind to the latent TGF-β complex and liberate the active TGF-β to interact with its receptors (Song et al., 2016). Integrin αvβ8 has been well characterized to be critical for TGF-β1

activation and in maintaining immune homeostasis; mice whose leucocytes are deficient in integrin αvβ8 develop a wasting inflammatory disorder (Aluwihare et al., 2009b; Mu et al., 2002; Travis et al., 2007b).

Type 1 diabetes mellitus (T1D) is a tissue-specific autoimmune disease where autoreactive T cells mediate progressive destruction of the islet cells of the pancreas resulting in insulin deficiency that can be fatal in the absence of therapy (Segovia-Gamboa et al., 2014). Tolerogenic DCs have been explored as therapy for autoimmune diseases and have been demonstrated to reverse diabetes in the non-obese diabetic (NOD) mice which is the murine model for T1D (Segovia-Gamboa et al., 2014). Previous experiments in our laboratory have shown that a chimeric fusion protein composed of the cholera toxin B subunit conjugate to the diabetes autoantigen proinsulin (CTB-INS) can suppress the activation of dendritic cells through suppression of DC costimulatory molecules, CD86 and CD80, and pro-inflammatory cytokines, and induction of an anti-inflammatory cytokine profile; a response that is significant in preventing the onset of type 1 diabetes (T1D) (Odumosu et al., 2011b). Recently our laboratory showed that CTB-INS upregulates gene expression of integrin αvβ8, TGF-β and increased Smad 2/3 signaling (Chapter 3). In this preliminary study we investigate the expression of integrin αvβ8 and TGF-β protein expression as a mechanism for CTB-INS induction of tolerance in human moDCs.

Materials and Methods

Monocyte Isolation and DC Culture

Experiments on peripheral blood mononuclear cells (PBMCs) were performed ex-

vivo, with aphaeresis blood provided by the Life Stream Blood Bank (San Bernardino, CA) according to Loma Linda University IRB requirements. Blood donor information was anonymized before initiation of the study. Human monocytes were isolated from peripheral blood, after red blood cell lysis, using LS separation columns and anti-CD14 magnetic MicroBeads (Miltenyi Biotec) as previously described (Mbongue et al., 2015) and maintained in culture at a density of 5×10^5 to 1×10^6 cells/ml. Immature DCs were differentiated from CD14 positive monocytes by incubation for 6 days in RPMI 1640 medium (Hyclone, GE Healthcare Life Sciences) containing 10% FBS and supplemented with human GM-CSF (50ng/ml) and human IL-4 (10ng/ml) (Miltenyi Biotec). Half the medium was replaced with fresh medium every 2 days being careful not to dislodge the cells from the substrate.

Synthesis and Isolation of CTB-INS Fusion Protein

CTB-INS fusion protein was synthesized in the *E. coli* expression vector, strain BL-21 (DE3) pLysS (Invitrogen, Carlsbad, CA) and purified as previously described (Mbongue et al., 2015; Odumosu et al., 2011b). Briefly, CTB-INS protein synthesis in the bacterial culture was stimulated with 2.0mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG), (Sigma Chemical Co. St Louis, Mo) at 3 hours of culture. Bacteria cell culture was harvested at density of 0.2-0.4 $O.D._{600}$, and the his-tagged CTB-INS protein was isolated from the bacterial cell homogenate using Maxwell Model 16 robotic protein purification system (Promega, Inc.) according to the manufacturer's instructions. The elution buffer was removed by dialysis at 4°C in phosphate buffered saline (PBS) and the purity of the isolated protein was determined by polyacrylamide gel electrophoretic

mobility analysis followed by immunoblotting (Western) with anti-Cholera Toxin as the primary antibody (Sigma, Inc).

Dendritic Cell Treatments

The differentiated human moDCs (assessed by observation of dendrite formation using phase contrast microscopy) were incubated with 5µg/ml of CTB-INS protein or vaccine vehicle (control) for 1hour, 3hours, and 6hours time points, or at separate experiments, for 24hours.

TGF-β1 ELISA

The TGF-β1 cytokine concentration in DC culture supernatants was assessed using the Human/Mouse TGF-β1 ELISA Ready-Set-Go kit (2^{nd} generation) (eBioscience) according to the manufacturer's protocol. Culture supernatants of human moDC cultures obtained from untreated DCs and DCs treated with CTB-INS for 1, 3, 6, and 24 hours were collected and immediately stored at -80°C until further analysis. A 100µl volume of the supernatants of each sample, was analyzed by the ELISA in duplicates. Active TGF-β1 induced by CTB-INS was assessed by assay of the free TGF-β1 present in the cell culture supernatants. Total TGF-β1 was obtained by acid activation of cell culture supernatants to release TGF-β1 to the immunoreactive form. Briefly, samples were activated by 1N HCl for 10 minutes and neutralized by 1N NaOH as directed by manufacturer of ELISA kit. Acidified samples were diluted three fold with manufacturer dilution buffer before assay. Controls were run to determine baseline concentrations of TGF-β1 present in culture media.

Flow Cytometry

Adherent and suspended DC were harvested from treated and untreated cell cultures, washed with PBS and labelled with fluorescent antibodies conjugated to DC cell surface targets. Antibodies and reagents used were anti-human CD11c PE, HLA-DR PerCP (Becton Dickinson), CD14-FITC (Biolegend), Human integrin β8-APC (R and D Systems), Fixable Viability Dye eFluor 450 (eBioscience). Samples were analyzed using Miltenyi MACSQuant flow cytometer (Miltenyi Biotec), and the data analyzed using FlowJo software (Tree Star, Ashland, OR). DCs were gated by Forward Scatter Area and Side Scatter Area (FSC-A/SSC-A), and cells with $CD14^{low}$ and $CD11c^{hi}$ phenotype were gated as DCs.

Statistical Analysis

Data analysis was performed using GraphPad prism 5 (GraphPad Software, San Diego, CA). Welch's *t*-Test (unpaired *t*-Test with Welch's correction) was used for each pairwise comparison and one-way analysis of variance (ANOVA) was used for multiple group comparisons.

Results

CTB-INS Induced Increased Expression of Active TGF-B1 in Vaccinated DCs

We have examined the effect of CTB-INS on increased biosynthesis of TGF-β1 mRNA in moDCs (Chapter 3). To better clarify the mechanism by which CTB-INS induced tolerance, preliminary study for TGF-β1 cytokine expression was conducted. Active TGF-β1 expression increased as analyzed by ELISA, however there was great

variability between subjects assessed and increase in active TGF-β1 was not significant (Fig. 4.1A). The increase in active TGF-β1 was highest at 3 hours of CTB-INS treatment and correlated with TGF-β1 mRNA biosynthesis in response to CTB-INS (Chapter 3). Total TGF-β1 did not vary much between treated and untreated moDCs, due to variability in the samples, as observed with assay of active TGF-β1 (Fig. 4.1B).

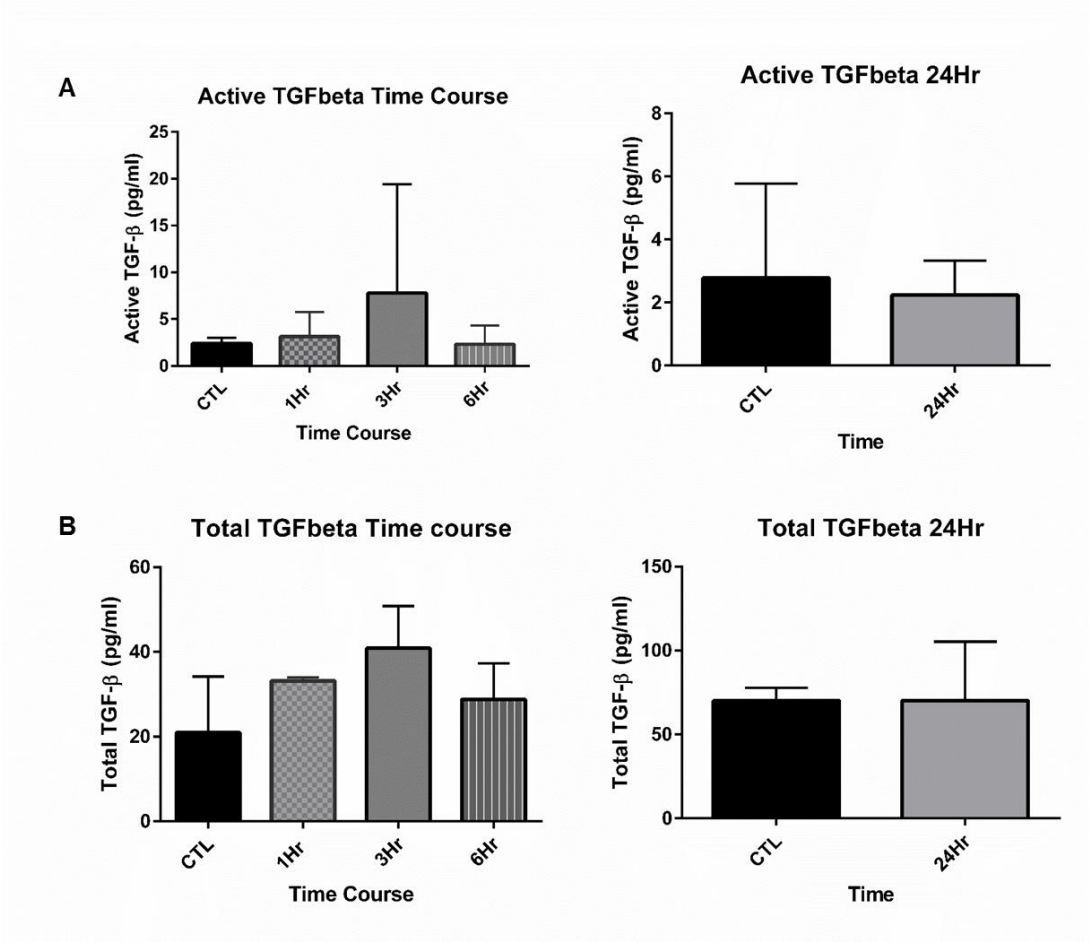

Figure 4.1. TGF-β1 cytokine expression in CTB-INS treated human moDCs. Human moDCs were cultured and treated with vaccine vehicle (dialysis buffer; CTL) or 5µg/ml of CTB-INS for 1hour, 3hours, 6hours, and 24hours. Thereafter, supernatants of cell cultures were obtained for ELISA quantification of TGF-β1 cytokine expression. Panel (A) graph of active TGF-β1 release in 1, 3, 6hr of CTB-INS time course treatment with cytokine expression at 24hr on *right* of panel. Panel (B) is total TGF-β1 in time course and 24hr treatments. Data are presented as mean ± SD from 3 independent experiments.

Furthermore, following increased gene expression of the known TGF-β1 activator, integrin αvβ8 (Chapter 3), CTB-INS treated moDCs were recovered after 24hours and labelled for cell surface analysis of integrin αvβ8 expression by flow cytometry. Integrin αvβ8 was increased in CTB-INS treated moDCs versus untreated moDCs (Fig. 4.2A and B).

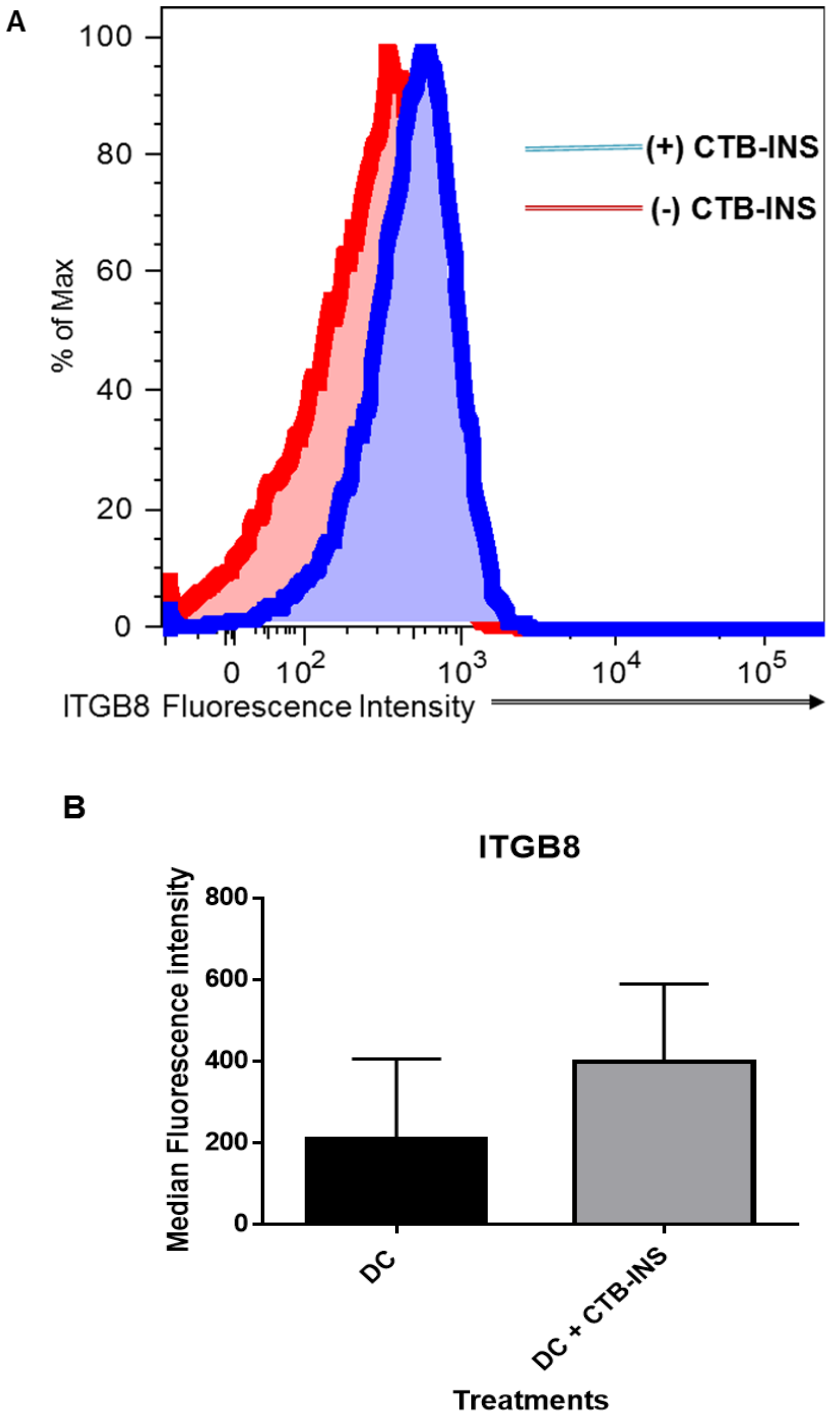

Figure 4.2. Integrin αvβ8 (ITGB8) expression in moDCs. Human moDCs were treated with 5µg/ml CTB-INS fusion protein vaccine for 24hrs and analyzed by flow cytometry to determine expression of integrin αvβ8. Untreated cells were used as control experiment. Data of mean fluorescence intensity (MFI) is represented here for two experiments. (A) Histogram overlay depicts expression values of integrin αvβ8 in moDCs with and without treatment of CTB-INS. (B) The graph compares the MFI of integrin expression in the treated DCs with the untreated moDCs.

Discussion

This chapter documents preliminary studies on the effect of CTB-INS fusion protein on TGF-β1 activation. We observed that CTB-INS upregulates active TGF-β1 expression. TGF-β1 activation is crucial for its biological functions, therefore increase of active TGF-β1 expression may be more significant than total or latent TGF-β1 production as observed in the present study (Hasegawa et al., 2004). Although these preliminary indications of increased active TGF-β1 were not statistically significant, an increase in the number of subjects sampled may increase the power of the study and yield more replicable data. Previous studies have observed the challenge of measuring TGF-β1 bioactivity, as only a few cells are able to secrete significant amounts of measurable active TGF-β1 in response to appropriate treatments (Glick et al., 1989; Mazzieri et al., 2000). The absence of abundant active TGF-β1 in the cell culture may not be indicative of lack of TGF-β1 activation, but may be due to either TGF-β1 activation occurring on the cell surface as a result of TGF-β1 binding to transmembrane cell surface proteins (Dennis and Rifkin, 1991; Mazzieri et al., 2000; Munger et al., 1999b), or due to active TGF-β1 removal from the cell culture solution as it binds to its cognate receptors, allowing only minute release of active TGF-β1 to the culture media (Mazzieri et al., 2000).The use of more sensitive assays like the Mink lung epithelial cells luciferase assay (Mazzieri et al., 2000), may yield more productive measurement of bioactive TGF-β1, than analysis by ELISA.

The analysis of integrin αvβ8 cell surface protein did not yield dramatic levels of expression in CTB-INS treated moDCs. Increasing sample size by repeating the experiments will be necessary to establish the validity of these preliminary findings. One

reason for the lack of statistically significant increase of Integrin αvβ8 during flow-cytometric analysis in CTB-INS treated DCs may be related to the lack of ample amount of active TGF-β1 in the DC culture stated above: LAP of latent TGF-β1 is a ligand for Integrin αvβ8. The binding of Integrin αvβ8 to LAP of the latent TGF-β1 in DCs may have impeded availability of integrin αvβ8 for cell surface analysis by flow cytometry (Cambier et al., 2005; Munger et al., 1999a) .

In conclusion, our preliminary studies suggest a role for TGF-β1 and integrin αvβ8 in the mechanism by which CTB-INS exerts tolerogenic functions on human moDCs. Further analysis of CTB-INS induction of integrin αvβ8 on moDC immunosuppression, that include CTB-INS suppression of DC costimulatory molecules CD80 and CD86 and induction of regulatory T cells (Tregs), will help to assess the significance of integrin αvβ8 upregulation and TGF-β1 activation in human moDCs.

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CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

In this body of work we sought to elucidate the mechanisms by which CTB-INS induces tolerogenicity of DCs as a means of evaluating its efficacy for clinical applications for therapy in autoimmune diabetes, and to understand the mechanistic processes involved in the induction of DC tolerance for broader application in tissue specific and systemic autoimmune disease conditions and immunosuppressive mechanisms involved in tissue rejection and cancer.

Chapter 1 discusses therapeutic strategies for autoimmune diseases and highlights current findings that indicate the prospects of tolerogenic DCs in the fight against immune dysregulation. This review establishes the need to identify key regulators or biomarkers of DC tolerogenicity so as to enable the generation and maintenance of tolerogenic DCs. Known indicators of DC tolerogenicity are further discussed and studies with the immunosuppressive chimeric CTB subunit autoantigen conjugate (CTB-INS) fusion protein are introduced. CTB-INS and its various autoantigen fusion proteins may induce tolerogenesis along multiple pathways. Previous studies in our laboratory have shown that CTB-INS and other CTB conjugated autoantigen fusion proteins can prevent and treat diabetes insulitis in the non-obese diabetes (NOD) mice (Carter et al., 2006b), prevent costimulatory molecule expression and DC activation of human moDCs (Odumosu et al., 2011a; Odumosu et al., 2011b), suppress pro-inflammatory cytokine expression, enhance anti-inflammatory cytokine profiles, modulate anti-inflammatory T cell morphogenesis (Odumosu et al., 2011b) (Odumosu, *et al.*, unpublished), and induce

biosynthesis of the immunoregulatory molecule, IDO1(Mbongue et al., 2015). Chapter 2 initiates investigation into the mechanism by which CTB-INS induces IDO biosynthesis in human moDCs and demonstrates the involvement of the non-canonical NF-κB pathway in CTB-INS-mediated IDO induction (Kim et al., 2016). TGF-β1 was shown to employ activation of the non-canonical NFκB pathway to stimulate IDO biosynthesis in murine DCs (Belladonna et al., 2008). Therefore, Chapter 3 documents our investigation into CTB-INS tolerogenic mechanisms by examination of the role of TGF-β1 in CTB-INS-mediated IDO biosynthesis in human moDCs. This chapter shows that CTB-INS functions in DCs by upregulating TGF-β1 expression and by stimulation of Smad2/3 signaling; however, no relationship between CTB-INS-induced TGF-β1 and IDO biosynthesis is identified. Further, identification of CTB-INS induction of immunoregulatory proteins activin-A and integrin αvβ8, are documented. Lastly, based on the potent immunosuppressive functions of TGF-β1 (Shurin et al., 2013), in Chapter 4 we continue to investigate the nature of CTB-INS upregulation of TGF-β1 and its biological activator, integrin αvβ8. The immunological implications of CTB-INS induction of integrin αvβ8 in human moDCs, will require elucidating the role of integrin αvβ8 in CTB-INS inhibition of DC costimulatory molecule expression and induction of regulatory T cells (Tregs).

Future Directions

Specific Aim 1

TGF-β1 is a potent immunosuppressive cytokine but requires activation to exert its biological functions (Li et al., 2006b). Integrin αvβ8 integrin is critical for TGF-β1

activation and is the only integrin expressed on mice $CD4^+$ T cells and dendritic cells (Travis et al., 2007b; Worthington et al., 2012). Recent studies in our laboratory have shown that the chimeric fusion protein consisting of the cholera toxin B sub unit conjugated to the diabetes autoantigen proinsulin (CTB-INS) induces upregulation of αvβ8 integrin biosynthesis. Previous studies in our laboratory have established the immunosuppressive function of CTB-INS on human monocyte-derived dendritic cell (moDC) activation. Further elucidation of the mechanism of CTB-INS induction of tolerance is required for validation of its efficacy and safety for therapy in autoimmune diabetes. Our long-term goal is to elucidate the immunoregulatory mechanisms involved in CTB-INS fusion protein modulation of human dendritic cell tolerance as an essential step in evaluating the vaccine efficacy and safety. The objective of the study proposed here is to elucidate the role of integrin αvβ8 on CTB-INS suppression of DC activation and for the induction of regulatory T cell (Treg) differentiation. The central hypothesis behind the proposed research is that CTB-INS fusion protein suppresses DC activation and DC-mediated Treg differentiation via integrin αvβ8 activation of TGFβ1. This hypothesis is based on documented studies and on data from this dissertation. First, integrin is crucial to TGFβ activation. Mice with a point mutation of the RGD integrin binding site to RGE in TGFβ1, exhibited complete phenotypic expression as TGFβ1-null mice and died from multi-organ inflammatory disease early in life (Yang et al., 2007). Second, integrin αvβ8 is highly expressed in mouse intestinal CD103+ DC subsets and is required for the generation of regulatory T cells by the intestinal DCs, for maintaining immune homeostasis in the intestine (Païdassi et al., 2011). Also, mice with Integrin αvβ8 deficient leukocytes develop an age-related wasting and inflammatory disorder (Travis et

al., 2007b). Third, CTB-INS induces increased expression and biosynthesis of integrin αvβ8 in human moDCs (Fig. 3.1E; Fig. 4.2B). Based on these observations, we will test our central hypothesis by the following specific aims:

Aim 1.1: Assess Increased Integrin αvβ8 Expression in moDCs following CTB-INS Inoculation

Our working hypothesis here is that increasing levels of integrin αvβ8 protein expression correlates with CTB-INS treatment of moDC culture. This will be achieved by repeating earlier experiments measuring the upregulation of surface levels of integrin by flow cytometry on the membrane of DCs following vaccine inoculation. We expect to detect increased integrin αvβ8 expression on CTB-INS vaccinated DCs assessed by comparing integrin αvβ8 expression in CTB-INS treated DCs versus untreated DCs.

Aim 1.2: Determine CTB-INS-mediated Induction of TGFβ1 Activation in moDCs by Integrin αvβ8

The working hypothesis is that CTB-INS stimulates activation of TGFβ1 by an integrin αvβ8-dependent mechanism to modulate suppression of moDC activation by inhibition of DC costimulatory molecule, CD80 and CD86 expression. We will repeat experiments showing quantification of TGFβ1 cytokine expression in CTB-INS treated DC cell culture supernatants, so as to increase the sample size, by conducting ELISA or Cytometric bead array. We will also measure the bioactivity of TGF-beta in CTB-INS treated DCs by mink lung epithelial cells luciferase assay. In addition, we will measure TGFβ activity after blocking integrin αvβ8 expression by a monoclonal antibody specific

to integrin αvβ8 in CTB-INS treated DC cultures, and assess the expression of DC costimulatory molecules by flow cytometry analysis.

Aim 1.3: Characterize CTB-INS Modulation of T cell Differentiation

The working hypothesis here is that CTB-INS modulates DCs to induce Treg differentiation by an integrin αvβ8-dependent mechanism. To test this hypothesis the phenotypic and cytokine profiles of T cell populations will be assessed by flow cytometry of mixed DC-T cell co-cultivation experiments in the presence or absence of CTB-INS and integrin αvβ8 neutralizing antibody.

Specific Aim 2

Smad2/3 signaling is required for the induction of tolerogenic DCs and is induced by members of the TGF-β superfamily ligand (Lan et al., 2012). Earlier studies have observed the occurrence of TGF-β-independent activation of Smad2/3 signaling pathway in rat vascular and renal cells (Rodríguez-Vita et al., 2005; Yang et al., 2009). Previous studies in our laboratory have established the immunosuppressive function of CTB-INS on the activation of human monocyte-derived dendritic cells (moDC). Further elucidation of the mechanism of CTB-INS induction of tolerance is required for validation of its safety for therapy in autoimmune diabetes. Our long term goal is to elucidate the suppressive mechanisms underlying CTB-INS fusion protein modulation of human dendritic cell activation as an essential step in evaluating the efficacy and safety of the vaccine. The objective of the proposed study is to elucidate the role of CTB-INS on modulation of Smad2/3 for the induction of tolerogenic DCs. The central hypothesis

behind the proposed research is that CTB-INS fusion protein activates Smad2/3 signaling independently of TGFβ superfamily members for the induction of DC tolerance. This hypothesis is based on data from this dissertation that shows that CTB-INS stimulates Smad2/3 phosphorylation in the presence of TGFβ1 neutralization. Based on this observation we will test our central hypothesis by the following aim:

Aim 2.1: Determine CTB-INS Induction of Smad2/3 Signaling Independently of TGF-β Superfamily Ligands

The working hypothesis is that CTB-INS fusion protein activates Smad2/3 signaling independently of TGFβ superfamily members for the induction of DC tolerance. This hypothesis will be tested by measuring Smad2/3 phosphorylation in human moDCs treated with CTB-INS, by Immunoblot analysis, after blocking all natural ligands of Smad2/3. We expect to detect Smad2/3 phosphorylation in DCs treated with CTB-INS in the presence of global blocking of TGFβ superfamily signaling, as assessed by comparing Smad2/3 phosphorylation in CTB-INS treated DCs with TGFβ superfamily treated DCs in the presence of neutralizing antibodies to the TGFβ superfamily members.

It is anticipated that the future studies proposed here will reinforce the detection of these novel mechanisms by which CTB-INS mediates its tolerogenic functions.

Conclusions

The experimental findings documented in this dissertation demonstrate that CTB-INS fusion protein vaccine induction of the immunoregulatory Smad2/3 signaling, TGF-β1 superfamily cytokines and the biological activator of TGF-β1, integrin αvβ8, may

represent plausible mechanisms utilized by the vaccine in inducing immune suppression of human moDCs. These studies have revealed new insights involving the identification of immunoregulatory proteins defining the mechanistic action of CTB-INS, which is essential for the validation of CTB-INS safety and efficacy as a tool for the induction of tolerance in clinical applications for autoimmune diabetes. Investigations into the immunosuppressive strategies described here may also enhance the understanding of the modalities for tolerance induction in other autoimmune conditions. Every incremental understanding of the observed and supposed mechanism of tolerance induction in DCs is required given the intricate combination of intrinsic and extrinsic factors, such as environmental triggers or cell types, that add complexity to the development of autoimmune conditions. Solid understanding of the potential and defined mechanisms involved in tolerance and in the immune response, will increase the opportunities for developing safe and effective therapeutic strategies for autoimmune conditions (Van Brussel et al., 2014). For example, a novel finding documented in this dissertation is that TGF-β1 may not mediate IDO1 biosynthesis in human moDCs (Chapter 3). This finding may contribute to the understanding of the specific modulators of DC tolerogenicity that may be required in translational studies and clinical applications that require *ex vivo* generation of human tolerogenic DCs, especially tolerogenic DCs with IDO phenotype. Another significant finding produced by this dissertation relates to mechanism of CTB-INS tolerance induction, and the interactions of the immunoregulatory molecules induced by CTB-INS in human moDCs. It is clear that CTB-INS may utilize multiple mechanisms in mediating tolerance; the vaccine induces a number of immunomodulatory factors including, IDO1, activin-A, TGF-β1, integrin αvβ8 and Smad2/3 signaling, some

acting synergistically and others acting independently of each other. It is important to understand these distinctions since the CTB-INS fusion protein is not a natural product of biological systems. Therefore, further incisive characterization of the mechanistic functions of CTB-INS is required to expedite its clinical application for the therapy of T1D. In Figure 5.1, the findings of the present study and prospective studies of immediate relevance are summarized.

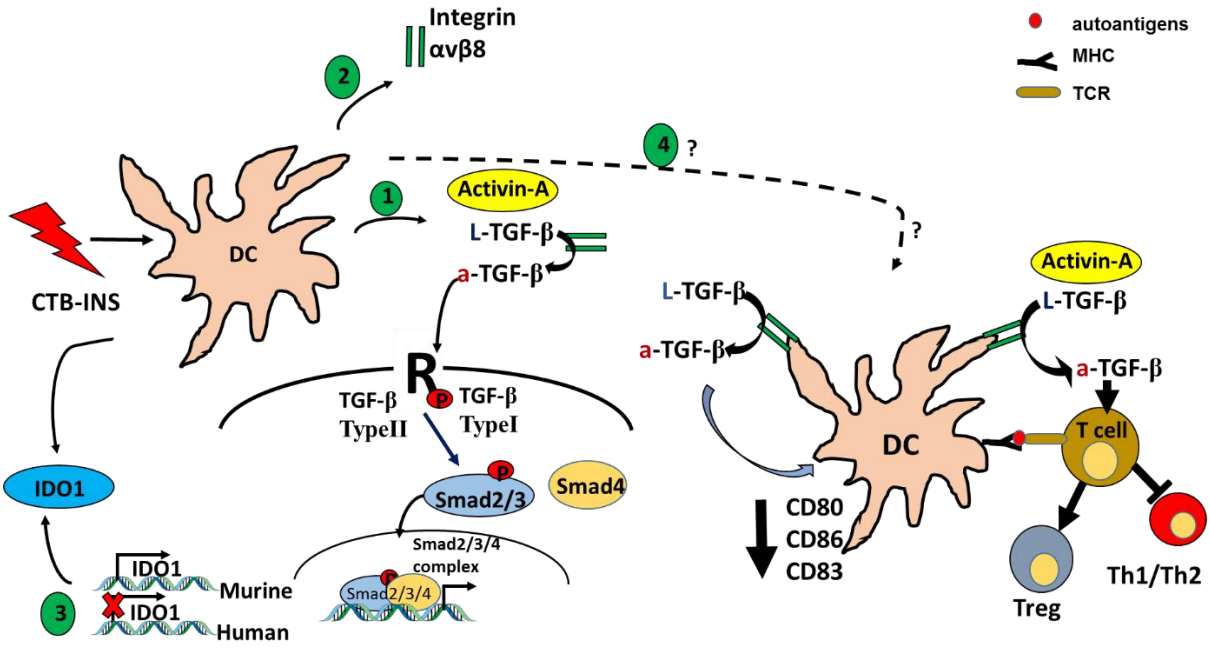

Figure 5.1. Overall summary of experimental findings presented in this dissertation: Projected functions of integrin αvβ8 and TGF-β superfamily members in CTB-INS induction of immune tolerance in human dendritic cells. In signaling pathways (1, 2) CTB-INS upregulates immunoregulatory proteins, TGF-β1, activin-A and integrin αvβ8 expression in human monocyte-derived dendritic cells (moDCs). The presence of CTB-INS fusion protein induces Smad2/3 signaling by increasing Smad2/3 phosphorylation. In pathway (3), increased TGF-β signaling appears unrelated to IDO1 biosynthesis in human moDCs. In signaling pathway (4), the mechanism by which CTB-INS suppresses DC activation is proposed to include stimulation of immunoregulatory proteins TGF-β1, activin-A and integrin αvβ8. Integrin αvβ8 is proposed to activate the latent complex of TGF-β complex to induce DC tolerance resulting in regulatory T cell (Treg) proliferation. a-TGF-β: Active TGF-β; L-TGF-β: Latent TGF-β; MHC: major histocompatibility complex molecules class II; TCR: T cell receptor.

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