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The Expression Of Connexin-43 By CD11c+ Dendritic Cells Is Required to Maintain CD4+ Foxp3+ Regulatory T Cell Population in Peripheral Lymphoid Organs

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ABSTRACT

THE EXPRESSION OF CONNEXIN-43 BY CD11c^{+} DENDRITIC CELLS IS REQUIRED TO MAINTAIN CD4^{+} Foxp3^{+} REGULATORY T CELL POPULATION IN PERIPHERAL LYMPHOID ORGANS

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Foxp3^{+} regulatory T cells (T_R) are an immunosuppressive subset of CD4^{+} T cells that maintain homeostasis of the immune system. They are sustained by the interaction between the Major Histocompatibility Complex (MHC) molecules present on antigen presenting dendritic cells and the T Cell Receptor (TCR) expressed on T_R cells that is specific for the MHC loaded with an antigenic peptide. Here, we show that in addition to MHC/TCR interaction, Connexin-43 (Cx43) expression by dendritic cells is required to maintain the T_R cell population. CD11c^{+} dendritic cells represent a major subset of antigen presenting cells. Using flow cytometry, we have observed that mice which lack Cx43 expression in CD11c^{+} dendritic cells (Cx43^{DC-}), have a lower percentage of T_R cells which express lower levels of Foxp3. These mice showed increased incidence of dermatitis as they age, even though we show that their dendritic cells can efficiently present antigen to naive T cells using proliferation inhibition assay. The decrease in the proportion of T_R cells were associated with an altered phenotype of these cells, demonstrated by lower expression of CD39 and higher expression of CD73, ectonucleotidases mediating T_R cell immunosuppressive function. We propose that the presence of Cx43 on the surface of dendritic cells is required for effective communication between T_R cells and dendritic cells so as to sustain T_R cell homeostatic expansion and Foxp3 expression.

This thesis is dedicated to the loving memory of my parents, John Titus Joseph and Lilly Titus Joseph, who I wish could have seen this moment.

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First and foremost, I would like to thank God Almighty for His grace throughout this process and for ordering my steps, so that I can be part of a spectacular laboratory and be under great mentorship. It has been a good journey.

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NOMENCLATURE

^{3}H	Tritium
AMP	Adenosine MonoPhosphate
ADP	Adenosine DiPhosphate
ATP	Adenosine TriPhosphate
APC	Antigen Presenting Cell
BCR	B cell receptor
cAMP	cyclic Adenosine MonoPhosphate
CFA	Complete Freund's Adjuvant
CTLA-4	Cytotoxic T lymphocyte Antigen-4
CTM	Complete Tumor Medium
Cx	Connexin
CXCL8	C-X-C motif Ligand 8
DC	Dendritic Cell
DNA	DeoxyriboNucleic Acid
FBS	Fetal Bovine Serum
FGL2	Fibrinogen-like protein
Foxp3	Forkhead-box P3
IL	Interleukin
Klrg1	Killer cell Lectin like Receptor G1
LAG-3	Lymphocyte-Activation Gene-3
MHCI	Major Histocompatibility Complex Class I

MHCII	Major Histocompatibility Complex Class II
min	Minute (s)
NAD	Nicotinamide Adenine Dinucleotide
PBS	Phosphate Buffered Solution
PD1	Programmed cell Death protein 1
PE	Phycoerythrin
rpm	Revolutions per minute
TCR	T-Cell Receptor
TGF-β	Tumor Growth Factor β
T_H1	T helper 1
T_H2	T helper 2
T_H17	T helper 17
TNF	Tumor Necrosis Factor
T_R cells	Regulatory T cells
TRAIL-DR5	Tumor-necrosis-factor Related Apoptosis-Inducing Ligand-Death Receptor 5
WT	Wild-Type

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Most multi-cellular organisms, if not all, face the constant threat of infection by a variety of microbial pathogens. The probability that the host organism will survive depends on its ability to overpower and eliminate the invading foreign material, by inducing defensive responses that are termed broadly as ‘immune responses’. Ideally, these responses target the pathogenic material and not the host cells and tissues. In this introduction, we will review antigen presentation by innate immune cells and the consequent activation of a few specific types of T cell subsets that play an important role in adaptive immunity and homeostasis. In addition, we will discuss the current understanding of the role of Connexin-43 in the immunological synapse, and how it relates to current research.

1.1. Antigen presentation

The first line of defense against foreign material is the physical and chemical barriers of the body like skin and antimicrobial enzymes secreted at mucosal surfaces, respectively. If foreign material or a pathogen were to successfully overcome the barriers and enter the body, the innate immune system responds to it in a multi-faceted way. Neutrophils, basophils, macrophages, monocytes, dendritic cells, natural killer cells and granulocytes bind foreign molecules through Pattern Recognition Receptors (PRRs), get activated and directly or indirectly facilitate the elimination of the pathogen *(1-3)*. In most cases, the pathogen or what is referred to as antigen, is digested and destroyed by resident phagocytes. All phagocytic cells like macrophages/monocytes, granulocytes and dendritic cells have the ability to internalize antigen, through a process called phagocytosis,

or take up large amounts of extracellular material through a process called pinocytosis and degrade the antigens that were endocytosed. Activated phagocytes secrete pro-inflammatory cytokines like Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), IL-6 and IL-18 and chemokines like CXCL-8 that recruit and activate cells of the adaptive immune system at the site of pathogen entry *(4, 5)*.

One of the main roles of phagocytes is to present antigenic peptides on the cell's surface in order to activate cytotoxic and helper T cells of the adaptive immune system. T cells are not able to recognize native antigens and conformational epitopes; therefore, they depend on recognition of processed antigen for activation. Antigen processing relies on proteolytic digestion of ingested proteins/antigens and binding of resulting peptides to the Major Histocompatibility Complex (MHC) molecules. MHCs are polymorphic transmembrane glycoproteins found on the cell's surface having a characteristic peptide-binding groove. MHC structure is stabilized by binding peptides derived from self or exogenous proteins. There are two types of MHC molecules. MHC Class I (MHCI) molecules are expressed by all nucleated cells and present antigenic peptides to cytotoxic $CD8^+$ T cells *(6).* It increases the targeting of abnormal cells by $CD8^+$ cytotoxic T cells that recognizes the mutated peptides, like those resulting from DNA damage or cancer associated mutations *(7)*. MHC Class II molecules are only present on phagocytic cells, of which macrophages and dendritic cells are referred to as "professional antigen presenting cells" *(8).* When the endocytosed material is broken down in acidified endosomes, it is loaded onto the MHCII molecule and transported to the cell membrane for antigen presentation. The helper $CD4^+$ T cells require this presentation of antigenic peptide in the context of MHCII molecules in order to be activated *(9).* CD11c is a type I transmembrane protein that is constitutively present on all dendritic cells and is used in conjunction with other markers to identify various subsets. CD11c is, therefore,

used in this study to identify the dendritic cell population, which are known to be efficient activators of regulatory T cell population. This is further discussed in 1.3.

1.2. Development and activation of $CD4^+$ T cells

The adaptive immune responses are carried out by B and T lymphocytes, two types of white blood cells that are differentiated from hematopoietic stem cells in the bone marrow. B cell differentiation occurs in the bone marrow, and T cell maturation takes place in the thymus. B cells and T cells have a large repertoire of B cell receptors (BCRs) or T cell receptors (TCRs) to enable these cell types to detect and eliminate a variety of pathogens *(10).* A TCR is a dimer and consists of one α and one β chain, each having a variable and a constant region. The variable regions of these chains have a unique sequence that can bind to a peptide in complex with an MHC molecule. In this way, T cells are MHC-restricted and cannot be activated by antigenic peptide alone. The development of T cells or thymocytes takes place in the thymus and is a very systematic process that ensures a low probability of self-reactivity. After the successful rearrangements of the V, D and J gene segments of the TCR's β-chain takes place, there is a rapid clonal expansion of the cells whose β-chain was successfully rearranged, accompanied by the expression of co-receptor proteins CD4 and CD8 that have the role of binding to a portion of the MHC Class II and MHC Class I molecule, respectively, to infer stability of the eventual TCR/MHC interaction *(11, 12)*. The cell proliferation is followed by α-chain rearrangement in each individual cell *(13).* The next step in ensuring that these cells will bind to an MHC molecule is called positive selection where thymic cortical epithelial cells express MHC molecules for thymocytes to bind, and during this step, the T cell will commit to expressing either CD4 or CD8 depending on the strength of the affinity by which the TCR binds to the MHCII or MHCI *(14)*. In addition, the T cells that do not bind in this

step undergo apoptosis. During negative selection, any thymocyte that binds with high affinity to an MHC molecule with a self-antigen peptide is eliminated by apoptosis *(15)*. This significantly reduces the chance of auto-reactive T cell clones. Elimination of self-reactive T cells is complemented by a dominant tolerance mechanism relying on the presence of suppressor $CD4^+$ T cells. These cells, called natural regulatory T cells (T_R), expressing transcription factor Forkhead box P3 (Foxp3) are generated in the thymus and recognize MHCII + self-peptides. Current data support the conclusion that T_R cells are generated in an instructive process when interactions between TCR and MHCII/peptide complex, stronger than that of conventional $CD4^+$ thymocytes, induce Foxp3 expression and launch a genetic program of T_R cell differentiation *(16)*. Immunosuppressive role of T_R cells relies on various mechanisms and depends on recognition of a self-peptide on MHCII molecule, which is further discussed in 1.3. The $CD4^+$ $Foxp3^+$ T_R thymocytes migrate out of the thymus and circulate to the lymphoid tissues through the blood stream. Professional antigen presenting cells like dendritic cells migrate to the lymphoid tissues to interact with circulating naïve $CD4^+$ conventional and T_R cells *(17).* These could be $CD11c^+$ migratory Langerhans cells or dermal dendritic cells that reside in the skin and sample antigens *(18)*. When the naïve $CD4^+$ T helper cell recognizes the specific antigen presented by a dendritic cell and co-stimulatory molecules, the T cell remains in the lymphoid tissue and undergoes clonal expansion with the production of cytokines that gives rise to effector T cells and memory T cells. The naïve and effector T cells migrate out of the lymph node and move toward the site of infection by chemotaxis. The $CD4^+$ T cells recognize antigen presented by APC at the site of infection, get activated and undergo clonal expansion and cytokine production *(19).* Depending on the cytokines in the environment, the effector T cells differentiate into subsets like T helper 1 (T_H1) cells that secrete IFN-γ *(20)*, T helper 2 (T_H2) cells that secrete IL-4 *(21)*, T helper 17 (T_H17) cells that

secrete IL-17 *(22)* or peripheral regulatory T (T_R) cells that secrete IL-10, as depicted in Fig. 1 *(23)*, and upregulate the expression of characteristic transcription factors and cytokines. T_H1, T_H2 and T_H17 cells release pro-inflammatory cytokines, whereas T_R performs an immunosuppressive role and controls the immune response *(22)*.

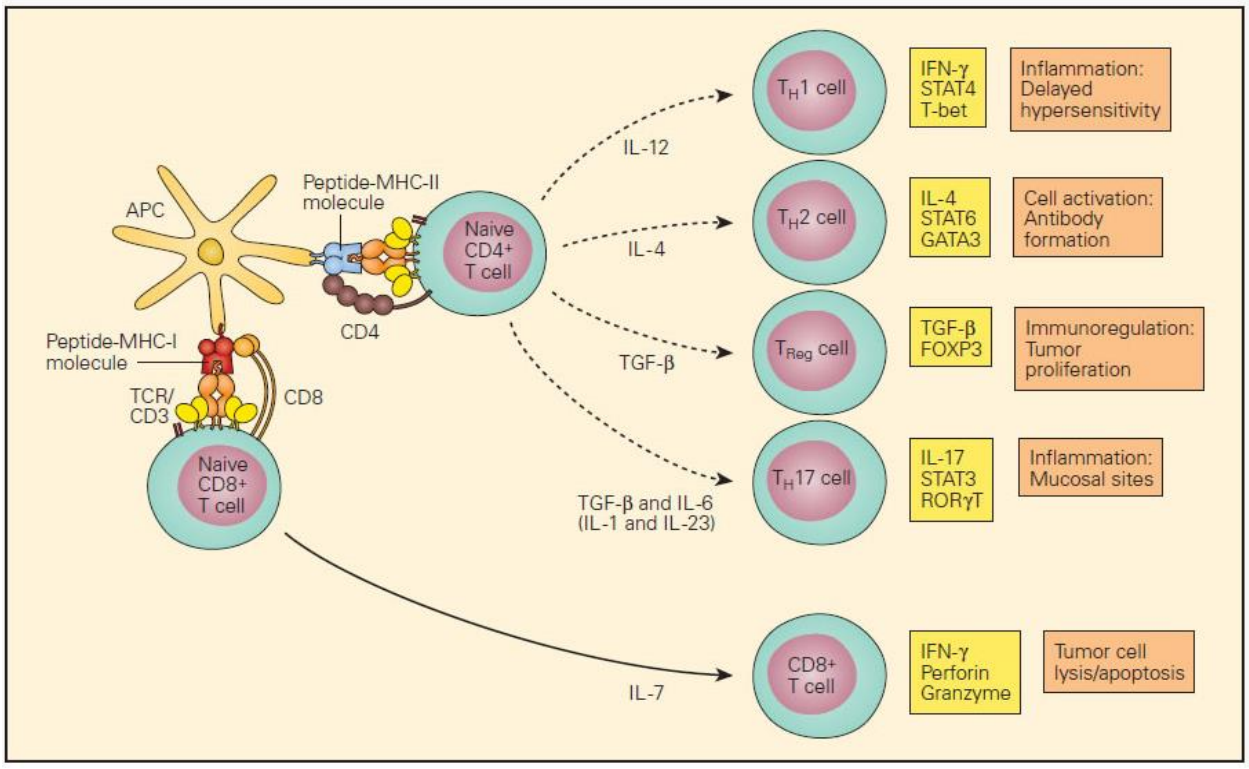

Fig. 1. Naïve T cells differentiate on activation based on the cytokines in its environment. Animated drawing depicting TCR/MHC interaction between an APC and $CD4^+$ T cell through MHC-II; and interaction between the APC and $CD8^+$ T cell through MHC-I. Based on the cytokines in the environment, the effector T cells differentiate into effector cells with pro-inflammatory, anti-inflammatory or cytotoxic roles specified within the orange boxes. The yellow boxes note the genes that are upregulated in the T cell subset. *(23).*

1.3. Immunosuppressive $CD4^+$ $Foxp3^+$ regulatory T cells

Maintenance of immune tolerance is largely carried out by $CD4^+$ $Foxp3^+$ T_R cells. Gene mapping and functional studies established that Foxp3 is an X-chromosome-encoded gene and a master regulator of $CD4^+$ T_R cell development in both humans and mice. Foxp3-deficient mice do not have functional T_R cell population and develop fatal lymphoproliferative autoimmune syndrome at 3-4 weeks of age *(24)*. T_R cells constitute up to 10-20% of the $CD4^+$ T cell compartment. Like other $CD4^+$ T cells, $CD4^+$ $Foxp3^+$ T_R cell population's development and activation are strictly dependent on the TCR/MHC interactions *(25)*.

All APCs, expressing class II MHC molecules, have the ability to induce antigen specific $CD4^+$ $Foxp3^+$ T_R cells in the presence of IL-2 and TGF-β. However, there is evidence that dendritic cells (DCs) are the most efficient activators of T_R cells *(26)*. In the presence of high concentrations of IL-2, the apoptotic signaling in T_R cells is inhibited. On recognition of the MHC/peptide complexes through their TCR, T_R cells undergo clonal expansion and make direct contact or produce effector molecules to enforce immune suppression. Some of the molecules by which these T_R cells maintain homeostasis have been outlined in Table 1 *(27)*. $Foxp3^+$ Regulatory T cells can be immunosuppressive in an antigen non-specific manner in multiple ways. Through the secretion of cytokines like TGF-β and IL-35 by T_R cells, $CD4^+$ effector cells are led to differentiate into immunosuppressive $Foxp3^+$ cells as discussed in 1.2 *(28, 29)*. Other cytokines like IL-10 have been shown to be required for effective immunosuppression at interfaces like the colon and lungs *(30)*. Regulatory T cells are also able to transfer cyclic-AMP (cAMP) into conventional cells in a contact-dependent manner through gap junctions to result in the induction of the expression of the transcription factor ICER (Induced Cyclic-AMP Early Repressor) in conventional $CD4^+$ T cells. ICER represses at the IL-2 and IL-4 gene loci and prevents the secretion of cytokines by the

activated $CD4^+$ T cells *(31)*. CD39 is an ectonucleotidase present on the surface of $Foxp3^+$ T_R cells, that converts adenosine diphosphate (ADP) or adenosine triphosphate (ATP) to adenosine monophosphate (AMP), where ATP is a known mediator of inflammation. CD73 is also an ectonucleotidase on the T_R cell whose function complements CD39 and converts AMP to adenosine which has been shown to have an immunosuppressive role *(32)*. T_R cells can also maintain homeostasis by secreting cytolytic molecules like granzymes and perforin to induce apoptosis in conventional $CD4^+$ T cells *(33)*. Certain dendritic cells express indoleamine 2,3-dioxygenase (IDO) that catalyzes the degradation of tryptophan to kynurenine which starves the effector T cells and puts them in cell cycle arrest. Via CTLA-4-induced signaling, T_R cells can increase IDO expression in dendritic cells and also direct T_R differentiation of naïve $CD4^+$ T cells *(34)*.

Table 1. Immunosuppressive effector molecules of T_R cells. The known effector molecules of T_R cells can be classified into secreted immunosuppressive cytokine molecules, molecules that inhibit metabolic signaling, secreted cytolytic molecules and membrane associated molecules that inhibit T effector cell activation.
IL- interleukin; TGF-β- Tumor growth factor β; FGL2- Fibrinogen-like protein; cAMP-inhibitory second messenger cyclic AMP; NAD- nicotinamide adenine dinucleotide as metabolized by CD38 and operative at P2X7; TRAIL-DR5 (tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5); CTLA-4- Cytotoxic T-Lymphocyte Antigen 4; LAG-3- Lymphocyte-activation gene 3 *(27)*.

Immunosuppressive cytokines	Metabolic signaling	Cytolytic molecules	Membrane-associated molecules
TGF-β	cAMP	Granzyme A/B	CTLA-4
IL-10	CD39	Perforin	LAG-3
IL-35	CD73	TRAIL-DR5	
FGL2	NAD	Galectin-1	

As a result of the effects of these molecules and direct interactions, activated T cells and antigen presenting cells are not able to carry out their immunological response effectively. T_R cell population consumes a large portion of IL-2 produced by the activated $CD4^+$ T cells and in doing so, carries out IL-2 deprivation and induces apoptosis of both cell populations *(35)*. For this reason, T_R cell populations were initially identified by the expression of CD25, which is the α-chain of the IL-2 receptor. However, the upregulation of CD25 on all activated CD4+ cells and the existence of non-immunosuppressive $CD4^+$ $CD25^+$ cells were observed, and Foxp3 expression was found to be a more precise marker for the immunosuppressive cells *(24)*. Immunoregulation is an essential process in order to avoid potentially damaging autoimmune responses by non-regulatory T cells and to maintain tolerance to self-antigens. $CD4^+$ $Foxp3^+$ T_R cells have a high turnover rate and respond to a range of signals from the environment that may make them anergic or persist as memory cells *(36)*. Therefore, maintenance of a stable population size of functional T_R is required for effective immune suppression.

1.4. Connexin-43

Connexins are a family of small transmembrane proteins that assemble into membrane complexes to create small channels called gap junctions in order to allow communication between cells or carry out paracrine functions as hemi-channels *(37, 38)*. Connexin-43 (Cx43) has a relatively long cytoplasmic domain with a number of phosphorylation sites that have been proposed to interact with cytoskeletal elements and signaling molecules like protein kinase C, mitogen-activated protein kinase and β-catenin *(39)*. Recent evidence has shown that Cx43 may have the ability to act as a transcription factor *(40)*. The expression of Cx43 is widespread among immunological

cells like dendritic cells, monocytes, B cells, T cells and Natural Killer cells *(41)*. On antigen activation, expression of Cx43 is upregulated in T cells *(42)*. Hemi-channel function of Cx43 is pro-inflammatory as it involves the release of ATP, a mediator of inflammation *(43)*. In contrast, channels can form at gap junctions between effector and T_R cells to facilitate the transfer of cAMP from T_R cells to effector cells *(31)*. In summary, modulating connexin-43 expression or gap junction formation may alter the course of adaptive immune response mediated by T cells. When the hemi-channel activity of Cx43 was reduced while simultaneously promoting gap junction plaque formation using a mimetic peptide- αCT1 in a non-obese diabetic mouse model, T_R suppressor ability was partially restored *(44)*. Phase III clinical trials of the use of αCT1 for wound healing during treatment of diabetic foot ulcers is currently underway *(45)*. Additionally, higher expression of Cx43 has been observed in human and murine thymic epithelial cells *(42)*. Although the mechanism of its function has not been elucidated, several reports conclude that Cx43 is a component of the immunological synapse.

1.5. Purpose of research and experimental design

To examine the role of gap junctions and communication between T_R cells and DCs, we used a mouse model in which *Gja1* gene that codes for Cx43 was knocked out in $CD11c^+$ dendritic cells alone. Several of these mice spontaneously developed dermatitis and a preliminary experiment showed a reduction in the number of $CD4^+$ $Foxp3^+$ T_R cells in both the lymph nodes and spleens of the $Cx43^{DC-}$ mice. This provided the initial motivation for the study of the role of connexin in maintaining the regulatory T cell population. This research contributes to the literature on the involvement of Cx43 in the maintenance of regulatory T cell population, with the hope of shedding light on novel targets for therapeutic treatments of auto-immune diseases. We look into the changes

caused in mice due to the lack of expression of Cx43 in dendritic cells. For this reason, phenotypic and functional analyses of peripheral populations of DC and $CD4^+$ T cells using flow cytometry were carried out. Flow cytometry data on the dendritic cell population in the skin of wild-type (WT) control mice and $Cx43^{DC-}$ mice were also analyzed. The ability of the $CD11c^+$ dendritic cells with no expression of Cx43 to activate $CD4^+$ naïve T cells and the ability of their T_R cells to suppress proliferation of activated $CD4^+$ T cells were measured through radioactive thymidine incorporation assays described in 2.6. and 2.7.

CHAPTER 2

METHODS

2.1. Generation of $Cx43^{DC-}$ mice

Conditional knockout of *Gja1* gene that codes for Connexin-43 ($Cx43^{loxP}$) were crossed with $CD11c^{cre}$ and $Foxp3^{GFP}$ reporter mice *(39, 46)*. These mice were compared to wild-type control mice during analyses. Mice were housed and sacrificed according to the guidelines of the Institutional Animal Care and Use Committee of Old Dominion University under IACUC protocol number 17-017. Subsequent cell work was carried out with the approval of the Institutional Biosafety Committee of Old Dominion University under IBC protocol number 19-007.

2.2. Isolation of dendritic cells from the skin

Dorsal skin from the mice were cut and incubated in 0.5 mL of 0.2 mg/mL Dispase I solution in a 24-well plate overnight at 4^{o}C. Hair was removed and incubated at 37^{o}C in 1 mL Collagenase IV-DNase solution (RPMI, 10% FBS, 1.5 mg/mL Collagenase IV and 0.5 mg/mL DNase) followed by addition of 1mL of Collagenase IV-DNase solution every 30 minutes for 2 hours. The solution was then filtered and centrifuged at 1200 rpm for 6 minutes. The pellet was resuspended in PBS + 2% FBS for antibody staining and subsequent flow cytometry analysis.

2.3. Cell purification, flow cytometry and cell sorting

Lymph nodes and spleens of $Cx43^{DC-}$ and WT mice were isolated and passed through nylon mesh to form cell suspensions in PBS + 2% FBS. Spleen suspensions were centrifuged at 1200 rpm for 6 minutes and resuspended with 0.16 M sterile ammonium chloride to lyse red blood cells in the

sample. This sample was incubated at room temperature for 6 minutes and centrifuged at 1200 rpm and resuspended in PBS + 2% FBS. Aliquots of the cell suspension were stained with eight color antibody sets with antibodies specific for CD4, CD8, CD44, CD62L, 4-1BB, Tim-3, PD-1, IL-7R,Klrg-1, BTLA, Lag3, CD25, CD39, CD73, Sirp1α, CD80, 4-1BBL, MHCII, CD24, CD86, PDL1, F4/80, Ox40L and Gr1 (BD Bioscience, BioLegend, eBioscience) for 30 minutes at 4ºC with subsequent washes with PBS + 2% FBS, and analyzed using a flow cytometer (BDFACSCanto). $CD4^+$ cells were negatively selected using streptavidin beads (mentioned in 2.4), while $CD11c^+$ cells were positively selected using PE magnetic beads (mentioned in 2.5). In addition to this, these cells were sorted from the cell suspensions using a flow cytometer (BD Influx) for increased purity of 98-99%.

2.4. Negative selection of $CD4^+$ cells using streptavidin beads.

Cell pellets were resuspended in 150 μL of PBS + 2% FBS and stained with biotinylated antibodies against CD8, Ter119, B220, CD49b (BD Bioscience, BioLegend, eBioscience) for 30 minutes at 4ºC. After a wash with PBS+ 2%FBS, the cells were incubated with streptavidin magnetic beads (Appendix) for 20 minutes at 4ºC, and the tube was placed in a magnet for 6 minutes. The clear solution was carefully pipetted out, centrifuged at 1200 rpm for 6 minutes and resuspended in PBS + 2% FBS as a $CD4^+$ sample. Purity of the sample was checked using flow cytometry.

2.5. Positive selection of $CD11c^+$ cells using PE beads.

Cell pellets were resuspended in 150 μL of PBS + 2% FBS and stained with CD11c-PE antibody for 30 minutes at 4ºC, followed by a wash with PBS + 2% FBS. The cells were incubated with PE beads for 20 minutes at 4ºC. Then the cells were washed and resuspended in 750 μL of PBS + 2

% FBS and loaded into the column attached on a magnetic plane. The flow through was disposed, and the column was detached from the magnet. PBS + 2% FBS was used to flush the $CD11c^+$ cells into a collection tube. Purity of the sample was checked by flow cytometry.

2.6. Proliferation assay

In a 96-well round bottomed plate, $3x10^4$ sorted $CD11c^+$ DCs and $2x10^4$ $CD4^+$ cells from a transgenic mouse whose $CD4^+$ T cells are specific for a peptide PCC50V54A were stimulated with soluble peptide (5 μM) in CTM (Appendix) and incubated at 37°C with 5% CO_2. Three days after stimulation, 1 μCi/well of ^{3}H-thymidine was supplied. The radioactivity of the resulting cells after washing were measured on Tri-Carb 3110TR Liquid Scintillation Analyzer.

2.7. Proliferation inhibition assay

In a 96-well round bottomed plate, $5x10^4$ sorted naïve ($CD44^{lo}$ $CD62L^{hi}$) $CD4^+$ T cells and $5x10^4$ WT DCs from lymphopenic mice ($TCR\alpha^-$) were stimulated using antibody against CD3 (BD Bioscience- 3 μg/mL) in CTM (Appendix) and incubated at 37°C with 5% CO_2. In different wells, T_R were added in increasing concentrations of 0%, 5%, 10% 20% and 40% of total naïve $CD4^+$ T cells. After three days of stimulation, 1 μCi/well of ^{3}H-thymidine was added, and radioactivity of the sample after washing was measured on Tri-Carb 3110TR Liquid Scintillation Analyzer.

2.8. Data analysis

Flow Cytometry data was analyzed in dot plots and histograms using FlowJo v10. The results of the proliferation assay and the proliferation inhibition assay were graphed using Exel.

CHAPTER 3

RESULTS

3.1. Development of dermatitis in $Cx43^{DC-}$ mouse

The $Cx43^{DC-}$ mice, having a lack of expression of Connexin-43 in $CD11c^{+}$ dendritic cells, seem to visually develop normally apart from the dermatitis observed. About 25% of mice aged 1 to 4 months begin to develop a form of dermatitis on their dorsal skin (Fig. 2), which is first seen as barbering. Moreover, 60% of the mice older than 5 months had dermatitis progressing from barbering to open skin lesions. This skin disease is likely caused by an autoimmune response that has been sustained due to lack of immune suppression. During an auto-immune response, the T_R population is usually lower than in healthy mice. Lymph nodes and spleens were isolated from these mice and T cell populations were analyzed to further investigate the cell populations affected by the lack Cx43 in DCs that may have caused the disease.

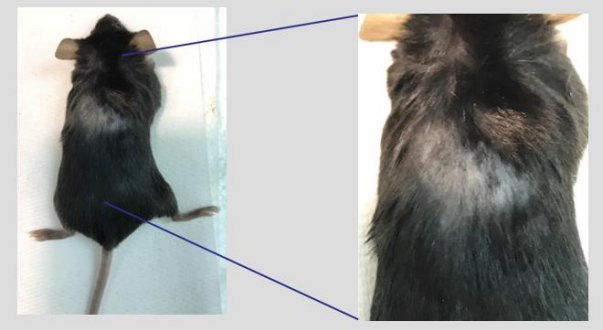

Fig. 2. $Cx43^{DC-}$ mice spontaneously develop dermatitis. Image of barbering seen on the dorsal skin of two-month old $Cx43^{DC-}$ mice

3.2. Dendritic cell population density in the skin of $Cx43^{DC-}$ mice varies compared to that of wild-type control mice

To investigate the changes in skin's dendritic cell population due to the lack of Cx43 on $CD11c^{+}$ DC cells, skin was digested, and the cells were isolated to be stained. There was a presence of $CD11c^{+}$ $MHCII^{+}$ $CD24^{-}$ $CD103^{-}$ dermal DCs in young 2-month old $Cx43^{DC-}$ mice, which was barely detectable in control mice (Fig. 3). These cells are usually only detectable at 3 months of age as the immune system matures. It is possible that the existence of larger proportion of dermal dendritic cells that are migrating to the skin, is the reason why $Cx43^{DC-}$ mice are predisposed to developing skin disease. The $CD11c^{+}$ $MHCII^{+}$ $CD24^{+}$ $CD11b^{+}$ Langerhans cell population was increased in $Cx43^{DC-}$ mice with respect to age, whereas $CD11c^{+}$ $MHCII^{+}$ $CD24^{+}$ $CD11b^{-}$ dermal dendritic cell population was decreased, also with respect to age compared with age-matched WT mice older than 3 months.

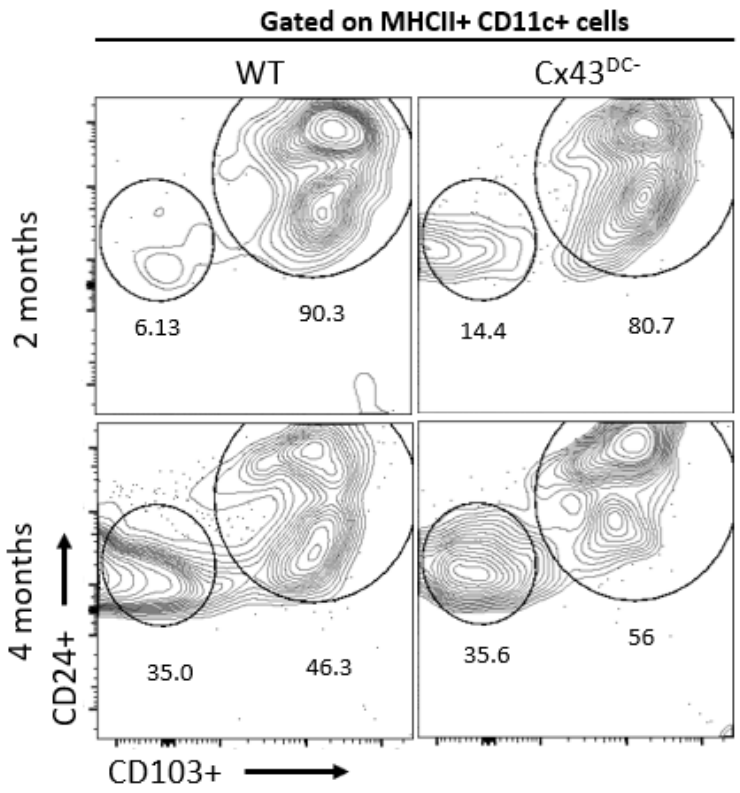

Fig. 3. Increased presence of dermal dendritic cells in the dorsal skin of 2 month old $Cx43^{DC-}$ mice. Mice lacking Cx43 expression by $CD11c^{+}$ dendritic cells have a larger proportion of $CD24^{-}$ $CD103^{-}$ dermal dendritic cells in the dorsal skin of 2 month old mice. Dermal dendritic cells usually are present in low numbers in the skin till 3 months of age when the immune system is mature.

3.3. Increased activation is seen in older $Cx43^{DC-}$ mice with dermatitis.

The flow cytometry data analysis of cells from lymph nodes showed that in $Cx43^{DC-}$ mice with dermatitis, more than 70% of the $CD4^{+}$ cells in the lymph nodes were activated, depicted as $CD44^{hi}CD62^{lo}$ cell population in Fig. 4. These activated cells from $Cx43^{DC-}$ mice had a decrease in activation markers 4-1BB and Klrg1 expression, accompanied by an increase in inhibitory molecule PD-1 in old mice, compared to age-matched WT (Fig. 5). The changes in population of activated cells and expression of activation markers shown in Fig. 4 and 5 were not seen in $Cx43^{DC-}$ mice without dermatitis or in the WT mice. Therefore, the increased activated population could be

attributed to the response to the dermatitis seen on the dorsal skin, while the changes in expression of 4-1BB and Klrg1 may be due to the way Cx43^{DC-} cell populations interact after the onset dermatitis.

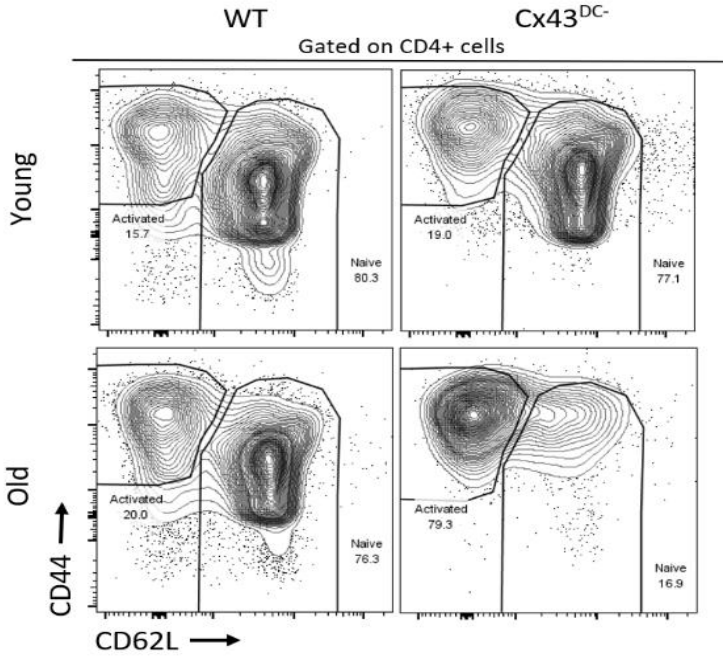

Fig. 4. Increased activation in Cx43^{DC-} mice with dermatitis**.** Total CD4^{+} cells can be separated into activated and naïve cells based on the expression of CD44 and CD62L. The plots show that old Cx43^{DC-} mice with dermatitis had almost 80% of CD4^{+} cells activated in the lymph nodes.

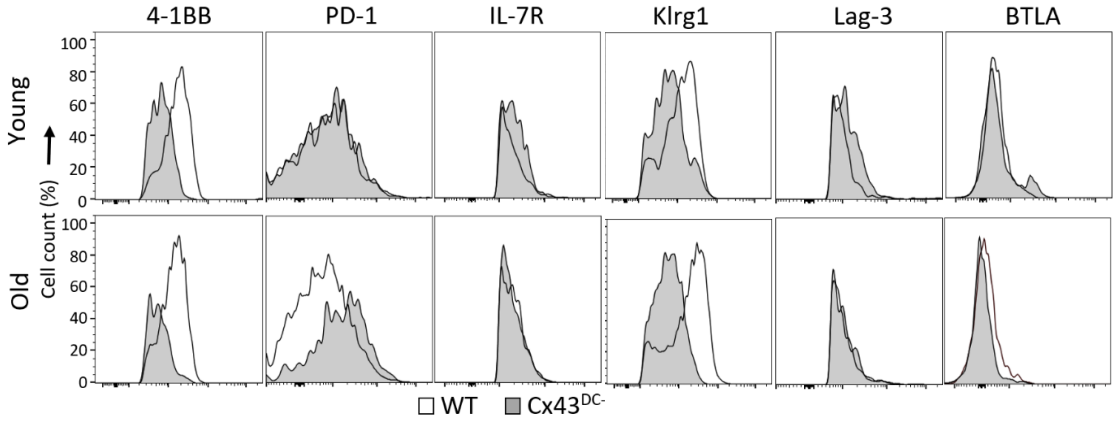

Fig. 5. Changes in expression of molecular markers for activation and inhibition in activated cells of $Cx43^{DC-}$. Histograms showing the expression of 4-1BB, PD-1, IL-7R, Klrg1, Lag3 and BTLA by activated $CD4^{+}$ $CD44^{hi}CD62^{lo}$ cells of WT and Cx43DC- mice.

3.4. Decrease of $CD4^{+}$ $Foxp3^{+}$ T_R population in $Cx43^{DC-}$ mice with relation to age.

Using flow cytometry, we analyzed the different T cell populations in the lymph nodes of the knockout mice alongside age-matched controls. We found that $Cx43^{DC-}$ mice had a smaller population of $CD4^{+}$ $Foxp3^{+}$ T_R cells compared to WT mice (Fig. 6, 7). Additionally, these T_R cells expressed Foxp3 with a lesser intensity (Fig. 8) as indicated by the decrease in mean fluorescence intensity measurement of the fluorophore by flow cytometry. The lower amounts of immunosuppressive regulatory T cells may have led to the development of dermatitis seen among $Cx43^{DC-}$ mice, due to the consequent failure to control immune response at the dorsal skin.

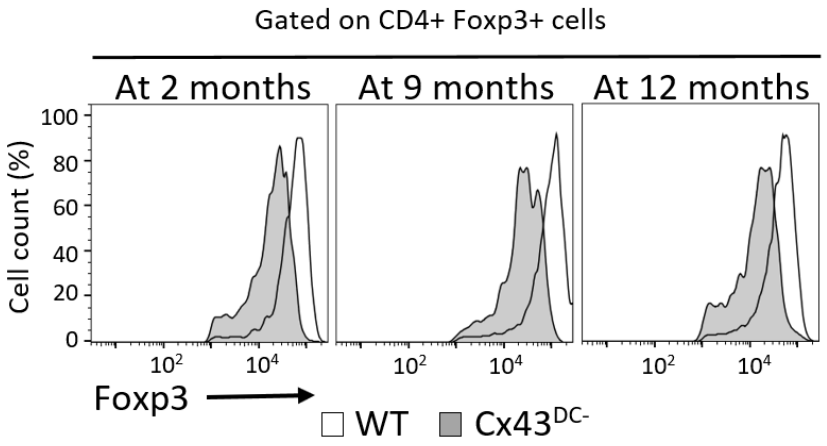

Fig. 6. Lower $CD4^+$ $Foxp3^+$ T_R cell population in $Cx43^{DC-}$ mice. Flow cytometry analysis of Foxp3 expression by gated $CD4^+$ $Foxp3^+$ cells showing that $Cx43^{DC-}$ mice had lower proportion of $Foxp3^+$ T_R cells compared to age-matched WT at 2 months, 9 months and 12 months.

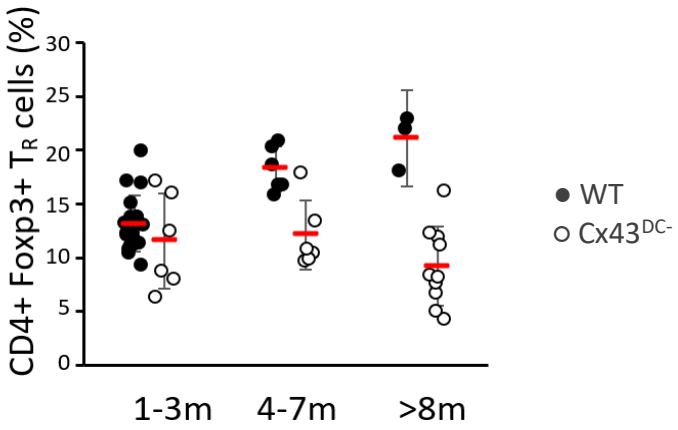

Fig. 7. Decrease in $CD4^+$ $Foxp3^+$ T_R population in $Cx43^{DC-}$ mice with respect to age. The percentage of T_R cells in lymph nodes of $Cx43^{DC-}$ mice decrease with respect to age categorized as 1-3 months, 4-7 months and greater than 8 months, based on flow cytometry data.

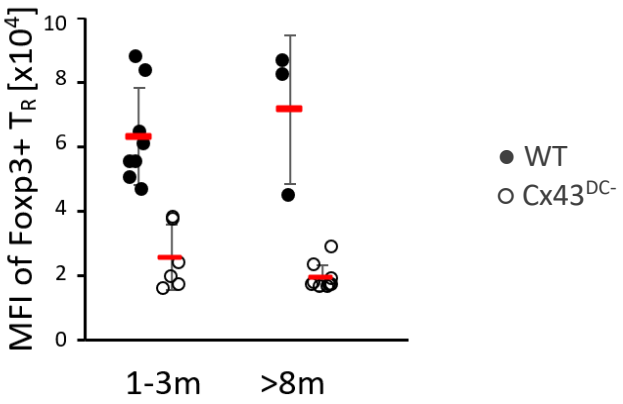

Fig. 8. Decrease in Foxp3 expression of $CD4^+$ $Foxp3^+$ T_R cells in $Cx43^{DC-}$ mice with respect to age. Mean fluorescence Intensity of Foxp3 expression of the T_R cells of $Cx43^{DC-}$ mice is also reduced, portraying a reduced expression of Foxp3 at 1-3 months of age and greater than 8 months of age based on flow cytometry data.

3.5. An altered phenotype of $CD4^+$ $Foxp3^+$ T_R is observed in $Cx43^{DC-}$ mice.

As mentioned earlier, CD39 and CD73 are ectonucleotidases on the surface of $Foxp3^+$ regulatory T cells. Flow cytometric data analysis of $Foxp3^+$ T_R cells of $Cx43^{DC-}$ mice showed that a large majority of the cells took on a $CD39^+$ $CD73^+$ phenotype (Fig. 9), unlike the populations of different level of expressions seen in WT mice. This population also had a decrease in CD39 expression and an increase in CD73 expression compared to the same population of WT mice (Fig. 10).

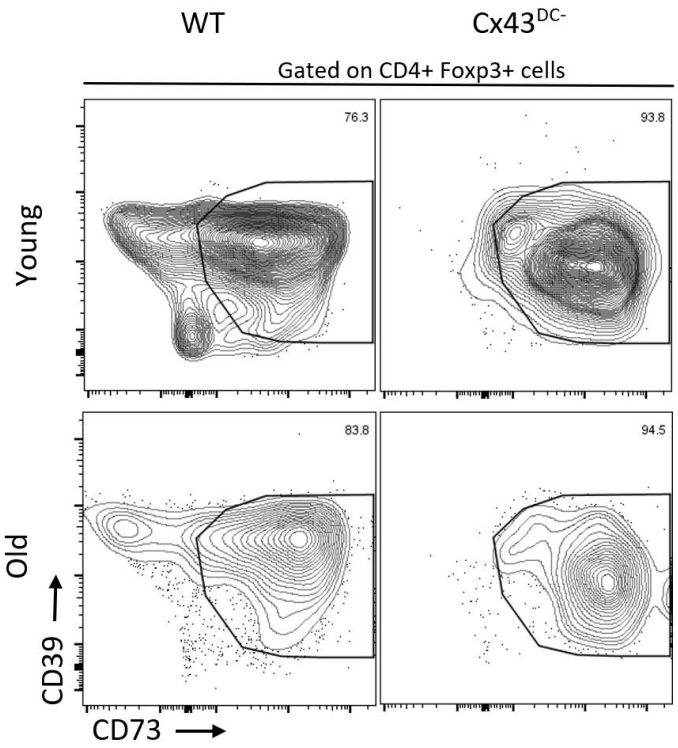

Fig. 9. Majority of $CD4^+$ $Foxp3^+$ cells of $Cx43^{DC-}$ mice have acquired $CD39^+$ $CD73^+$ phenotype. Flow cytometry data that shows that more than 90% of $CD4^+$ $Foxp3^+$ T_R cells from $Cx43^{DC-}$ mice have acquired $CD39^+$ $CD73^+$ phenotype at young (2 months) and old (12 months) age compared to age-matched WT.

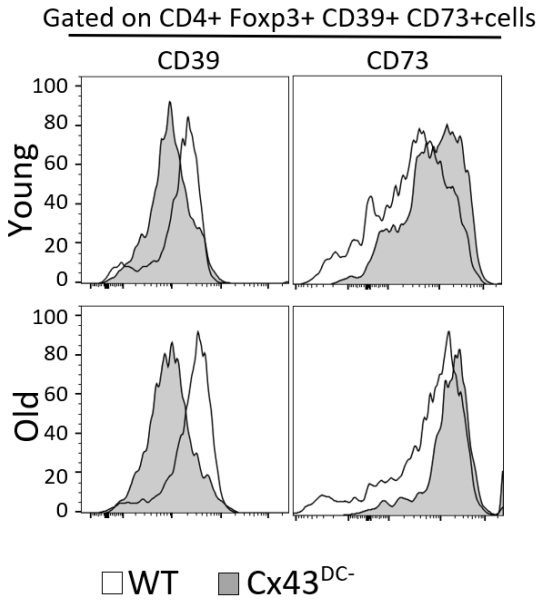

Fig. 10. $CD4^+$ $Foxp3^+$ T_R cells of $Cx43^{DC-}$ mice have altered expression of CD39 and CD73. The $CD4^+$ $Foxp3^+$ $CD39^+$ $CD73^+$ T_R population gated in Fig. 7 displays varied expressions of CD39 and CD73 among $Cx43^{DC-}$ and WT. There is reduced expression of CD39 and increased expression of CD73, measured by flow cytometry in both young and old mice.

3.6. $CD4^+$ $Foxp3^+$ T_R cells from $Cx43^{DC-}$ mice suppress the proliferation of $CD4^+$ cells.

It was proposed that presence of $CD4^+$ $Foxp3^+$ $CD39^+$ $CD73^+$ with altered phenotype might off-set the conversion of pro-inflammatory ATP to immunosuppressive adenosine and be responsible for a possible lack of immune suppression leading to the dermatitis seen on the mice. To check this, we set up a proliferation inhibition assay with APCs and $CD4^+$ naïve T cells along with increasing proportions of $CD4^+$ $Foxp3^+$ T_R cells. The splenocytes were taken from TCRαKO mice which did not have functioning T cells, and used as APCs in this assay. Soluble CD3 antibody was supplied to ensure activation of the $CD4^+$ naïve T cells. The amount of tritium labelled thymidine (^{3}H-thymidine) incorporated was used to measure proliferation of the cells. The results from this experiment show that the $CD4^+$ $Foxp3^+$ cells from the $Cx43^{DC-}$ mice suppressed the proliferation of $CD4^+$ naïve WT cells at a comparable level as $CD4^+$ $Foxp3^+$ cells from WT mice, with only a

slight decrease, as the readings were within one standard deviation of the WT and considered not significant (Fig. 11). Although the phenotype of the regulatory cells developed in $Cx43^{DC-}$ mice had altered levels of CD39 and CD73 expression, perhaps they were able to suppress using mechanisms of immunosuppression.

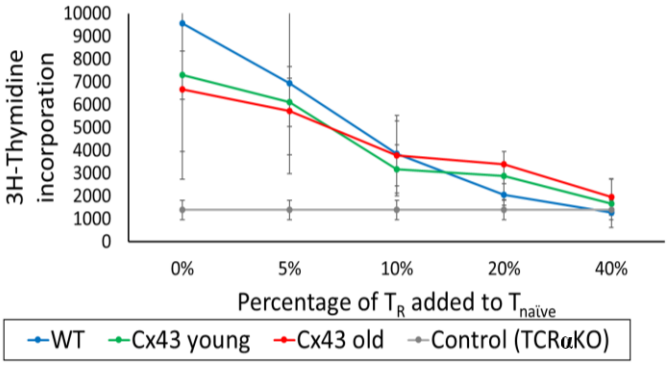

Fig. 11. Proliferation inhibition assay results. Data from the scintillation counter showing the proliferation of $CD4^+$ T cells on activation decreases when $CD4^+$ $Foxp3^+$ T_R cells are added to the well.

3.7. Prominent $CD4^+$ $Foxp3^-$ $CD25^+$ cell population is seen in $Cx43^{DC-}$ mice

Flow cytometry analysis showed that WT mice have less than one percent of $Foxp3^-$ $CD25^+$ cells at 2 months and at 12 months, whereas $Cx43^{DC-}$ have about 4% at 2 months and about 10% when they are 12 months or older (Fig. 12). CD25 is the IL-2 receptor required for differentiation into $CD4^+$ $Foxp3^+$ T_R cells in the presence of IL-2. There is a possibility that $Foxp3^+$ cells lose their Foxp3 expression while still expressing CD25 but are not immunosuppressive. Increased population of $CD25^+Foxp3^-$ cells is present in mice where T_R cell ontogeny is disrupted by deletion

of connexin gene in double positive thymocytes. Increased proportion of this population in $Cx43^{DC-}$ mice indicates that there is impaired communication between T_R and DC cells that affects T_R cell stability in the peripheral tissues. Thus, transition of $Foxp3^+$ cells to $Foxp3^-$ $CD25^+$ cells could be the cause for decreased proportion of $CD4^+$ $Foxp3^+$ T_R population in $Cx43^{DC-}$ mice. Cx43 expression by dendritic cells may be involved in triggering $CD4^+$ $Foxp3^+$ T cells that do not lose their Foxp3 expression.

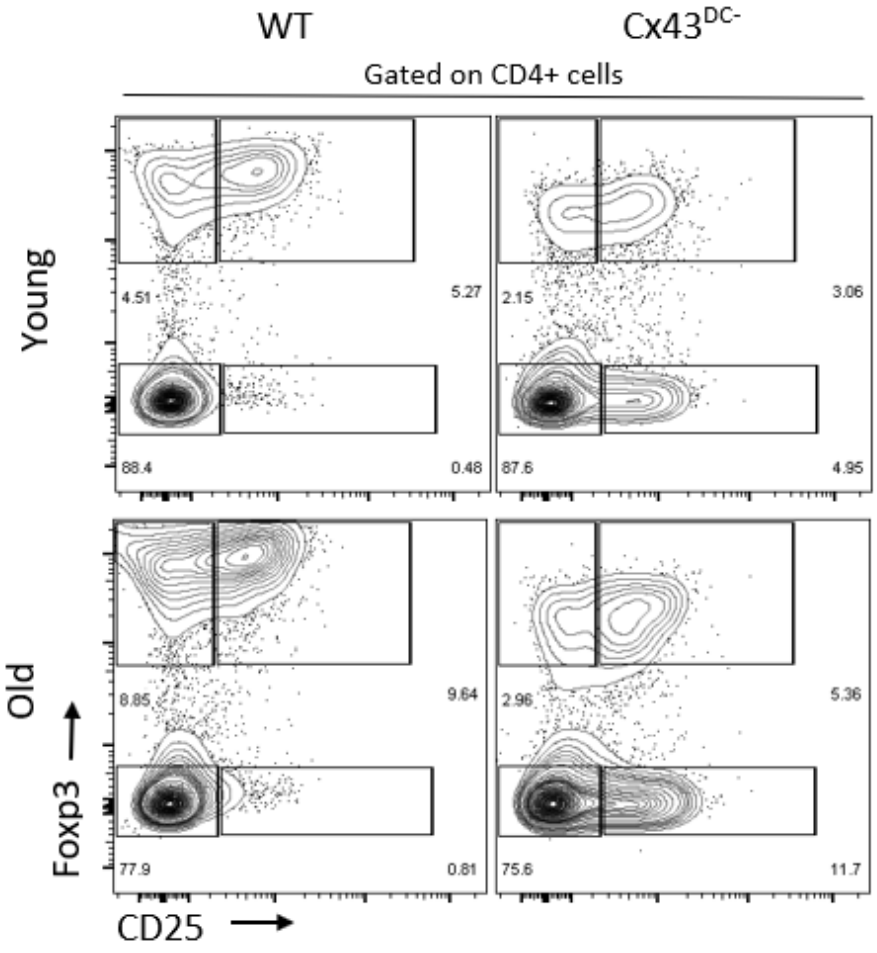

Fig. 12. Prominent presence of $CD4^+$ $Foxp3^-$ $CD25^+$ cells in $Cx43^{DC-}$ mice. Age-matched WT and $Cx43^{DC-}$ have differing proportions of $CD4^+$ $Foxp3^-$ $CD25^+$ cells shown by flow cytometry at young (2 months) and old (12 months) age.

3.8. $CD11c^+$ dendritic cells from $Cx43^{DC-}$ mice can present antigen effectively

$CD11c^+$ dendritic cells sorted from WT or $Cx43^{DC-}$ mice were added to a well with $CD4^+$ naïve T cells sorted from a mouse with transgenic TCR for a peptide PCC50V54A in a proliferation assay. After four days of incubation with the peptide, ^{3}H-Thymidine was added. The incorporation of ^{3}H-Thymidine was used to measure proliferation of $CD4^+$ that were successfully activated by $CD11c^+$ dendritic cells. Fig. 13 shows that $CD4^+$ T cells activated by DCs from $Cx43^{DC-}$ mice proliferate more vigorously than the same cells activated by wild type dendritic cells. This data shows that inhibition of communication between T_R cells and DCs also affect DCs by making them more effective antigen presenting cells. This further suggests that T_R cells modulate antigen presenting cells. Enhanced function of antigen presenting cells in $Cx43^{DC-}$ mice likely affects organs which are exposed to contact with environment like skin and gut airways. Enhanced responses to antigen could be a factor that promotes the skin inflammation observed in $Cx43^{DC-}$ mice. In the current assay, this could result in the enhanced proliferation of effector $CD4^+$ T cells.

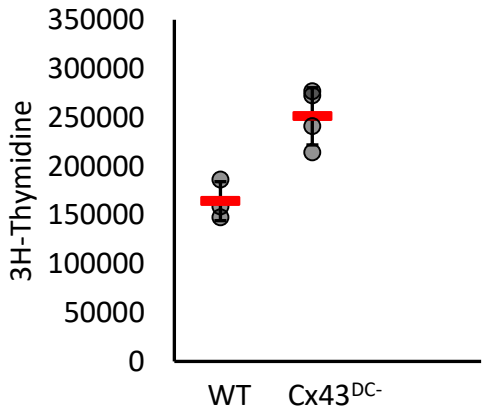

Fig. 13. Proliferation assay results. Data from the proliferation assay with $CD11c^+$ APCs from WT and $Cx43^{DC-}$ mice with $CD4^+$ T cells from transgenic mice specific for peptide PCC50V54A. ^{3}H-Thymidine measurements show that $CD11c^+$ DC from $Cx43^{DC-}$ triggered greater proliferation.

CHAPTER 4

DISCUSSION

4.1. Cx43 expression by $CD11c^+$ dendritic cells maintains $CD4^+$ $Foxp3^+$ cell population

Lack of Cx43 expression in $CD11c^+$ dendritic cells alone resulted in decrease in population of $CD4^+$ $Foxp3^+$ T_R, and a possible formation of dermatitis on the dorsal skin of the mouse. These findings support that of Yamazaki et al. (2003) in that it confirms that $CD11c^+$ dendritic cells maintain the T_R population *(26).* However, our data shows that in addition to the interactions between TCR on T_R cells and MHC on DCs are important for both cell populations and that the communications through gap junctions is most likely required for sustaining functions of both cell subsets.

CD25 is the IL-2 receptor required for differentiation into T_R cells. A prominent population of $CD4^+$ $Foxp3^-$ $CD25^+$ cells were seen in the lymph nodes of $Cx43^{DC-}$ mice. An explanation could be that the T_R cells that were formed in the periphery during activation lose their Foxp3 expression and may assume features of exT_R cells *(47, 48).* These cells are known to produce pro-inflammatory cytokines and promote autoimmune diseases. It is currently not known what promotes loss of Foxp3 and generation of exT_R cells. It is possible that the loss of Foxp3 by T_R cells, and thereby its immunosuppressive function, is the reason for the increase in proliferation seen in the proliferation assay where $CD11c^+$ DC from $Cx43^{DC-}$ mice presented PCC50V54A antigen to $CD4^+$ naïve T cells. In case of T_R cells that interacted with the peptide + MHCII on $CD11c^+$ DC that did not have Cx43 for proper signaling, T_R cells may not receive some, at present unknown, signal dependent on gap junction formation, leading to loss of Foxp3

expression. At the same time, Cx43 deficient DCs also loose T_R cell mediated tonic signal which conditions these cells to be over-stimulatory. Overtime, lack of interactions between DCs and T_R cells which involve Cx43 affects both populations altering their response to homeostatic interactions and antigenic stimulation.

We hypothesized that the $CD4^+$ $Foxp3^+$ T_R cell from $Cx43^{DC-}$ mice were not able to suppress proliferation due to the changes in CD39 and CD73 expression which may offset the conversion of pro-inflammatory ATP to immunosuppressive adenosine. However, the results of the proliferation inhibition assay show that these T_R cells suppressed the proliferation of activated $CD4^+$ T cells with only a slight decrease in immunosuppressive function. However, the proliferation inhibition assay used to examine the suppressor function of T_R cells from $Cx43^{DC-}$ mice may not be the most suitable assay. This was the first assay used to probe T_R cell function *in vitro* and there has been multiple examples when the short term suppressor function of T_R cells is preserved *in vitro* but *in vivo* functions are impaired.

Taken together, our results show that lack of Cx43 expression by dendritic cells does not affect the ability of these cells to present antigen to $CD4^+$ naïve T cells and activate them. However, the $CD4^+$ $Foxp3^+$ population was significantly reduced. Knowing that dendritic cells maintain the T_R cell population *(26)* and that Cx43 signaling enhances the generation of $Foxp3^+$ T_R cells *(39)*, we propose that Cx43 expression by $CD11c^+$ dendritic cells is required for maintaining $CD4^+$ $Foxp3^+$ T_R cell population.

4.2. Future Research

It is possible that $CD11c^+$ DC interact with an intermediary component that directly or indirectly maintain the T_R cell population, but there is no evidence of such a component as of now. We were not able to test the antigen-specific activation of regulatory T cells by antigen presenting cells because T_R cells with transgenic TCR do not make it through thymic selection. Thus, we were unable to run an in vitro test of aspects of the interaction between APC and antigen specific T_R cells. The findings of a study like this would be of great importance to our proposal of the role of Cx43.

Cx43 is undeniably a part of the immunological synapse and this has been proposed by multiple research studies. The results of application of αCT1, a mimetic peptide of Cx43, to increase wound healing mentioned earlier is a big example of this association. Our results implicate gap junction protein, Cx43 in the communication between $CD11c^+$ dendritic cells and $CD4^+$ $Foxp3^+$ T_R cells. It is possible that Cx43 is used by $CD4^+$ effector T cells or $CD4^+$ $Foxp3^+$ T cells to execute their immunological roles and this could be a topic of interest for future research. In addition, it is very likely that Cx43 is not the only connexin involved in maintaining regulatory T cells. Furthermore, looking into the Cx43 signaling pathway may shed some light onto the precise changes taking place at a molecular level that leads to maintenance of regulatory T cells.

Undeniably, understanding the cells that use or require Cx43 to directly or indirectly control auto-immune responses will open up pathways for targeted therapies that may correct one of the underlying factors of autoimmune diseases which is the low amount of $CD4^+$ $Foxp3^+$ T_R at the site of immune response.

CHAPTER 5

CONCLUSION

In the $Cx43^{DC-}$ mouse model, Connexin-43 molecule is not expressed by $CD11c^{+}$ dendritic cells and a higher probability of developing dermatitis was seen in these mice. The low population of T_R in these mice, support the notion that the skin disease may be autoimmune in nature. The ability of $CD11c^{+}$ dendritic cells to present antigen to naïve T cells were not affected by the loss of Cx43. However, the drastic decrease in T_R cell population and Foxp3 expression, combined with the altered $CD39^{+}$ $CD73^{+}$ phenotype, indicate that Cx43 is an important player in the communication between antigen presenting cells and the T_R cell subset. These results also suggest that TCR/MHC interaction along with defined co-stimulatory processes are not enough in itself, but require Cx43 expression by CD11c dendritic cells, to maintain immunosuppressive $CD4^{+}$ $Foxp3^{+}$ T_R cell population in the peripheral lymph nodes.

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APPENDIX

REAGENTS

0.16M Ammonium Chloride
NH_4Cl: 8.3g, KH_3CO: 1g, MiliQ added up to 1L with pH at 7.2.

Antibody Stains (eBioscience, BD Bioscience, BioLegend)

Fluorophores	**Antibodies for**					
APC	BTLA	CD11c	Tim3			
APC-Cy7	CD4	CD8	CD62L			
Biotinylated	CD8	CD39	CD49b	4-1BB	Klrg1	Ter119
BV421	CD8	CD25				
BV510	CD4					
FITC	MHCII					
PE	CD4	CD11c	CD44	CD73		
PE-Cy5	CXCR3	IL-7R	CD4			
PE-Cy7	CD4	PD1	Lag3			

BSS
NaCl 8g, KCl 400mg, $CaCl_2$ 140mg, $MgSO_4$.7H20 100mg, $MgCl_2$.6H_2O 100mg, Na_2HPO_4.2H_2O 60mg, KH_2PO_4 60mg, Glucose 1g, $NaHCO_3$ 350mg, MiliQ added up to 1L.

Collagenase IV (Worthington)

Complete Freund's Adjuvant (CFA) (Sigma-Aldrich)
Mixed with 1x PBS at 1:1 before administration

Complete Tumor Medium (CTM)
αMEM 500mL, "tumor cocktail" 50mL, FBS 50mL, L-glutamine (200mM) 5mL.
Tumor cocktail- αMEM 630mL, dextrose 7.5g, essential amino acids 50x (Cellgro) 200mL, endogenic amino acids 100x (Cellgro) 100mL, Sodium pyruvate 100x (Cellgro) 100mL, adjust pH to 7.0, NaHCO3 8.5g, Gentamycin (Gemini Bioproducts) 0.5g, Penicillin G 0.6g, Streptomycin 1g, β-mercaptoethanol 34uL.

Dispase I (Sigma-Aldrich)

DNase (Sigma-Aldrich)

FBS(Atlanta Biologicals)
Fetal Bovine Serum

1X PBS (Cellgro)
Dulbecco's Phosphate Buffered Saline without calcium and magnesium

PE Magnetic beads (Miltenyi Biotec)

RPMI (HyClone)
Roswell Park Memorial Institute growth medium 1640 with L-Glutamine

Streptavidin Magnetic beads (BD Bioscience)

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EDUCATION

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Certified Training Modules (2 month programs)
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Construction of Reporter Vectors- Wnt5a-EGFP and WNT5a-d2EGFP 2012
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Plant Tissue Culture 2011
Sangenomics Research Labs, Bengaluru, Karnataka, India
DNA Forensics 2010
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PUBLICATIONS

Keogh, M. B., Castro-Alférez, M., Polo-López, M. I., Fernández Calderero, I, Al-Eryani, Y. A., **Joseph-Titus, C**., Sawant, B., Dhodapkar, R., Mathur, C., McGuigan, K. G., Fernández-Ibáñez, P. *Capability of 19-L polycarbonate plastic water cooler containers for efficient solar water disinfection (SODIS): Field case studies in India, Bahrain and Spain.* 2015. Solar Energy **116**: p. 1-11

PRESENTATIONS

"**Expression of Connexin-43 by CD11c+ Dendritic Cells is Required for Maintaining CD4+ Foxp3+ Regulatory T Cell Population."** (Poster)
Old Dominion University's Graduate Research Achievement Day 2019
"**Connexin-43 expression by CD11c+ dendritic cells is required for maintaining CD4+ Foxp3+ regulatory T cell population"** (Oral)
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