EXPANSION AND CHARACTERIZATION OF HUMAN DOUBLE NEGATIVE REGULATORY T CELLS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science

University of Toronto

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Master of Science, 2018

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ABSTRACT

A subset of αβ-T cell receptor (TCR) positive, NK lineage marker negative, CD4−CD8− double negative regulatory T cells (DN Tregs) comprise only ~1% of peripheral blood T lymphocytes. Clinical applications of DN Tregs in humans are limited by their scarce number and the lack of effective expansion method. Herein is described a protocol for large-scale ex vivo expansion of functional and pure human DN Tregs. In vitro, expanded DN Tregs induce a cell-contact dependent immunosuppression of autologous T cells and B cells, and are cytotoxic towards various lung cancer, and leukemic cells. In vivo, infusion of DN Tregs delays an onset of xenogeneic graft-versus-host disease (GVHD) in humanized mouse model. Furthermore, short treatment of expanded DN Tregs with rapamycin augments their suppressive function. Taken together, these results indicate a dual function of ex vivo expanded DN Tregs and suggest their therapeutic potential in suppression of allograft rejection and treatment of malignancies.
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CONTRIBUTIONS

Paulina Achita designed the experiments, acquired, analyzed and interpreted the data under direct supervision from Dr. Li Zhang. Jong Bok Lee, Betty Joe and Tabea Huag contributed to the acquisition of the data. Dr. Dzana Dervovic and Dr. Dalam Ly contributed to the analysis and the interpretation of the data. Canadian Institute of Health Research has funded this study, and Paulina Achita was a recipient of Ontario Graduate Scholarship.
TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................................................. iii

CONTRIBUTIONS ....................................................................................................................... v

TABLE OF CONTENTS ................................................................................................................ vi

LIST OF ABBREVIATIONS .......................................................................................................... xi

LIST OF FIGURES ....................................................................................................................... xiv

LIST OF TABLES ........................................................................................................................ xvi

CHAPTER 1. INTRODUCTION ................................................................................................. 1

1.1. Overview of the Immune System ......................................................................................... 2

1.2. Tolerance Mechanisms ...................................................................................................... 4

1.2.1. Central Tolerance ........................................................................................................... 4

1.2.2. Peripheral Tolerance ..................................................................................................... 5

1.3. Overview of Tregs ............................................................................................................... 7

1.3.1. nTregs ........................................................................................................................... 8

1.3.1.1. Development of nTregs .......................................................................................... 9

1.3.1.2. Mechanisms of nTreg Suppression ....................................................................... 9

1.3.2. CD4+ iTregs .................................................................................................................. 14

1.3.2.1. Th3 Cells ................................................................................................................ 15

1.3.2.2. Tr1 Cells ................................................................................................................ 15
CHAPTER 2. METHODS

2.1. Blood Samples .................................................................................................................. 37
2.2. Cell Isolation and Magnetic Sorting ................................................................................. 37
2.3. Freezing and Thawing of Cells ......................................................................................... 38
2.4. Expansion of DN Tregs .................................................................................................... 38
2.5. Antibodies and Flow Cytometry ....................................................................................... 39
2.6. Detection of Cytokines and Chemokines Secreted by DN Tregs ......................... 41
2.7. *In Vitro* T cell and B cell Suppression Assays .............................................................. 41
2.8. *In Vitro* Suppression Assays with Rapamycin-treated DN Tregs ...................... 42
2.9. Transwell® Experiments ................................................................................................ 42
2.10. Lymphocyte Cytotoxicity Assay .................................................................................... 43
2.11. Cancer Cells Cytotoxicity Assay ................................................................................... 43
2.12. Mice and Xenogeneic GVHD Model ............................................................................ 44
2.13. Monitoring of Lymphocyte Migration and Proliferation *In Vivo* .................... 44
2.14. Data Analysis ................................................................................................................ 45

CHAPTER 3. RESULTS .............................................................................................................. 46

3.1. Frequencies and Phenotypic Analysis of TCRαβ⁺ CD3⁺ CD4⁻ CD8⁻ DN Tregs in the Peripheral Blood of Healthy Adults .................................................... 47
3.2. Human DN Tregs Can Be Expanded *Ex Vivo* .............................................................. 51
3.3. Supplementation of IL-7 During Expansion Enhance Proliferation and Suppressive Function of DN Tregs ................................................................. 54
3.4. *Ex Vivo* Expanded DN Tregs are Potent Suppressors *In Vitro* ....56

3.5. Phenotype of *Ex Vivo* Expanded DN Tregs .........................................58

3.6. Cytokine Profile of *Ex Vivo* Expanded DN Tregs ..................................60

3.7. DN Treg-mediated Suppression Is Not Facilitated by IFN-γ or IL-10 Cytokines and Requires Cell-to-Cell Contact .................................................................62

3.8. DN Tregs Do Not Suppress by Killing Responder Cells ......................69

3.9. *Ex Vivo* Expanded DN Tregs Kill Human Cancer Cells .....................71

3.10. Spatial and Temporal Dynamics of Human DN Tregs *In Vivo* ............73

3.11. *Ex Vivo* Expanded DN Tregs Delayed Onset of Xenogeneic GVHD in NSG Mice ........................................................................................................75

3.12. Rapamycin Augmented Immunosuppressive Function of DN Tregs *In Vitro* and *In Vivo* .........................................................................................78

CHAPTER 4. DISCUSSION ..................................................................................82

4.1. General Discussion ..................................................................................83

4.2. Future Directions ....................................................................................93

4.2.1. To Identify Markers That Are Critical for DN Treg Function ..........94

4.2.2. To Determine the Effects of DN Treg-Immunosuppressive Agents Combination Therapy on the Treatment of Xenogeneic GVHD ....95

4.2.3. To Determine Whether Human DN Tregs Suppress DCs .................96

4.2.4. To Determine Whether Trogocytosis is Critical for Human DN Treg Function .................................................................................................97
4.2.5. To Determine Signalling Pathways That Govern DN Tregs ...............98

4.3. Conclusion .........................................................................................99

REFERENCES ............................................................................................101
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>aAPC</td>
<td>Artificial antigen presenting cell</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-chloride-potassium</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cellular therapy</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>Allo</td>
<td>Allogeneic</td>
</tr>
<tr>
<td>ALPS</td>
<td>Autoimmune lymphoproliferative syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell or Allophycocyanin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B cell lymphoma extra-large</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplant</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>5,6-carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>Fgl-2</td>
<td>Fibrinogen-like protein 2</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence-minus-one</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft-versus-leukemia</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplant</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible co-stimulator</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immunodysregulation, polyendocrinopathy, enteropathy, X-linked</td>
</tr>
<tr>
<td>iTreg</td>
<td>Inducible regulatory T cells</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte-activation gene 3</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LPR</td>
<td>Lymphoproliferation</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MST</td>
<td>Median survival time</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NOX2</td>
<td>NADPH oxidase 2</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD/SCID IL-2Rgnull</td>
</tr>
<tr>
<td>nTreg</td>
<td>Naturally-occurring regulatory T cells</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated-molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Peptide-derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll A protein</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SOT</td>
<td>Solid organ transplant</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Tconv</td>
<td>Conventional T cell</td>
</tr>
<tr>
<td>Teff</td>
<td>Effector T cell</td>
</tr>
<tr>
<td>T1D</td>
<td>Type one diabetes mellitus</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>Th3</td>
<td>T helper type 3</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TREC</td>
<td>T-cell receptor excision circles</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>Tr1</td>
<td>Type 1 regulatory T cell</td>
</tr>
<tr>
<td>Tsup</td>
<td>Suppressor T cell</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical cord blood</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Phenotypic characteristics of DN Tregs isolated from peripheral blood. ........ 48

Figure 2. Cell surface expression of memory markers CCR7 and CD45RO on peripheral blood T cells. ........................................................................................................50

Figure 3. Schematic representation of the method for ex vivo expansion of DN Tregs.... 52

Figure 4. Isolation and expansion of DN Tregs. .......................................................... 53

Figure 5. The effect of supplementation of IL-7 and IL-15 during the expansion on DN Treg proliferation and function. ................................................................. 55

Figure 6. DN Tregs suppress proliferation of autologous T cells, and CD19+ B cells..... 57

Figure 7. Phenotypic characteristics of ex vivo expanded DN Tregs. ......................... 59

Figure 8. Cytokine profile of DN Tregs. ........................................................................ 61

Figure 9. Addition of IL-2 and/or IL-7 directly to the suppression assay does not impair functionality of DN Tregs. ................................................................. 63

Figure 10. DN Tregs produce IL-10 and IFN-γ............................................................. 64

Figure 11. Role of IFN-γ and IL-10 in the mechanism of DN Treg suppression........... 65

Figure 12. Mechanism of inhibition mediated by DN Tregs requires cell-to-cell contact. .................................................................................................................. 67

Figure 13. DN Tregs suppress secretion of IFN-γ by CD4+ T cells and CD8+ T cells. .... 68

Figure 14. DN Tregs do not kill autologous CD4+ or CD8+ T cells. ......................... 70

Figure 15. Ex vivo expanded DN Tregs can kill human cancer cells. ....................... 72
Figure 16. Tracking and proliferation of human lymphocytes adoptively transferred to NSG mice................................................................. 74

Figure 17. Schematic protocol of the xenogeneic GVHD experiment................................. 76

Figure 18. Treatment with DN Tregs delayed the onset of xenogeneic GVHD. .............. 77

Figure 19. Rapamycin-treated DN Tregs manifest augmented regulatory function. ........ 80

Figure 20. Rapamycin-treated DN Tregs delayed the onset of xenogeneic GVHD......... 81
LIST OF TABLES

Table 1. List of the antibodies used in this study. .............................................................. 40
CHAPTER 1. INTRODUCTION
Chapter 1

Introduction

1.1. Overview of the Immune System

The immune system encompasses many biological structures and processes that are fundamental for protecting the organism from pathogens, and for distinguishing those pathogens from the body’s healthy tissue. The immune system has been simplistically subdivided into two “lines of defense”: the innate immunity and the adaptive immunity. Although the two classes of the immune system have evolved independently, they are not mutually exclusive, but rather complementary. Defects in either system, although rare, result in host vulnerability to infections, malignancy, autoimmunity and immunodeficiency disorders.

The innate subdivision of the immune system represents the first line of defense against intruding pathogens and environmental agents. The underlying defense mechanisms include anatomical barriers such as skin and mucosal tissues, and physiological barriers such as acidic pH of the stomach, the onset of fever during the infection, or activation of the complement system. The innate response evolved to non-specifically recognize molecules that are evolutionary conserved amongst the broad groups of microbes, referred to as pathogen-associated-molecular patterns (PAMPs), through pattern recognition receptors (PRRs) (Mogensen, 2009). These receptors are present on the cell surface of innate immune cells, such as dendritic cells, macrophages and neutrophils, and the most extensively studied are Toll-like receptors (TLRs) (Kawai and Akira, 2010). Activation of immune cells via PRRs leads to synthesis of pro-inflammatory cytokines, chemokines, cell adhesion molecules, and other anti-microbial agents, which ultimately lead to elimination of pathogens, and priming of the adaptive immune response (Mogensen, 2009). Traditionally, the innate immune system was classified as incapable of providing immunological memory. However, a growing body of evidence suggests that the innate immune memory may be initiated upon priming
event during the first exposure (Netea et al., 2011), but preserves for a much shorter time period than the second division of the immune system (Netea et al., 2015), known as adaptive or acquired immunity.

The adaptive immune system has evolved sophisticated mechanisms to recognize and fight a broad range of pathogens. The cells of paramount importance to the adaptive immune system are T lymphocytes and B lymphocytes, which are capable of specific elimination of pathogens via recognition of their antigens, which are unique molecular structures distinctive for each pathogen. These cells express unique receptors on their cell surface: T cell receptors (TCR) on T cells and B cell receptors (BCR) on B cells. TCR and BCR possess enormous repertoire diversity due to somatic recombination that occurs during the development of T cells in the thymus, and B cells in the bone marrow, allowing specific recognition of antigens from nearly all pathogens. At the initial contact with a pathogen, there is a lag time between exposure and mounting maximal immune response, because naïve lymphocytes must first encounter the antigen in periphery, followed by activation of lymphocytes and differentiation into effector, or long-lived memory cells. Thanks to the immunological memory, subsequent encounters with the same pathogen will enable the host to mount a more rapid and efficient response.

Activation of the innate immunity plays a crucial role in stimulating the adaptive immunity (Kumagai and Akira, 2010; Netea et al., 2015). Innate immune cells migrate towards the source of infection and discharge a vast array of antimicrobial weaponry, which in turn facilitates recruitment of other immune cells to the injury site. The key mediators between the innate and the adaptive immunity are dendritic cells (DC), which together with macrophages and B cells, belong to a class of professional antigen presenting cells (APCs). APCs digest antigens into smaller fragments and display the peptide fragments coupled with major histocompatibility complexes (MHC) on their cell surface in a process known as ‘antigen presentation.’ All nucleated cells present endogenous peptides on MHC class I molecules, whereas APCs may also internalize extracellular peptides and present it on MHC class II molecules. T cells can then recognize these complexes using TCR on their cell surface.
T cells can be divided into two main groups based on the type of MHC-peptide complexes they recognize, which is concurrent with differential expression of TCR co-receptors on their cell surface. Thus T cells that express CD8 co-receptor recognize antigens that are presented by MHC class I molecules. These cells differentiate into cytotoxic T cells whose function is to destroy infected cells by killing. On the other hand, T cells that express CD4 co-receptor recognize peptides presented by MHC class II molecules and release cytokines, and growth factors that regulate other immune cells. Depending on the cytokine milieu that CD4\(^+\) T cells encounter during TCR activation, naïve CD4\(^+\) T cells may differentiate into several lineages of T helper (Th) and T regulatory (Treg) cells, which are defined by their function and pattern of cytokine production.

However, the TCR-MHC engagement may not be robust enough to effectively activate T cells. In order to decrease the activation threshold APCs must present a broad range of co-stimulatory molecules that will bind to the corresponding receptors on the T cells. The activation, however, must be tightly regulated to ensure that activation is resolved following the combat of pathogens by the T cells. Furthermore, T cells that recognize self-antigens must be eliminated to avoid mounting an attack against the host organism. Fortunately, the immune system has evolved multiple mechanisms to ensure immune homeostasis and suppression of the immune responses against self-antigens.

### 1.2. Tolerance Mechanisms

#### 1.2.1. Central Tolerance

Numerous tolerance mechanisms exist to prevent detrimental self-reactivity. The first checkpoint of self-tolerance happens in the thymus during the final stages of T cell development, but before the maturation and circulation of T cells. The primary mechanism of central tolerance is ‘negative selection’, also known as ‘clonal deletion,’ which was first proposed by Frank MacFarlane Burnet who ultimately shared the Noble
Prize in 1960 with Peter Medawar for their work on immunological tolerance - a phenomenon explained by clonal deletion (Liston, 2011). Since somatic recombination produces TCRs of enormous diversity, it would be virtually impossible to avoid production of self-reactive TCRs. Therefore, a vast majority of the T cell precursors that express TCRs with high avidity for self-MHC complexes are eliminated via apoptosis; only those T cell precursors that express low avidity TCRs for self-peptides will undergo maturation and reach the periphery (Starr et al., 2003). Furthermore, some thymocytes with high-avidity TCRs for self-peptides may avoid apoptosis by internalization of TCR in order to undergo a secondary gene rearrangement to change the specificity of their TCRα locus (McGargill et al., 2000; Wang et al., 1998). This process is known as ‘receptor editing’ and replaces a self-reactive TCR with a new, non-reactive one.

### 1.2.2. Peripheral Tolerance

Some autoreactive T cells may not have sufficient affinity for self-antigens and the thymic epithelial cells may not express all the possible self-antigens. Therefore, these low-avidity autoreactive T cells may escape central tolerance mechanisms and migrate to the periphery. When left unchecked, the activation of these cells may lead to the development of autoimmune disease. Fortunately, several peripheral tolerance mechanisms evolved to restrain both the number and the function of autoreactive T cells. These mechanisms include anatomical isolation, cellular inactivation, deletion by activation-induced cell death, or direct suppression of self-reactive T cells by specialized cells (Mueller, 2010).

*Immune-privileged sites.*

Certain specialized tissues in the human body are able to tolerate the introduction of antigens without evoking an inflammatory response against them. In these sites tissue allografts are readily accepted, and thus termed ‘privileged sites’ for having an advantage over other sites, where usual immune response would be mounted (Forrester et al., 2008). The privileged sites offer necessary protection to tissues where the inflammatory response could cause permanent damage as it is in the case of brain, eyes, testes, placenta
and fetus. The anatomy of these sites limits the access to lymphatic drainage, thus restraining the immune cells from entering such sites. Although the immune cells can access these sites through circulation, markedly fewer cells from these sites can reach the lymphatic organs. Some sites may be characterized by low expression of classical MHC class I molecules, expression of immunoregulatory molecules and secretion of immunosuppressive mediators that aid in the maintenance of immune privilege (Forrester et al., 2008). An interesting phenomenon became evident through animal models, where tolerance to antigen introduced in the privileged sites, such as brain or eye, was transferable to other sites in an antigen (Ag)-specific manner (Galea et al., 2007; Medawar, 1948).

**Clonal anergy.**

Effective activation of T cells requires simultaneous delivery of Ag-specific signals and co-stimulatory signals. Anergy occurs when TCR recognizes peptide-MHC complexes on tissues that lack co-stimulatory molecules, or display co-inhibitory molecules. Instead of activation, such cells are induced into a state of long-term hypo-responsiveness characterized by active repression of TCR signalling and thus the inability to perform any effector functions (Wells, 2009).

**Activation-induced cell death.**

Autoreactive T cells chronically engaged by self-peptide-MHC complexes can also die by apoptosis. This activation-induced cell death is a result of the combination of two molecular mechanisms: Fas receptor engagement with FasL, and Bim-dependent sequestering of a B cell lymphoma 2 (Bcl-2)- and B cell lymphoma extra-large (Bcl-xL)-regulated mitochondrial death pathway (Mueller, 2010). Activated T cells upregulate Fas on their cell surface and apoptosis is triggered upon engagement with cells expressing FasL. Activated T cells that upregulate both Fas and FasL may also kill each other directly.

**Control by regulatory T cells.**
Specialized immune cells termed regulatory T cells (Tregs) can actively suppress self-reactive cells through many different mechanisms depending on the cell type involved (discussed in the subsequent chapters). Tregs can be described as highly heterogeneous population of cells of different developmental origins. They are crucial in controlling adaptive immune responses ranging from autoimmune diseases to inflammatory conditions.

Many mechanisms are responsible for eliciting immunological tolerance. However, this state of unresponsiveness may be deleterious under certain circumstances. The best examples of undesired tolerance are observed in the tumour microenvironments, where excessive tolerance weakens immune response against tumour antigens, or during chronic infections, where microbial or viral agents can be no longer eliminated. On the other hand, these tolerance mechanisms can be harnessed to restrain autoimmune diseases, graft rejections, and allergies. Considering all the different mechanisms discussed earlier, Tregs represent the most feasible component of the immune system to be exploited for clinical applications.

1.3. Overview of Tregs

In 1971, Gershon and Kondo published the first paper suggesting that thymus-derived lymphocytes were required for tolerance induction (Gershon and Kondo, 1971). The adoptive transfer of such cells from mice that were tolerated to the particular antigen (sheep red blood cells) to naïve mice prevented the latter to respond to immunizing doses of such antigens. The authors named this phenomenon “infectious immunological tolerance” and suggested that these cells were Ag-specific, and produced “immunosuppressive substances” as their mode of action (Germain, 2008). Many subsequent studies and compelling “logic of the argument that the immune system needed suitable brakes to prevent excessive activity” resulted in recognition of the importance of this subset of cells by the scientific community, and these cells were termed suppressor T cells (Tsup) (Germain, 2008; Kapp, 2008). However, interest in
Tsup fell drastically in the mid 1980s leading to downfall of the field for many reasons, starting with the inability to generate stable long-term Tsup lines, the incapacity to identify soluble factors mediating immunosuppression, negative findings regarding identifying unique surface markers and transcription factors, and lastly, the shift in the interests of immunological community to rather identify pivotal molecular elements of the immune system (Ligocki and Niederkorn, 2015; Moller, 1988).

Sakaguchi resurrected interest in the suppressive lymphocytes in 1995 demonstrating that a small subset of CD4+ T cells that co-express IL-2Rα (CD25) function as Tsup (Sakaguchi et al., 1995). Depletion of CD4⁺CD25⁺ cells in the normal mice resulted in the spectrum of autoimmune diseases, when remaining CD4⁺CD25⁻ cells were transferred to the immunodeficient mice (Sakaguchi et al., 1995). Further studies demonstrated the immunosuppressive activity of CD4⁺CD25⁺ in vitro and multiple groups corroborated presence and function of these cells in humans (Baecher-Allan et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Ng et al., 2001). With the ability to finally isolate these cells and to avoid the negative connotation around Tsup, these cells came to be known as Tregs.

Tregs can be classified into two groups based on their developmental origin: naturally occurring (n) Tregs that differentiate in the thymus and inducible (i) Tregs that differentiate in the periphery.

1.3.1. nTregs

Subsequent studies showed that the forkhead box P3 (Foxp3) transcription factor is not only an essential intracellular marker but also an absolute requirement for function and developmental fate of nTregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). The importance of Foxp3 in the development of Tregs, as well as the importance of Tregs themselves, have been shown in scurvy mice and in human with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome who develop lethal autoimmune disease as the result of mutation in Foxp3 and thus Treg deficiency (Bennett et al., 2001; Brunkow et al., 2001). Majority of IPEX patients expect to die
within the first year of life as uncontrolled activation and clonal expansion of CD4+ cells results in autoimmune enteropathy, type-1 diabetes mellitus, infections and cutaneous manifestations, such as lesions and severe eczema (Barzaghi et al., 2012).

Undeniable evidence that CD4+CD25+Foxp3+ cells are committed to regulatory lineage during development in the thymus gave rise to term ‘naturally occurring Tregs’ (nTregs). The importance of nTregs was evident in mice who received neonatal thymectomy three days after birth, which resulted in abrogated production of nTregs and multi-organ autoimmune disease (Sakaguchi et al., 2007).

1.3.1.1. Development of nTregs

Since the function of nTregs is drastically different from that of Tconv cells, it should be no surprise that nTregs follow developmental pathway different from that of Tconv cells. During the development, most of double-positive thymocytes are unable to recognize self-MHC complexes and thus die by apoptosis. Those cells whose TCR engages with self-MHC complexes mature and become single-positive thymocytes expressing either CD4 or CD8 co-receptor. The next step involves negative selection (reviewed in section 1.2.1.), where the cells with high avidity or specificity for self-peptides are eliminated (Hogquist et al., 2005). Only small proportion of the cells that have low avidity for self-peptides leave the thymus to become Tconv cells; however, cells at the single-positive stage with intermediate avidity for TCR complexes may become nTregs (Kronenberg and Rudensky, 2005).

1.3.1.2. Mechanisms of nTreg Suppression

nTregs use numerous tools to suppress a large number of different target cells including T cells, B cells, DCs, macrophages, osteoblasts, mast cells, NK cells and NKT cells (Shevach, 2009). Shevach suggested that these mechanisms can be broadly divided to those that target T cells, such as production of anti-inflammatory cytokines, killing the target cells and cytokine consumption, and those that target APCs, which includes decreasing co-stimulation or antigen presentation (Shevach, 2009). The mechanisms
listed below are not mutually exclusive and many mechanisms may be used simultaneously.

**Inhibitory Cell Surface Molecules.**

nTregs may express on their cell surface a variety of inhibitory molecules whose role is to dampen immune response. One of the most extensively studied molecules on nTregs is a constitutively expressed cytotoxic T cell lymphocyte 4 (CTLA-4) receptor, but on other T cell subsets only gets upregulated upon activation (Read *et al.*, 2000; Salomon *et al.*, 2000; Takahashi *et al.*, 2000). Interestingly, it is the Foxp3 transcription factor that controls the expression of CTLA-4 in Tregs (Hori *et al.*, 2003; Marson *et al.*, 2007; Ono *et al.*, 2007; Wu *et al.*, 2006; Zheng *et al.*, 2007). Wing *et al.* has found that CTLA-4 is crucial for Treg function as its deficiency impairs the regulatory function of nTregs *in vitro*, and *in vivo* has been shown to induce systemic lymphoproliferation, lethal autoimmune disease mediated by T cells and more effective immunity against tumours (Wing *et al.*, 2008). CTLA-4 acts as an immune checkpoint by direct binding with CD80 and CD86 on Teff cells to inhibit expansion of these cells *in vivo* (Paust *et al.*, 2004), or by down-regulating CD80 and CD86 expression on DCs and thus inhibiting the activation of Teff cells by DCs (Cederbom *et al.*, 2000).

PD-1:PD-ligand 1 (PD-L1) pathway has also been strongly associated with nTreg function, control of multiple tolerance checkpoints and is indispensable in delivering inhibitory signals in persistent antigen stimulation, tumours, chronic infections, and play an important role in T cell activation, tolerance and inflammatory-induced tissue damage (discussed in great detail by (Francisco *et al.*, 2010)). Since PD-1:PD-L1 pathway is commonly used by a multitude of different cells, the intricacies of this pathways will not be discussed here. However, it is important to mention that PD-1 ligation in the presence of TCR stimulation and TGF-β may transform naïve T cells into Tregs. This is especially detrimental in cancer setting where nTregs perpetuate highly immune-suppressive environment preventing the body to fight cancer on its own. Blockade of this pathway in cancer patients by administrating the antibodies against PD-1 or PD-L1, as well as CTLA-4, show promising results in the treatment of various cancers, started being used
routinely in the clinic and there are multiple ongoing clinical trials around the world (Iwai et al., 2017).

Lymphocyte-activation gene 3 (LAG-3), a CD4-related molecule, is another inhibitory cell surface marker that has been associated with suppressive mechanisms of nTregs through direct Teff-Treg interactions or by modulation of APC function. The latter mechanism, involves binding of LAG-3 to MHC class II molecules on DCs, which results in downregulation of the expression of co-stimulatory molecules on DCs (Liang et al., 2008). Furthermore, CD4\(^+\)CD25\(^+\) Tregs from LAG-3 knockout mice manifest reduced regulatory activity, which can be restored by ectopic expression of LAG-3 via retroviral transduction on CD4\(^+\) T cells (Huang et al., 2004). However, a recent study suggests that LAG-3 may also negatively impact proliferation of Tregs and their function at the sites of inflammation by downregulation of CD25 (Zhang et al., 2017).

An inhibitory molecule TIGIT were found to be expressed on activated Tregs and directly suppress immune responses of Th1 and Th17 cells, as well as induce production of fibrinogen-like protein 2 (Fgl-2) (Joller et al., 2014). Fgl-2 production also contributes to nTreg-mediated immunosuppression as Fgl2/- knock out mice develop autoimmune glomerulonephritis due to inability of Tregs to mount immunosuppression despite their higher frequency (Shalev et al., 2008). Binding of TIGIT with the DC surface ligand CD155 (also known as poliovirus receptor) can also inhibit T cell responses (Yu et al., 2009).

**Killing.**

Both human and murine nTregs may also directly kill autologous CD4\(^+\) and CD8\(^+\) T cells, CD14\(^+\) monocytes and mature, and immature DCs using perforin/granzyme-dependent pathway (Grossman et al., 2004). However nTreg do not kill by Fas/FasL or TNF-related apoptosis-inducing ligand (TRAIL) (Shevach et al., 2006). nTregs were also found to preferentially kill Ag-presenting B cells, but not bystander B cells (Zhao et al., 2006).

**Ectonucleotidase Activity.**
Deaglio et al. identified that co-expression of ENTPD1 and ecto-5'-nucleotidase, known as CD39 and CD73, respectively, is unique to nTregs population among T cells (Deaglio et al., 2007). CD39 is critical to nTreg function, as CD39-deficient mice showed diminished immunosuppressive function in vitro and were unable to prevent allograft rejection in vivo (Deaglio et al., 2007). Together, CD39 and CD73 degrade extracellular ATP, ADP, and AMP to generate pericellular adenosine. Adenosine reduces activation in Teff cells by binding to A2A receptor, which is upregulated in T cells upon antigenic stimulation, and thus the presence of adenosine reduces production and release of pro-inflammatory cytokines by Teff cells (Ohta and Sitkovsky, 2001). This process can be viewed as “immunological switch” that shifts ATP-driven pro-inflammatory state to an anti-inflammatory state driven by the presence of adenosine (Antonioli et al., 2013). Moreover, adenosine may participate in a positive feedback loop as nTregs also upregulate A2A receptor expression upon TCR stimulation (Deaglio et al., 2007). In contrast to Teff cells, adenosine binds to A2A receptor on nTregs, which in turn drives the proliferation of Tregs and further influences their immunoregulatory function (Ohta et al., 2012; Zarek et al., 2008). In humans, 90% of Foxp3+ Tregs have surface expression of CD39 and although surface expression of CD73 on Tregs is insignificant, it is abundantly present in the cytoplasm (Mandapathil et al., 2010).

Cytokine Depletion.

nTregs constitutively express CD25, a subunit of IL-2R, but are unable to produce IL-2 (Chinen et al., 2016). On this basis alone, researchers suspected that consumption of IL-2 produced by Teff could be a major force behind Treg suppression (Pandiyan et al., 2007; Thornton et al., 2004). However, equally many studies emerged that disputed this view and have shown that IL-2 consumption is not critical for Treg function. For example, in a transwell setting where a membrane separates Tregs from Teff cells abrogates regulatory function of Tregs (Thornton and Shevach, 1998). Hofer et al. argues that it likely all comes down to spatio-temporal logistics of IL-2 consumption competition between Tregs and Teff. Depending how quickly activated T cells can upregulate CD25 on their cell surface to consume autocrine/paracrine IL-2, within this
window Tregs may reduce activation by consuming IL-2 given they are in close proximity and high density (Hofer et al., 2012).

**Anti-inflammatory Cytokines.**

Upon activation, nTregs produce an arsenal of potent anti-inflammatory cytokines with IL-10, TGF-β and IL-35 playing pivotal roles.

The role of TGF-β in immune homeostasis has been long known, as addition of anti-TGF-β antibody abolishes suppression mediated by nTregs (Nakamura et al., 2001) and mice deficient in TGF-β or molecules required for TGF-β signalling develop systemic autoimmune disease (Li et al., 2006). However, Tregs themselves do not have to produce TGF-β, because they can induce TGF-β production in other immune cells (Kullberg et al., 2005). Furthermore, TGF-β produced by nTregs may also induce expression of Foxp3 in Tconv cells rendering them regulatory (Chen and Konkel, 2010). Inactive form of TGF-β is maintained on the surface of nTregs through binding with latency-associated peptide (LAP) (Annes et al., 2003). This latent TGF-β is responsible for converting Foxp3− to Foxp3+ cells upon TCR stimulation (Andersson et al., 2008).

IL-10 producing Tregs have been found indispensable in many in vivo models of inflammation and homeostatic expansion, and have also been implicated in maintaining the balance between pathogen elimination and immunopathology in viral, fungal and parasitic infections (Couper et al., 2008). For example, administration of anti-IL-10 Ab abrogates the ability of Tregs to inhibit intestinal colitis (Asseman et al., 1999), but the suppression is dependent on the differentiation status of Teff cells with naïve Teff cells being unresponsive to IL-10 (Asseman et al., 2003). Interestingly, nTregs may control production of IFN-γ by Th1 cells without suppressing Th1 differentiation via IL-10, but not TGF-β or IL-35 (Sojka and Fowell, 2011).

Collison et al. identified IL-35 as novel inhibitory cytokine, which is produced by murine nTregs and is required for maximal suppression of proliferation of responder cells (Collison et al., 2007). In vitro, nTregs from mice deficient in the genes encoding for subunits of IL-35: IL-12α and IL-27β were unable to supress responder cells and in vivo
they were unable to ameliorate inflammatory bowel disease. nTregs may also induce naïve T cells to express IL-35 rendering them immunosuppressive. However, IL-35 is not produced by human nTregs (Bardel et al., 2008).

1.3.2. CD4⁺ iTregs

In contrast to nTregs that develop in the thymus, Tregs may also be generated extrathymically upon antigen stimulation in tolerogenic conditions. These induced Tregs (iTregs), also known as adaptive Tregs, can be generated in peripheral lymphoid tissues such as GALT, spleen, lymph nodes and even inflamed tissues, from naïve Tconv cells that are often Foxp3⁻.

The de novo generation of functional iTregs has been observed in cases where the antigens are encountered in the presence of TGF-β. To generate iTregs in vivo antigens can be delivered through intravenous injection, oral administration, administration of non-depleting CD4 antibodies or tolerogenic DCs (Schmitt and Williams, 2013). The minimal requirement of iTreg generation in vitro is TCR stimulation of naïve CD4⁺ T cells in the presence of TGF-β and IL-2. Additionally, retinoic acid, a vitamin A metabolite, promotes development and function of CD4⁺ iTregs.

Many iTregs share similar phenotype with nTregs, as they are also CD4⁺CD25⁺Foxp3⁺ and share many of the mechanisms of suppression as nTreg cells. The extent to which these two populations have redundant versus complimentary roles in the immune system remains unclear (Bilate and Lafaille, 2011). However, the two populations have different TCR β-chain repertoires with the overlap between the two being 10-42% suggesting different functions of these two types of Tregs (Haribhai et al., 2011). There is a popular notion that the major function of nTregs is to prevent autoimmunity, whereas that of iTregs is to raise the activation threshold of immune responses directed against innocuous environmental antigens (Curotto de Lafaille and Lafaille, 2009). However, both nTregs and iTregs were required to induce complete tolerance in the mouse model of colitis (Haribhai et al., 2011), and both played active
roles in asthma tolerance (Huang et al., 2013). Generation of iTregs in the periphery may also be one of the main barriers to the eradication of tumours and clearance of pathogens.

There are also many iTreg types that do not express Foxp3 transcription factor. These include Th3, Tr1, most of CD8+ Tregs, and a unique population of TCRαβ+CD4−CD8− cells. Other types of cells that may have a regulatory function under certain conditions are TCRγδ+ T cells and NKT cells, but these will not be further discussed.

1.3.2.1. Th3 Cells

A unique subset of TGF-β-secreting CD4+ cells was identified in the mucosal tissues of mice and humans during studies concerning identifying mechanisms of oral tolerance, and these cells were termed Th3 cells (Chen et al., 1998a; Fukaura et al., 1996; Inobe et al., 1998). Although the developmental lineage of Th3 cells is still unclear, they can be induced in the gut by DCs via TCR signalling and CD86 co-stimulation (Weiner, 2001b). Th3 induction can be further enhanced by the presence of TGF-β, IL-4, IL-10 or anti-IL-12 (Inobe et al., 1998; Seder et al., 1998). Similarly to nTregs, Th3 cells constitutively express CTLA-4 on their cell surface, initiation of which results in the production of copious amounts of TGF-β, which in turn suppresses activation and proliferation of Th1 and Th2 cells, and provides help for IgA production (Chen et al., 1998b). The non-specific suppression mediated by Ag-specific Th3 cells gave rise to the term ‘bystander suppression’ and is recognized as an important mechanism in the induction of tolerance to exogenous antigens from food proteins and bacterial flora (Weiner, 2001a).

1.3.2.2. Tr1 Cells

Type 1 Tregs (Tr1) are also a subset of CD4+, however, they do not express Foxp3. Tr1 cells can be distinguished from other CD4+ cells by the co-expression of LAG-3 and CD49b, in both mice and humans (Gagliani et al., 2013). Tr1 cells inhibit immune-mediated inflammation via cytokine-dependent mechanisms by secreting
abundant amounts of anti-inflammatory IL-10, which diminishes the function of APCs and Ag-specific Teff cells (Gagliani et al., 2013). Additionally, human Tr1 can selectively kill APCs via a perforin/granzyme B-dependent mechanism that requires recognition of MHC class I, CD2, and CD226 (Magnani et al., 2011). Similar to nTregs, Tr1 cells can inhibit T cell responses in a cell contact-dependent manner facilitated by CTLA-4 and PD-1 (Akdis, 2008), and by the disruption of metabolism of Teff cells via production of the ectoenzymes CD39, and CD73 (Bergmann et al., 2007).

Since Tr1 cells belong to a family of inducible Tregs they can be induced in vitro by the chronic antigen stimulation in the presence of IL-10 (Groux et al., 1997). In vivo, Tr1 cells were generated in diabetic mice that underwent pancreatic islet transplantation by exogenous administration of IL-10 in conjunction with rapamycin (Battaglia et al., 2006a). Additionally, IL-27 production by tolerogenic DCs is crucial for in vivo differentiation of Tr1 cells in both mice (Awasthi et al., 2007; Fitzgerald et al., 2007; Stumhofer et al., 2007) and humans (Murugaiyan et al., 2009). Interestingly, IL-6 in the absence of IL-27 also drives differentiation of murine Tr1 cells from naïve CD4+ T cells in vitro (Jin et al., 2013).

In 1994 Bacchetta et al. published the first clinical evidence regarding the tolerogenic role of Tr1 cells in severe combined immunodeficiency patients who underwent HLA-mismatched fetal liver hematopoietic stem cells transplant (HSCT) (Bacchetta et al., 1994). High endogenous production of IL-10 by Tr1 cells contributed to the development of split chimerism characterized by the presence of T and NK cells of donor origin, whereas B cells and APCs were host-derived. Interestingly, this split chimerism was devoid of GVHD. Serafini et al. and Andreani et al. also have shown that Tr1 cells were involved in induction of mixed chimerism in patients undergoing HSCT for the treatment of thalassemia major and played a role in sustaining long-term allograft tolerance despite the chronic donor-host allo-Ag stimulation (Andreani et al., 2014; Serafini et al., 2009).
1.3.3. CD8⁺ Tregs

Gershon’s original suppressor T cells were defined as CD8⁺ (Gershon and Kondo, 1971). Thus in the 1970s and 1980s this population of Tregs was the most extensively studied. However, since the discovery in 1995 of highly potent CD4⁺CD25⁺Foxp3⁺ cells, there had been reluctance in the scientific community to re-explore the regulatory role of CD8⁺ Tregs, particularly because nTregs suppress activation and proliferation of CD8⁺ T cells (Shevach, 2006). Despite the decrease in interest to study these cells, several subpopulations of CD8⁺ Treg have been characterized in the past 40 years (Ligocki and Niederkorn, 2015). Many of the CD8⁺ subtypes share functional and phenotypic similarities to nTregs, and these may include (i) CD8⁺CD122⁺ Tregs (Rifa'i et al., 2004); (ii) CD8⁺Foxp3⁺ Tregs (Mahic et al., 2008); (iii) CD8⁺LAG-3⁺Foxp3⁺CTLA-4⁺ Tregs (Boor et al., 2011); (iv) CD8⁺Foxp3⁺CCR7⁺ (Wen et al., 2016); (v) CD8⁺IL-10⁺CCR7⁺CD45RO⁺ Tregs (Wei et al., 2005) and (vi) CD8⁺CD122⁺PD-1⁺ Tregs (Dai et al., 2010) and many more.

Because of the highly diverse population of CD8⁺ Tregs, it should be no surprise that CD8⁺ Tregs may either develop in the thymus or differentiate in the periphery from naïve T cells. Murine and human CD8⁺CD28low Tregs arise in the thymus, despite the fact that the lack of CD28 expression on their cell surface that would otherwise suggest differentiation of chronically activated Tconv cells to Tregs in the periphery (Vuddamalay et al., 2016). In vitro, human CD8⁺ Tregs were induced from naïve CD8⁺CD25⁻CD45⁺ T cells by co-culture with IL-2 and TGF-β, or by stimulation with allogeneic mDCs even in the absence of these cytokines. However, the combination of IL-2 and TGF-β yielded the most potent suppressive phenotype (Bjarnadottir et al., 2014). Murine CD8⁺ Tregs may also be induced in the presence of retinoic acid in addition to the aforementioned conditions (Lerret et al., 2012).

CD8⁺ Treg suppression is Ag-specific and may be mediated by contact-dependent mechanisms, or via secretion of immunosuppressive molecules and inhibitory cytokines such as IL-10 and TGF-β (Endharti et al., 2005; Lerret et al., 2012; Zhang et al., 2009). Contact-dependent suppression involves the direct killing of CD4⁺ T cells by perforin-
mediated cytotoxicity (Lu and Cantor, 2008), or by induction of apoptosis via Fas/FasL interaction (Chen et al., 2013), as well as direct inhibition via inhibitory molecules CTLA-4 or PD-1 (Boor et al., 2011). Interestingly, CD8⁺ Tregs can modify the suppressive mechanisms they use based on whether or not they also interact with CD4⁺ Teff cells. Upon contact with Teff, CD8⁺ Tregs predominantly produce IFN-γ and Fgl-2, whereas in the absence of contact with CD4⁺ cells, CD8⁺ Tregs induce Indoleamine 2,3-dioxygenase (IDO) (Li et al., 2010). IDO is the first and rate-limiting enzyme in tryptophan catabolism, thus causing depletion of tryptophan, which is essential for protein synthesis to sustain life. In the recent report by Wen et al. new suppressive mechanism of human CD8⁺Foxp3⁺CCR7⁺ Tregs has been identified that restrains activation and proliferation of CD4⁺ T cells (Wen et al., 2016). CD8⁺ Tregs release NADPH oxidase 2 (NOX2)–containing microvesicles into target CD4⁺ cells, which in turn inhibit TCR signal transduction by increasing ROS and thus reducing phosphorylation of the TCR-associated kinase ZAP70. Furthermore, donor-specific CD8⁺Foxp3⁺ Tregs facilitated de novo generation of CD4⁺Foxp3⁺ Treg via a TGF-β dependent mechanism in a phenomenon known as ‘infectious tolerance’ (Lerret et al., 2012).

In humans, CD8⁺CD25⁺Foxp3⁺ Tregs may be involved in the prevention of asthma (Eusebio et al., 2015). Moreover, in patients with systemic lupus erythematus (Zhang et al., 2009), inflammatory bowel disease (Brimnes et al., 2005), or multiple sclerosis (Baughman et al., 2011) defective functions and/or reduced numbers of circulating CD8⁺ Tregs have been reported. Additionally, CD8⁺ Tregs may be one of the key players in immunoregulatory processes to restrict recognition of foreign antigens in the immune privileged anterior chamber of the eye and cornea (Niederkorn, 2006), may play a major role in allograft tolerance in the kidney (Derks et al., 2007) and liver-intestine (Sindhi et al., 2005), and they have been associated with fewer rejection episodes in the heart-transplanted patients (Dijke et al., 2009).
1.3.4. DN Tregs

1.3.4.1. Phenotype of DN Tregs

In addition to Tregs mentioned above there are T cells that express TCRαβ, but do not express CD4 or CD8 co-receptor, nor the conventional NK markers NK1.1 (mouse), or CD56 (human). These TCRαβ⁺CD4⁻CD8⁻ cells, known as double negative T regulatory cells (DN Tregs) have been shown to play an important immunoregulatory function in the variety of settings. Murine and human DN Tregs can be found in lymphoid and non-lymphoid tissues such as thymus, spleen, lymph nodes, skin, bone marrow, peripheral blood and lung (Reimann, 1991). DN Tregs also constitute a substantial component of TCRαβ⁺ cells in the intestinal epithelium (Hamad, 2010) and compose 70-90% of TCRαβ⁺ cells in the murine female genital tract (Johansson and Lycke, 2003). DN Tregs remain a rare population of cells in the peripheral blood and represent only 1-5% of total circulating CD3⁺ lymphocytes in mice and 1-2% in humans (Fischer et al., 2005; Zhang et al., 2001).

The first phenotypic and functional characterization of human DN Tregs by Fischer et al. revealed many similar properties to their murine counterparts (Fischer et al., 2005). DN Tregs isolated from peripheral blood consist of naïve, as well as Ag-experienced cells, and upon stimulation by allo-DCs they acquire effector memory phenotype (Fischer et al., 2005; Voelkl et al., 2011). Although freshly isolated DN Tregs are negative for the activation marker CD25, activation can induce its expression (Fischer et al., 2005). Furthermore, both human and murine DN Tregs do not express Foxp3 or CTLA-4, the two markers that are associated with other types of Tregs (Fischer et al., 2005; Gao et al., 2011; Hillhouse et al., 2010). In contrast to NKT cells, DN Tregs carry a polyclonal TCR repertoire and do not express CD16 or CD56 (Fischer et al., 2005). Upon stimulation with αCD3/CD28 mAbs or allogeneic DCs, DN Tregs secreted IFN-γ, IL-5, IL-4, IL-10 but no IL-2 (Fischer et al., 2005; Voelkl et al., 2011).

DN Tregs were shown to display high proliferative potential, which was further enhanced by supplementing the culture medium with IL-2 (Fischer et al., 2005). Analysis
of T-cell receptor excision circles (TRECs) revealed that DN Tregs had undergone a higher number of cell divisions as compared to CD4+ and CD8+ T cells, suggesting that DN Tregs “are not recent thymic emigrants but rather an expanded T-cell subset” (Fischer et al., 2005).

1.3.4.2. Overview of DN Treg Function

The first evidence that TCRαβ+ T cells that do not express CD4 and CD8 co-receptors may have suppressive function comes from a study conducted by Strober et al. in 1989. In this study, clonally expanded DN T cells obtained from murine spleens were able to successfully suppress responder cells in allogeneic mixed lymphocyte reaction (Strober et al., 1989). However, one of the major limitations of the study was the lack of staining for NK markers, thus, it may have been possible that NK cells were responsible for the observed suppression. However, a subsequent study by Bruley-Rosset et al. showed that DN Tregs were responsible for protection against GVHD if the host was pre-immunized with donor spleenocytes before the minor histocompatibility antigen (MHA)-mismatched bone marrow transplant (Bruley-Rosset et al., 1990). Although the suppressor cells were shown to have low expression of NK marker asialo-GM1, the possibility that they may be NK cells can be excluded, because depletion of asialo-GM1+ cells removed all NK activity in vitro, but only slightly affected suppression of GVHD in vivo.

In 2000, Zhang’s group was the first to phenotypically identify DN Tregs as TCRαβ+CD4−CD8−NK1.1− cells that display regulatory function in vitro and in vivo (Zhang et al., 2000). Since then, multiple rodent models had been adapted to study DN Treg function in vivo. The adoptive transfer of DN Tregs induced donor-specific tolerance to allogeneic islet, skin, and heart, as well as xenogeneic heart grafts (Chen et al., 2003b; Chen et al., 2007; Chen et al., 2005; Ford et al., 2002; Lee et al., 2005; Ma et al., 2008; Zhang et al., 2007; Zhang et al., 2000); infusion of DN Tregs attenuated GVHD (He et al., 2007; Juvet et al., 2012; Young et al., 2003a); and DN Tregs successfully controlled autoimmune diabetes (Duncan et al., 2010; Ford et al., 2007; Hillhouse et al., 2010; Liu et al., 2016), lymphoproliferative syndrome (Ford et al.,
2002; Juvet et al., 2012) and infection (Cowley et al., 2005; Hossain et al., 2000; Johansson and Lycke, 2003; Kadena et al., 1997).

1.3.4.3. Role of DN Tregs in GVHD

GVHD is a common, life-threatening complication that occurs after hematopoietic stem cell transplant (HSCT). GVHD follows when donor T cells recognize the host antigens as non-self. This results in the activation and rapid expansion of donor T cells that culminates in severe organ damage (further discussion of GVHD in chapter 1.4.2.). Adoptive transfer of murine DN Tregs has been shown to prevent GVHD after bone marrow transplant (BMT) (Juvet et al., 2012; Young et al., 2003a). Furthermore, infusion of murine DN Tregs after allogeneic BMT promoted induction of tolerance to donor antigens and establishment of mixed chimerism in the absence of GVHD (He et al., 2007).

Perhaps the most important clinical evidence suggesting the value of harnessing DN Tregs for immunotherapy comes from a study conducted by McIver et al. (McIver et al., 2008) that followed a group of 40 patients who received allogeneic HSCT. Significant difference was observed in the percentage of DN Tregs in peripheral blood of patients who developed GVHD and those who did not. Interestingly, the inverse correlation between the severity of GVHD (grade 1-4) and the percentage, as well as the absolute number of DN Tregs was observed. As a matter of fact, all patients whose DN Tregs expanded to more than 1% of peripheral T cells did not develop GVHD, strongly suggesting that the expansion of DN Tregs may prevent the development of GVHD after allogeneic HSCT. Additionally, the deficiency of DN Tregs was concomitant with increased clonal T cell expansion, which in turn was positively correlated with the occurrence of GVHD. Although the study did not offer any mechanistic explanation as the role of DN Tregs in suppressing allo-reactivity and thus the results represent correlation and may not necessarily mean causation. Nevertheless, before the publication of this study only the nTregs were shown to correlate inversely with GVHD in humans (Hess, 2006).
In a separate study, Ye et al. examined reconstitution of DN Tregs after allo-HSCT (Ye et al., 2011). In patients that received HLA-mismatched transplant, the rate of DN Treg reconstitution was reversely correlated with acute GVHD. Thus the patients who did not develop GVHD were characterized by significant expansion of DN Tregs in the peripheral blood. Moreover, the absolute counts of DN Treg 60 days post allo-HSCT and the grade of GVHD were inversely correlated – results similar to those observed by McIver et al. (McIver et al., 2008).

1.3.4.4. Role of DN Tregs in Cancer

Apart from the tolerogenic mechanisms, DN Tregs demonstrated anti-tumour activity, even though the notion is counterintuitive to the established understandings of Treg function. In mice, adoptive transfer of allogeneic DN Tregs has been shown to prevent A20 lymphoma tumour growth without causing GVHD (Young et al., 2001; Young et al., 2003b). Merims et al. have shown that human DN Tregs can effectively kill allogeneic and autologous CD34+ leukemic blasts in a dose-dependent manner (Merims et al., 2011). In this study, CD3−CD4−CD8−CD56− cells were isolated from the peripheral blood of acute myeloid leukemia patients during chemotherapy-induced remission. These cells underwent two weeks of ex vivo expansion using two rounds of stimulation with anti-CD3 monoclonal antibody and IL-2. Since both TCRαβ+ and TCRγδ+ cells were present in the ex vivo expanded cohort, the cells were sorted based on their TCR expression. Both subsets of TCRαβ+ and TCRγδ+ showed similar cytotoxicity against primary leukemic blasts (Merims et al., 2011).

1.3.4.5. DN Tregs in Lymphoproliferative Syndrome

Although DN Tregs have been recognized for more than three decades, ill understanding of their pathophysiologial roles led to many misconceptions and general dismissal among immunologists that these cells were legitimate constituents of the immune system (Martina et al., 2015). Historically, DN Tregs have been associated with lpr and gld mice that have the loss-of-function mutation in genes encoding Fas and FasL, respectively (Cohen and Eisenberg, 1991) and will be referred to in this section as lpr
DN Tregs. Since FasL is a death factor that binds to its receptor Fas and induces apoptosis in the Fas-expressing cells, the defects in either one lead to lymphadenopathy and splenomegaly, respectively, and systemic autoimmune disease characterized by immense lymphoproliferation, and accumulation of lpr DN Tregs (Shirai et al., 1990).

Some studies have shown that a large abundance of lpr DN Tregs in lpr and gld mice may be the result of conventional T cells down-regulating their co-receptor (Bristeau-Leprince et al., 2008). However, the lpr DN Tregs have abnormal phenotype in comparison with wild-type DN Tregs: lpr DN Tregs do not respond to antigenic stimulation (Davignon et al., 1988), express an unusual B-cell-specific CD45RA isoform called B220 (Cohen and Eisenberg, 1991), and overexpress and are dependent on the transcription factor Eomes (Kinjyo et al., 2010). However, even lpr DN Tregs have been shown to have potent immunoregulatory function against allo-antigens in vivo and in vitro (Ford et al., 2002), and that their functions is dependent on autocrine IFN-γ secretion (Juvet et al., 2012). Together, these results indicate that DN Tregs wrongfully had bad reputation within the scientific community.

### 1.3.4.6. DN Tregs in Diabetes

Diabetes mellitus is an autoimmune disease characterized by destruction of insulin-producing beta cells in the pancreas by autoreactive T cells and autoantibodies, which results in life-long dependence on insulin injections (Xie et al., 2014). If left untreated, diabetes results in death. The first report that DN Tregs may also alleviate autoimmune disease comes from study conducted by Ford et al. where they showed that peptide-activated transgenic DN Tregs prevented development of autoimmune diabetes by killing autoreactive CD8+ T cells (Ford et al., 2007). In another study, adoptive transfer of splenic DN Tregs obtained from non-obese diabetic (NOD) mice provided long lasting protection against diabetes (Duncan et al., 2010). Furthermore, transferred DN Tregs proliferated and differentiated into IL-10 secreting Tr1-like cells (Duncan et al., 2010). Lastly, adoptive transfer of DN Tregs converted from CD4+ cells in combination with anti-thymocyte serum administered to deplete pathogenic T cells have shown to
reverse autoimmune diabetes in NOD mice that recently developed diabetes (Liu et al., 2016).

1.3.4.7. Mechanisms of DN Treg-mediated Suppression

DN Tregs can suppress immune responses via various mechanisms. Similar to nTregs, murine DN Tregs suppress responder T cell proliferation 
\textit{in vitro} and \textit{in vivo} in a cell contact-dependent manner (Sakaguchi et al., 2008; Zhang et al., 2000), may express CTLA-4 on their cell surface and thus modulate co-stimulatory molecule CD80/CD86 expression on DCs (Gao et al., 2011; Wing et al., 2008), and require activation to carry out their suppressive function. Unlike nTregs which can suppress immune responses in an Ag-specific or non-specific manner, murine DN Tregs employ only Ag- or allo-Ag-specific suppression, both \textit{in vitro} and \textit{in vivo} (Fischer et al., 2005; Gao et al., 2011; Hillhouse and Lesage, 2013; Voelkl et al., 2011; Zhang et al., 2007). DN Tregs use an interesting process to acquire antigen specificity called trogocytosis, in which DN Tregs acquire allo-peptide-MHC complexes from APCs, which are then re-expressed on their cell surface (Juvet and Zhang, 2012).

Mackensen’s group shed the light on the function of human DN Tregs, which in many aspects is similar to their murine counterparts. In the first report by Fischer et al. DN Tregs were shown to acquire allo-antigen peptide from APCs, and suppress CD8$^+$ responder cells activated with the same peptide (Fischer et al., 2005). In a subsequent report, Voelkl et al.

extended suppression of allo-Ag-specific DN Tregs to autologous CD4$^+$ and CD8$^+$ T cells activated by allo-APCs or anti-CD3/CD28 microbeads (Voelkl et al., 2011). However, the mechanism of suppression contrasted those observed in murine models. Murine DN Tregs mediate suppression of responder T cells by eliminating them via Fas/FasL interaction or perforin/granzyme B pathway (Chen et al., 2003a; Ford et al., 2002; Young et al., 2002; Zhang et al., 2007; Zhang et al., 2006; Zhang et al., 2000); however, blocking the same pathways in human DN Tregs failed to abolish their suppressive capacity and, unlike murine DN Tregs, human DN Tregs did not induce apoptosis in the responder T cells, even though the first report suggested otherwise (Fischer et al., 2005). Even more striking is the fact that suppression was reversible as
responder T cells sorted from the mixed lymphocyte reaction (MLR) were able to proliferate upon removal of DN Tregs (Voelkl et al., 2011). Unlike nTregs, suppressive activity of DN Tregs is not modulated by the competition of growth factors or modulation of APCs (Sakaguchi et al., 2008; Wing et al., 2008). However, DN Treg-mediated allogeneic suppression is TCR-dependent, requires de novo protein synthesis and cell-to-cell contact (Voelkl et al., 2011).

In the most recent study conducted by A. Mackensen group, the addition of IL-7 to MLR was found to impair suppressive function of DN Tregs and that the effects of IL-7 were contributed to activation of Akt/ mechanistic target of rapamycin (mTOR) pathway, which is directly related to cell proliferation, differentiation, and metabolism (Allgauer et al., 2015). IL-7-treated DN Treg showed more activated phenotype, increased proliferation and down-regulation of anergy-associated genes, which was inversely correlated with their suppressive function, but can be reversed by selective inhibition of Akt or mTOR protein (Allgauer et al., 2015). The researchers suggest that DN Tregs, similarly to Tr1 cells (Roncarolo et al., 2014), require an anergic phenotype for their suppressive activity.

1.3.4.8. Development of DN Tregs

The origin and development of DN Tregs remain elusive. Based on the current evidence, DN Tregs may develop in the thymus, in the periphery, or they may ascend from Tconv cells. Given that DN Treg population is heterogeneous, it is likely that more than one if not all of these pathways are plausible (Juvet and Zhang, 2012).

During T cell development thymocytes progress through a series of maturation stages as defined by their phenotype. These include four double negative (DN) stages and double positive (DP) stage before committing to a CD4+ or CD8+ lineage (Koch and Radtke, 2011). Therefore, DN Tregs may represent a subset of thymic DN precursors that avoided development into single positive Tconv cells by escaping clonal deletion (Egerton and Scollay, 1990), or they may represent thymic cells that went through all the stages of the development and downregulated both co-receptors at the DP stage (Landolfi
et al., 1993). Based on TREC counts, human DN Tregs appear to have proliferated to a larger extent than Tconv cells (Fischer et al., 2005), which supports the notion of thymic development followed by expansion in the periphery. However, DN Tregs in the murine genital tract that represent 70-90% of the total pool of T cells appear to have completely developed extrathymically (Johansson and Lycke, 2003).

DN Tregs may also be generated de novo in the periphery from CD4+ or CD8+ Tconv cells that have downregulated its co-receptor (Petrie et al., 1990). For example, murine DN Tregs can be generated by stimulating CD4+ T cells with allogeneic DCs in the presence of IL-2 or IL-15 (Zhang et al., 2007). The disappearance of CD4 molecule was a result of gene silencing and resulted in stable lineage even after re-stimulation. These cells were potent suppressors and resistant to AICD. Crispin et al. demonstrated that the subset of human DN Tregs could derive from transformed CD8+ T cells, which have down-regulated the CD8 co-receptor (Crispin et al., 2008; Crispin and Tsokos, 2009). However, Ford et al. has shown that murine DN Tregs can arise in the absence of CD8+ or thymus, and that antigen stimulation of CD8+ cells in vivo does not convert CD8+ cells to DN Tregs (Ford et al., 2006).

1.4. Immunotherapy with Tregs

In organ transplantation and autoimmune diseases the in vivo induction of proliferation or adoptive transfer of Tregs would be beneficial, whereas in cancer setting Treg depletion and/or functional blockade of Tregs would enhance the immune responses against tumour antigens. The idea that Tregs could be used for immunotherapy was first proposed by Gershon back in the 1970s (Gershon, 1975). Since then the efficacy of Tregs has been shown in many preclinical models and the improvements in the methods for ex vivo expansion of Tregs over the past years made it possible to use Tregs in adoptive cellular therapy (ACT). In general, ACT involves isolation of cells of interest from a patient or donor, followed by ex vivo expansion of the cellular product and reinfusion back into the patient. Understanding the role of the different subset of Tregs in each
disease will allow for the development of highly effective immunotherapies specifically tailored to combat particular illness.

### 1.4.1. Treg Expansion Methods

There are many steps involved in the successful expansion of Tregs and each step comes with its own unique challenges. The first problem lies in obtaining immune tissue from which Tregs could be isolated. For this purpose, peripheral blood of healthy adults, banked umbilical cord blood (UCB) and discarded human thymus can serve as Treg sources (Dijke et al., 2016; Singer et al., 2014). The next challenge involves identification and isolation of Tregs from the bulk population. Unfortunately, the task is challenging, because there are many phenotypes of Tregs (as discussed in the earlier chapters) and no definitive cell surface markers that would distinguish Tregs from Tconv have been identified. Therefore, staining for multiple cell surface markers is required and the combination of the selected markers would ensure a relatively pure population of Tregs. These cells can then be isolated by magnetic sorting, which is probably the most common method employed by the researchers, or via fluorescence activated droplet cells sorting, microfluidics switch device, or microchip based sorting (Trzonkowski et al., 2015). The last step involves expansion of these cells in culture and as with any Tconv cells it includes activation and provision of growth factors, and nutrients. The easiest method is to use anti-CD3/CD28 beads, which will generate a population of polyclonal Tregs due to non-specific TCR stimulation. However, generation of Ag- or allo-Ag-specific Tregs would significantly reduce the numbers of Tregs that need to be infused into a patient, as they would be more potent as seen in the pre-clinical studies (Golshayan et al., 2007; Sagoo et al., 2011).

#### 1.4.1.1. Role of IL-2, IL-7 and IL-15 Growth Factors in Treg Maintenance and Proliferation

Interleukin 2 (IL-2), interleukin 7 (IL-7) and interleukin 15 (IL-15) belong to the family of common gamma chain (γc) cytokines that are essential growth factors for T cell development and homeostasis. All of these cytokines bind to receptors that use the γc-
receptor (CD132) to signal through two major signalling pathways the JAK-STAT pathway and the phosphoinositide 3 kinase (PI3K)-AKT pathway to induce expression of genes associated with T cell survival and proliferation. Mutations in the genes associated with γc-receptor lead to severe combined immunodeficiency (SCIDX1) characterized by severe reduction in circulating peripheral T cells, severe infections and patients urgently need BMT within first year of life (Di Santo et al., 1995).

IL-2 has been long known as one of the most important cytokines for adaptive immune response and has been the first cytokine used for propagation of T cells in vitro (Smith, 1988). Therefore, it has been baffling that the absence of IL-2 does not interfere with proliferation, or composition of T cells in the periphery (Schorle et al., 1991), but rather hyperactivation of CD4+ cells (Sadlack et al., 1993). Mice deficient in IL-2Rα (CD25), or IL-2Rβ (CD122) also succumb to hyperplasia and systemic inflammation (Suzuki et al., 1995; Willerford et al., 1995). The paradox that IL-2 seems to limit rather than enhance immune responses has been resolved with the discovery that IL-2 is critical for Treg development and expansion (Nelson, 2004; Sakaguchi et al., 2008). Since nTregs express high levels of CD25 and dysregulation of IL-2, or its receptor, is associated with autoimmunity, it was reasonable to predict that administration of low doses of IL-2 may have positive effect on the function and expansion of Tregs in the periphery (Klatzmann and Abbas, 2015). Several clinical trials have shown promising results in reducing incidence of GVHD (Kennedy-Nasser et al., 2014; Koreth et al., 2016), T1D (Hartemann et al., 2013) and SLE (He et al., 2016).

Under biological conditions, IL-7 regulates homeostasis of naïve and memory T cells and preserves T cell repertoire diversity, as it is responsible for V(D)J rearrangement during early T cell development (ElKassar and Gress, 2010). IL-7 is continuously produced by epithelial and stromal cells in the thymus and bone marrow, and by fibroblastic reticular cells in the T cell zones of secondary lymphoid organs (Rochman et al., 2009). IL7-Rα (CD127) is downregulated upon T cell activation, thus most likely IL-7 does not have an effect on activated T cells. This in turn decreases IL-7 consumption by activated T cells increasing the availability of IL-7 for other cells poised to receive survival signals. IL-7 impacts T cells homeostasis in at least two different ways
(Rochman et al., 2009): first, IL-7 promotes survival of T cells by activating PI3K-AKT signalling pathway, increasing the expression of survival factors and inhibiting the expression of the pro-apoptotic factors (Mazzucchelli and Durum, 2007; Surh and Sprent, 2008); and second, levels of circulating IL-7 are enhanced in lymphopenic conditions (Surh and Sprent, 2008) and it may promote expansion of T cells in tumours (Dummer et al., 2002), GVHD (Dean et al., 2008) and autoimmunity (Katzman et al., 2011; Monti et al., 2008). But even in the autoimmune setting IL-7 signalling had positive effect on Tregs as seen in an experimental model of autoimmune encephalomyelitis (EAE) in which IL-7 signalling pathway has been found to drive accelerated differentiation and proliferation of Tregs in the thymus leading to increased output of thymic and self-regulation of EAE (Chen et al., 2009). Although one of the characteristics of Tregs is low expression of IL7Ra, low levels of IL-7 in the periphery are required for Treg survival and to support Foxp3 expression to sustain CD25 expression on the Treg cell surface in vivo and modulate the ability of Tregs to efficiently bind IL-2, and transduce IL-2 signalling (Kim et al., 2012; Simonetta et al., 2014).

Although IL-15 induces similar signalling pathways as IL-2 or IL-7, its role in Treg homeostasis is not well established. However, beneficial effects of IL-15 on the expansion of Tregs have been reported previously. IL-15 has been found to promote de novo generation of Tregs in the thymus and to enhance their regulatory function (Ben Ahmed et al., 2009). In cell culture, IL-15 has been used in expansion of nTregs from UCB (Asanuma et al., 2011) and adult peripheral blood, however, it offered no advantage as compared to IL-2 alone (Lin et al., 2014). Additionally, IL-2 along with IL-15 was used for the expansion of allo-specific Tregs (Veerapathran et al., 2013).

1.4.1.2. Role of Rapamycin in Treg Expansion and Suppressive Function

The mammalian target of rapamycin (mTOR) is an evolutionary conserved 289-kDa serine/threonine protein kinase. mTOR is a key regulator of metabolism that integrates environmental cues in terms of nutrients, energy and growth factors, and is involved in cell growth, proliferation, and survival. The activation of mTOR is tightly regulated, induced by multiple stimuli and signals through PI3K-AKT-dependent fashion,
which leads to an increase in glucose uptake, and a switch from oxidative phosphorylation to glycolysis (Fox et al., 2005).

As the name suggests mTOR can be inhibited by rapamycin (sirolimus). Other mTOR inhibitors are referred to as rapalogs (analogs of rapamycin). These potent inhibitors of metabolism are commonly used as immunosuppressants to prevent organ rejection. Rapamycin promotes generation of Tregs as evident in the induction of de novo expression of Foxp3 in naïve T cells, thus it may be used to expand Tregs in vitro (Battaglia et al., 2006b; Battaglia et al., 2005; Golovina et al., 2011). Addition of rapamycin directly to nTreg culture also aids in their in vitro expansion, and these nTregs have amplified suppressive function relative to non-treated cells (Battaglia et al., 2006b; Battaglia et al., 2005; Golovina et al., 2011; Singh et al., 2012). Their improved regulatory function is at least in part due to upregulation of CD25 and CTLA-4 on their cell surface (Singh et al., 2012). Allgauer also reported that human DN Tregs increase their suppressive activity when treated with mTOR or AKT inhibitors (Allgauer et al., 2015).

1.4.2. Treatment of GVHD

One particular situation where the infusion of Tregs would be exceptionally beneficial therapeutically is graft-versus-host disease. GVHD is a major complication after allogeneic hematopoietic stem cell transplant (HSCT). HSCT is a life-saving treatment for many haematological malignancies and is beneficial in the treatments of benign disorders, such as autoimmune diseases. GVHD occurs when the donor T cells recognize the recipient (the host) as ‘non-self’ and attack the recipient tissues. The immune response results in potent inflammatory reaction, despite the routine administration of immunosuppressive drugs. Systemic inflammation eventually exhausts all the immunoregulatory mechanisms and culminates in severe organ damage. The global incidence of GVHD is related to HLA disparity and the larger the degree of mismatch, the more likely the occurrence of developing GVHD (Choi et al., 2010). Although recent advances in HLA typing allow for a much safer procedure,
approximately half of the patients that undergo allo-HSCT will develop acute GVHD and about half of those patients will eventually progress to chronic GVHD (Jacobsohn and Vogelsang, 2007). Overall, more than 10% of GVHD cases will result in death (Jamil and Mineishi, 2015). Worldwide, more than 20,000 allogeneic HSCT are performed annually, and the number continues to increase mainly due to advances in the safety profile, such as improved HLA matching, but also by extending HSCT for treatment of autoimmune diseases (Burt et al., 2015). In contrast to solid organ transplants, recipients of HSCT will ultimately develop systemic tolerance, because the donor’s lymphocytes and APCs will eventually replace the host’s leukocytes. Since the risk of GVHD is the highest in the first few months after HSCT, adoptive transfer of Tregs would be particularly suitable for the prevention and treatment of acute GVHD. Moreover, the feasibility of Treg immunotherapy is improved with the availability of Tregs from donors.

Trzonkowski et al. conducted the first-in-man clinical trial in the treatment of two cases of GVHD (Trzonkowski et al., 2009). Tregs were isolated from the HSCT donors, polyclonally expanded ex vivo and transferred to the recipients. The patient with chronic GVHD significantly improved, whereas the patient with grade IV acute GVHD only transiently experienced amelioration of the symptoms. The authors of this study argue that the lack of significant improvability of the latter was most likely due to the relatively late administration of Tregs.

In another Phase I dose-escalation clinical trial, 23 patients received a dose of 1-3×10⁷ Tregs/kg after partially HLA-matched UCB transplantation, which ensued in significant reduction in the incidence of Grade II-IV GVHD in comparison to 108 controls (Brunstein et al., 2011). In another trial conducted by Di Ianni et al., the HSCT transplant patients received ex vivo expanded Tregs together with Tconv cells isolated from the HSCT donor (Di Ianni et al., 2011). Less than 10% of patients developed chronic GVHD in the absence of any post-transplantation immunosuppression and maintained graft-versus-leukemia (GVL) effect. In the subsequent Phase II clinical trial Treg-Teff adoptive immunotherapy was found to prevent post-transplant leukemia relapse and significantly decreased the incidence of GVHD, and out of 43 patients only 15% developed GVHD (Martelli et al., 2014).
1.4.3. Treatment of Autoimmune Diseases

There are over 80 identified autoimmune diseases and the most common include rheumatoid arthritis (RA), type one diabetes mellitus (T1D), multiple sclerosis (MS) and systemic lupus erythematosus (SLE). In autoimmune diseases, immune cells recognize self-antigens as foreign and thus attack own healthy cells. The incidence of 29 autoimmune diseases is rising globally with an estimated prevalence of 7.6-9.4% (Cooper et al., 2009). Currently, there are no curative therapies for autoimmune diseases. Standard treatment involves administration of immunosuppressive drugs, which attempt to alleviate the symptoms by restraining the magnitude of immune response. However, the use of immunosuppressants is associated with severe and debilitating long-term side effects, such as increased risk of infection and cancer. The improved understanding of pathophysiology of autoimmune disease allowed for the development of new therapeutic interventions that are highly specific, with minimal off-target side effects.

In the first-in-man treatment of T1D with Tregs, recently diagnosed young patients received either one or two doses of ex vivo expanded autologous Tregs (Marek-Trzonkowska et al., 2014). Administration of Tregs was safe and effective, because in two-thirds of patients the therapy prolonged survival of beta cells and reduced the requirement for exogenous insulin. A clinical trial in newly diagnosed adult T1D patients is ongoing.

More ongoing and scheduled clinical trials are underway involving infusion of autologous/donor polyclonal/Ag-specific fresh/expanded Tregs, or Tr1 cells, for the treatment of lupus, autoimmune hepatitis, acute/chronic/steroid dependent/refractory GVHD, prevention of GVHD and complications after liver, and kidney transplantation (Gliwinski et al., 2017). There are also many planned and ongoing clinical trials in a large collaborative project named The ONE Study, which will evaluate safety, efficacy, and feasibility of Treg therapies for SOT tolerance (Gliwinski et al., 2017; Schliesser et al., 2012).
1.5. Hypothesis and Specific Research Aims

Adoptive cellular therapy using Tregs is one of the most promising non-pharmacological approaches for prevention of GVHD, induction of tolerance to transplanted organs and treatment of autoimmune diseases. Over the past few decades, multiple Tregs populations have been identified. Although the different subtypes of Tregs share many similarities, the subtle differences in the mechanisms of action and the preferences for particular tissues make them more, or less suitable for different clinical applications. So far the focus in the scientific community to harness the immunoregulatory function of Tregs in the clinic has been mostly on CD4^+CD25^−Foxp3^+ Tregs, as their phenotype, mechanism of action and their role in many diseases have been more extensively studied in comparison to other Tregs. However, exploiting the Treg type that has been associated with the improved outcome in certain diseases may lead to the development of therapies tailored to the specific needs of patients - a hallmark of personalized medicine.

The population of TCRαβ^+CD4^−CD8^− NK lineage negative cells (DN Tregs) has been identified in mice, rats, monkeys and humans, and has been shown in rodent models to attenuate GVHD, control autoimmune diabetes, lymphoproliferative syndrome, and infection, and to prolong survival of heart and skin allo-, and xeno-grafts. In humans, a higher frequency of DN Tregs in the peripheral blood of patients undergoing HSCT has been correlated with less severe GVHD. The current therapies for GVHD involve systemic administration of immunosuppressive drugs. However, the risk of developing GVHD remains high and the use of immunosuppressive agents are associated with high toxicity. Therefore, new treatments that would induce transplantation tolerance without the debilitating toxicity are needed. Despite the extensive animal studies demonstrating regulatory function of DN Tregs in various diseases, studying DN Tregs in humans has been hampered due to their low frequency in peripheral blood and the lack of adequate expansion method.
Therefore, I instigated a hypothesis that human DN Tregs can be expanded *ex vivo* and exhibit regulatory function both *in vitro* and *in vivo*. The specific objectives of this study were:

1. *To develop a protocol for ex vivo expansion of human DN Tregs.* To date, there was no established protocol for a large-scale *ex vivo* expansion of human DN Tregs. Therefore, I tested a combination of variety of expansion methods previously used for large-scale expansion of conventional and regulatory T cells. Once the protocol for producing the largest yield of DN Treg was established, I tested the addition of growth factors IL-7 and IL-15 to the expansion cell culture to determine whether supplementation of these cytokines had any effect on the number, or function of DN Tregs.

2. *To determine the phenotype of expanded DN Tregs.* The limited studies involving human DN Tregs described the phenotype of activated Ag- or allo-Ag-specific DN Tregs. In this study, I generated DN Tregs with polyclonal reactivity, thus it was important to determine whether the *ex vivo* expanded DN Tregs had the phenotype comparable to what had been previously observed, both in mice and humans. To this end, I performed a detailed analysis of cell surface markers expression pre- and post-expansion, as well as cytokine profile of the *ex vivo* expanded cells.

3. *To assess the function of expanded DN Tregs in vitro and in vivo.* In mice and humans, DN Tregs were found to suppress CD4$^+$ and CD8$^+$ T lymphocytes in an Ag-specific manner. Furthermore, human DN Tregs were found to be cytotoxic towards leukemic cells. Since the expanded DN Tregs have polyclonal specificity, I evaluated whether DN Tregs can also mediate non-specific suppression. To test this, I sorted and used autologous CD4$^+$, CD8$^+$ and CD19$^+$ cells as responder cells and *ex vivo* expanded DN Tregs as putative suppressors in the CFSE-based *in vitro* suppression assay. Since *ex vivo* expanded DN Tregs exerted suppressive activity *in vitro*, I adapted a xenogeneic GVHD model to assess their regulatory
function in vivo. Lastly, DN Treg cytotoxicity against leukemic and lung cancer lines was evaluated by a flow-based killing assay.

4. To investigate the mechanisms of DN Treg-mediated suppression in vitro. It has been shown that murine DN Tregs can suppress Ag-specific responses via various mechanisms. The mechanisms of human DN Treg-mediated suppression of CD4\(^+\) and CD8\(^+\) T cells have not been fully dissected, and seem to differ from their murine counterparts. Therefore, I first determined whether any soluble factors possibly identified in Objective 3 would mediate regulatory function. Next, I determined whether cell-to-cell contact is important. Since there has not been a consensus regarding the ability of human DN Tregs to induce apoptosis in responder cells, I also attempted to determine whether ex vivo expanded DN Tregs suppressed responder cells by killing.

5. To evaluate the effect of rapamycin treatment on DN Treg function in vitro and in vivo. Treatment of nTregs, as well as human naïve or Ag-specific DN Tregs, with mTOR inhibitors has been shown to increase their suppressive activity. Therefore, I treated DN Tregs post ex vivo expansion with rapamycin, an mTOR-inhibitor, to determine whether DN Treg with polyclonal specificity can be rendered more potent. Since DN Tregs showed augmented regulatory function following rapamycin treatment in vitro, the role of treated DN Tregs was also evaluated in vivo and compared to untreated DN Tregs.
CHAPTER 2. MATERIALS AND METHODS
Chapter 2

Materials and Methods

2.1. Blood Samples

This study was approved by Research Ethics Board (REB# 05-0221-T). Peripheral blood samples were obtained from 11 healthy individuals (6 females and 5 males) upon receiving informed consent from the study subjects. The volume of 50 to 100 ml of blood was collected in BD Vacutainer® blood collection tubes with sodium heparin.

2.2. Cell Isolation and Magnetic Sorting

Mononuclear cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation. Cells recovered from the gradient interface were washed three times in PBS, counted and immediately enriched for DN Tregs using magnetic-activated cell sorting technology (MACS), according to the manufacturer’s instructions (Miltenyi Biotec). Briefly, DN Tregs were purified from peripheral blood mononuclear cells (PBMCs) with LD column by negative selection using FITC-conjugated monoclonal antibodies (mAbs) directed against CD4, CD8, CD56 and TCRγδ cell surface markers, and anti-FITC magnetic beads. The remaining monocytes temporarily played a role of supporting cells. Purity ranged from 96% to 99% as measured by flow cytometry. For the isolation of CD4⁺, CD8⁺ and CD19⁺ cells, positive selection was performed by direct magnetic labeling and separation with LS column. Cells were washed, counted and cultured immediately or cryopreserved for later use.
2.3. Freezing and Thawing of Cells

Cells were cryopreserved if they were not used immediately for cell culture. To this end, cells were counted, centrifuged, gently resuspended in freezing medium (90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO)) and aliquoted to cryogenic storage vials. The cryogenic vials containing the cells were placed in an isopropanol chamber and stored at −80°C overnight. The cells were transferred the next day to the liquid nitrogen tank and stored in the gas phase above the liquid nitrogen.

To thaw frozen cells, the cryogenic vials containing cells were placed in water bath at 37°C. The cells were then diluted slowly in preheated RPMI medium and centrifuged at 400g for 6 min. The supernatants were carefully decanted without disturbing the cell pellets and washed again to remove residual DMSO. The cells were gently resuspended in the complete growth medium (RPMI media supplemented with 10% FBS, penicillin, and streptomycin).

2.4. Expansion of DN Tregs

Sorted cell fractions were resuspended in the complete growth medium supplemented with recombinant human (rh) IL-2 (250 U/ml). To activate DN Tregs, 2-3×10⁶ cells/well were seeded in a 24-well tissue culture plate coated with 2.5 μg/well of anti-CD3 mAb (OKT3, eBioscience). On day 3 of culture, cells were harvested, washed and cultured for another 4 days in the presence of rhIL-2. DN Tregs were restimulated on days 7, 12 and 17 with lethally irradiated (150 Gy) artificial antigen presenting cells (aAPCs) (human K562 cell line with surface expression of a transduced membranous form of anti-CD3 mAb, CD80, CD83, CD86 and 4-1BBL; a gift from Dr. Naoto Hirano) (Butler et al., 2012) in the presence of rhIL-2 and/or rhIL-7 (10 ng/ml, PeproTech) and/or rhIL-15 (10 ng/ml, PeproTech). DN Tregs were used for functional assay and phenotypic studies on day 21. Viability and purity of DN Tregs were regularly assessed by flow cytometry and the cells were purified via MACS if purity was found to be less than 90%.
2.5. Antibodies and Flow Cytometry

*Cell surface staining*

For investigation of the cell surface proteins by flow cytometry, cells were stained with fluorochrome-coupled Abs. Briefly, cells were harvested, placed in FACS tubes and washed twice with the staining media (PBS supplemented with 0.05% BSA). All centrifugation steps were performed at 500g at 4°C for 5 min. The supernatants were decanted, the pellet resuspended with the staining media and the amount of antibodies specified by the manufacturer was added, followed by 15 min incubation at 4°C. After the final wash step, cells were resuspended in 300 µl staining media or fixation buffer (2% paraformaldehyde in PBS) and filtered. For some experiments, cells were stimulated for 4 hours with Cell Stimulation Cocktail (2 µl/ml, eBioscience) containing PMA and ionomycin.

*Intracellular staining*

For intracellular cytokine staining, cells were activated with Cell Stimulation Cocktail (2 µl/ml, eBioscience) in the presence of monensin (1 µl/ml, GolgiStop, BD Pharminogen) for 4 hours. After washing cells were stained for surface proteins, as well as stained with fixable viability dye eFluor450 for live/dead recognition, fixed, permeabilized (all reagents were included in the Fixation and Permeabilization Buffer Set, eBioscience), and finally stained for intracellular cytokines.

The antibodies listed in Table 1 were used to label cells. Dead cells were excluded with PI (Sigma-Aldrich), DAPI (Biolegend) or 7-AAD (eBioscience). Flow cytometry data were acquired using LSR II (Becton Dickinson) or Accuri C6 (Accuri Cytometers), and analyzed using FlowJo 10 software (Tree Star).
Table 1. List of the antibodies used in this study.

<table>
<thead>
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<th>Fluorochrome</th>
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2.6. Detection of Cytokines and Chemokines Secreted by DN Tregs

DN Tregs and CD8\(^+\) T cells obtained from the same donors were grown using DN Treg expansion protocol. On day 21, cells were stimulated for 4 hours with Cell Stimulation Cocktail (eBioscience). Cell supernatants were collected and stored at \(-20^\circ\)C, thawed and processed using the Luminex\textsuperscript{®} platform and the Bio-Rad Human Cytokine 27-plex Array (FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-\(\gamma\), IL-1\(\beta\), IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1 (MCAF), MIP-1\(\alpha\), MIP-1\(\beta\), PDGF-BB, RANTES, TNF-\(\alpha\), VEGF).

For IL-10 and IFN-\(\gamma\) analyses, the same aliquots used for Luminex cytokine assays, and the aliquots of supernatants collected from suppression assays after 4 or 5 days of co-culture were assayed by enzyme-linked immunosorbent assays (ELISA).

2.7. In Vitro T cell and B cell Suppression Assays

DN Treg suppressive activity was measured by quantifying inhibition of proliferation of autologous lymphocytes under polyclonal stimulation, as previously described (Mond and Brunswick, 2003; Venken et al., 2007). Briefly, purified CD4\(^+\), CD8\(^+\) or CD19\(^+\) cells (responders) were labeled with 1\(\mu\)M CFSE (5(6)-Carboxyfluorescein N-hydroxysuccinimidy ester) and seeded in a 96-well U-bottom plate (2.5\(\times\)10\(^4\) cells/well). T cells and B cells were stimulated with anti-CD3/CD28-coupled (\(\alpha\)CD3/CD28) magnetic beads (Dynabeads, Life Technologies) or F(ab\(^\prime\))\(_2\) fragment of goat anti-human IgM (Jackson Laboratories), respectively, in the presence or absence of autologous DN Tregs at increasing effector-to-responder ratios. After 4 to 5 days of co-culture, cells were harvested and stained with Abs against different surface markers to discriminate between responders and suppressors. CFSE signal of gated responder cells was analyzed by flow cytometry. The suppressive capacity of DN Tregs towards responders in co-culture was expressed as the relative inhibition of the percentage of proliferating cells as assessed by CFSE dilution using the formula:
\[
\% \text{Inhibition of proliferation} = 100 - \left( \frac{\% \text{proliferation with DNTregs}}{\% \text{proliferation without DNTregs}} \times 100 \right)
\]

and expressed as mean ± SD of 3 replicates.

For the IFN-γ and IL-10 blocking studies, suppression assays were prepared as described above, except that CD4\(^+\) and CD8\(^+\) cells were blocked for 15 min with human serum (from human male AB plasma, Sigma-Aldrich) prior to co-culture. 10 μg/ml of purified mAbs to IFN-γ (MD-1, Biolegend), IL-10 (JES3-9D7, Biolegend) or both were added to the co-cultures. Purified rat IgG1, κ Isotype Ab (RTK2071, Biolegend) was used as control.

2.8. In Vitro Suppression Assays with Rapamycin-treated DN Tregs

Ex vivo expanded DN were treated with 2 μM rapamycin (Sigma) added to the culture media incubated at 37°C. After 2 hours, cells were harvested and washed three times with large volume of warmed PBS to ensure rapamycin had been washed away completely. Rapamycin-treated DN Tregs or untreated DN Tregs were used as suppressor cells in the suppression assay described above, with the exception of shortened duration to 3 days.

2.9. Transwell® Experiments

Transwell® experiments were performed in 24-well plates with 0.4 μm pore size (Millipore). Purified CD4\(^+\) and CD8\(^+\) T cells (5×10\(^5\)) were CFSE labeled and cultured in the bottom chamber with αCD3/CD28 beads. DN Tregs were placed directly into the bottom chamber or placed in the top chamber with αCD3/CD28 beads. For some co-culture experiments, CD4\(^+\) or CD8\(^+\) T cells were also added to the top chamber. After 4 days, the proliferation of responders was measured by CFSE dilution using flow cytometry.
2.10. Lymphocyte Cytotoxicity Assay

Flow-based Assay

Naïve CD4\(^+\) T cells were activated with αCD3/CD28 beads for 4 days. Activated cells were harvested, washed and co-cultured either alone or in the presence of varying ratios of DN Tregs. After 24 hours, the presence of AnnexinV, an early apoptotic marker was quantified on the surface of CD4\(^+\) T cells by flow cytometry.

\( ^{51} \text{Cr-release Assay} \)

Naïve CD4\(^+\) or CD8\(^+\) T cells were activated for 4 days with αCD3/CD28 beads. Activated cells were labeled with \(^{51}\text{Cr}\) and co-cultured with DN Tregs for 4 or 22 hours. The amount of \(^{51}\text{Cr}\) released was quantified using scintillation counter. The percentage of specific lysis was calculated using the standard formula and expressed as the mean of triplicates:

\[
\% \text{Specific killing} = 100 \times \frac{(\text{experimental} - \text{spontaneous release})}{(\text{maximum load} - \text{spontaneous release})}
\]

2.11. Cancer Cells Cytotoxicity Assay

To assess DN Treg-induced killing of cancer cell lines, a flow cytometry-based killing assay was adapted in which target cells were stained with PKH-26 before culturing with DN Tregs. Human leukemic and lung cancer cell lines were cultured for 2 and 16 hours, respectively, in triplicates, at increasing effector/target ratios. Cell death was assessed by staining with AnnexinV-FITC, an early apoptotic marker, and 7-AAD, a cell viability dye. The percentage of specific killing mediated by DN Treg in the PKH-26-gated cell population was calculated by subtracting non-specific AnnexinV-FITC- and 7-AAD-positive target cells measured in targets co-cultured in the absence of DN Tregs using the formula:
\[
\% \text{Specific killing} = 100 \times \frac{\left( \% 7AAD^+ \text{Annexin V}^+ \text{with DNTreg} - \% 7AAD^+ \text{Annexin V}^+ \text{without DNTreg} \right)}{100 - \% 7AAD^+ \text{Annexin V}^+ \text{without DNTreg}}
\]

2.12. Mice and Xenogeneic GVHD Model

Animal experiments were approved by University Health Network (UHN) Animal Care Committee (AUP\#322.21).

NSG mice were obtained from Jacksons Laboratory and bred in the pathogen-free colony at UHN Animal Facility. 6 to 8 week old mice received whole body irradiation (250 cGy) from \(^{137}\)Cs source 24 hours prior to tail vein injection. For xenogeneic GVHD induction, mice were injected with \(5 \times 10^6\) freshly isolated human PBMCs in 100 µl of PBS using an insulin syringe. Treated mice received either 1 or 3 injections of \(10^7\) autologous DN Tregs on day 0, or on day 0, 3 and 7, respectively. Some mice were injected with only \(10^7\) DN Tregs, or with PBS. All mice were monitored daily for the signs of GVHD including weight loss, hunched posture, ruffling of the fur, diarrhoea, reduced mobility or tachypnea. At the time of severe GVHD, defined as weight loss greater than 20% of initial weight, or presence of two or more symptoms described above, mice were sacrificed in accordance to the local animal welfare regulations, and an end point of survival was recorded.

2.13. Monitoring of Lymphocyte Migration and Proliferation \textit{In Vivo}

\textit{Ex vivo} expanded human DN Tregs or freshly isolated PBMCs were labeled with 5 µM CFSE. NSG mice were irradiated at 250 cGy. \(5 \times 10^6\) CFSE labeled DN Tregs or PBMCs were intravenously injected into the lateral tail vein 24 hours later. At days 1, 3, 5, 7 and 10 post-injection, 2-3 mice per group were sacrificed. Peripheral blood, spleen, lymph nodes, bone marrow, liver, lungs, and kidneys were harvested. Single cell suspensions were obtained from all organs by crushing, and Ficoll-Hypaque
centrifugation was performed to obtain mononuclear cells. Cells from bone marrow were obtained by flushing femurs and tibiae. The peripheral blood samples was depleted of red blood cells using ACK lysis buffer. The cells were counted using Vi-CELL cell counter (Beckman Coulter) and analyzed by flow cytometry.

2.14. Data Analysis

Graphical presentation and statistical analysis of data were performed with Prism 6 (GraphPad) or SPSS 22 (IBM). All results are presented as ‘means ± SD,’ unless otherwise specified. Survival analysis was performed using the log-rank test. Results were assessed for normal Gaussian distribution and then variance between the groups was assessed with either Student’s t test or ANOVA, or the Mann-Whitney, or Kruskal-Wallis test in the case of non-parametric data. $p$ values were considered statistically significant, when $p<0.05$, $p<0.01$, or $p<0.001$ represented in the figures as *, **, or ***, respectively.
CHAPTER 3. RESULTS
Chapter 3

Results

3.1. Frequencies and Phenotypic Analysis of TCRαβ⁺ CD3⁺ CD4⁻
CD8⁻ DN Tregs in the Peripheral Blood of Healthy Adults

To validate that DN Tregs are naturally present in the peripheral blood of healthy adults, PBMCs were collected from 4 donors and analyzed for the expression of cell surface markers: TCRαβ, CD4 and CD8. As shown in Figure 1a the DN Treg population, as identified by the lack of expression of CD4 and CD8 co-receptors, comprised on average only 1.48 ± 0.54% (n=4, Figure 1b) of the total TCRαβ⁺ cells, which is similar to what has been observed in the previous reports (Allgauer et al., 2015; Fischer et al., 2005; Voelkl et al., 2011). To further characterize the phenotype of peripheral blood DN Tregs cell surface expression of common activation markers, memory markers and selected cytokine associated receptors were assessed by flow cytometry. As shown in Figure 1c & d, DN Tregs have a slightly different pattern of activation and cytokine receptor expression, when compared to CD4⁺ and CD8⁺ T cells obtained from the same donor. Markedly, DN Tregs had significantly lower expression of CD62L (49.48 ± 15.11%), a homing receptor to secondary lymphoid organs rapidly shed after TCR triggering or following lymph node migration (Chao et al., 1997; Kishimoto et al., 1990), when compared to CD4⁺ T cells (87.28 ± 5.42%) and CD8⁺ T cells (75.38 ± 5.50%), suggesting that DN Tregs represent a more Ag-experienced T cell lineage. DN Tregs had significantly lower expression of CD25 (3.34 ± 2.03% vs. 9.47 ± 3.6%) and CD127 (77.45 ± 12.15% vs. 94.9 ± 1.01%), as compared to CD4⁺ T cells and significantly lower expression of CD122 (11.93 ± 3.76% vs. 23.58 ± 5.42%) in comparison to CD8⁺ T cells.
Figure 1. Phenotypic characteristics of DN Tregs isolated from peripheral blood. (a) Representative histogram of CD4⁺, CD8⁻ or CD4⁻CD8⁻ cell frequency in the TCRαβ⁺ population from one donor. The numbers refer to frequencies of cells in each quadrant. (b) Summary of percent expression of DN Tregs in peripheral blood from 4 different healthy donors, as defined by TCRαβ⁻CD4⁻CD8⁻ phenotype. (c) Evaluation of the expression of cell surface proteins shown as a gating strategy from one donor. All cells were gated on 7-AAD TCRαβ⁺ population and on CD4⁺, CD8⁻ or CD4⁻CD8⁻ (DN Tregs). Each gate was based on FMO control. (d) Summary of the cell surface markers expression from 4 different donors. Bars represent mean ± SD percentage expression of indicated markers. *p<0.05. **p<0.01. ***p<0.001.
Memory is a hallmark of the acquired immune system as it results from clonal expansion and differentiation of Ag-specific lymphocytes (Sallusto et al., 2004). Effector memory T (T$_{EM}$) cells migrate to peripheral tissues and display immediate effector functions, while central memory T (T$_{CM}$) cells home to secondary lymphoid organs and have little to no effector function, but readily proliferate and differentiate into effector cells in response to antigenic stimulation (Lanzavecchia and Sallusto, 2000). The two common markers that differentiate between naïve and memory phenotypes are CCR7 and CD45RO (Figure 2a). Therefore, naïve T cells can be considered as CCR7$^+$ and CD45RO$^-$, T$_{CM}$ cells as CCR7$^+$ and CD45RO$^+$, while effector memory cells are CCR7$^-$ and can be either CD45RO$^+$ (T$_{EM}$) or CD45RA$^+$ (T$_{EMRA}$). As seen in Figure 2b, the majority of CD4$^+$ and CD8$^+$ T cells show a naïve phenotype (66.1% and 54%, respectively). In contrast, only a small proportion of DN Tregs (15.1%) display a naïve phenotype. The majority of DN Tregs can be considered Ag-experienced cells with T$_{EM}$ and T$_{EMRA}$ phenotype (45.3% and 31.1%, respectively).
Figure 2. Cell surface expression of memory markers CCR7 and CD45RO on peripheral blood T cells. (a) Schematic of T cell differentiation stages from naïve to effector memory phenotype based on the surface expression of CCR7 and CD45RO. T\textsubscript{CM}, central memory T cells. T\textsubscript{EM}, effector memory T cells. T\textsubscript{EMRA}, effector memory CD45RA T cells. (b) Flow cytometry detection of T cell differentiation stages of CD4\textsuperscript{+}, CD8\textsuperscript{+} or CD4\textsuperscript{-}CD8\textsuperscript{-} (DN Tregs) cells. A representative figure is shown. Similar results were observed in different donors.
3.2. Human DN Tregs Can Be Expanded Ex Vivo

It has been shown that human DN Tregs can be generated by stimulation with allogeneic mDCs (Fischer et al., 2005). However, the process is complex and therapeutically relevant numbers of DN Tregs cannot be generated. In order to explore the potential of clinical use of DN Tregs, we developed a novel protocol that allows for large-scale expansion of polyclonal human DN Tregs, with the steps summarized in Figure 3.

Briefly, PBMCs were isolated from peripheral blood of healthy donors. The population of CD4⁺, CD8⁺ and TCRγδ⁺ T cells, and CD56⁺ NK cells was depleted by magnetic beads sorting to enrich for the DN Treg population (Figure 4a). From 50 to 100 ml of blood, we were able to obtain 5-25×10⁵ DN Tregs (n=8). After sorting, cells were activated with anti-CD3 mAb in the presence of rhIL-2, followed by co-culture with lethally irradiated aAPCs every 5 days. aAPC delivered optimal stimulation through the expression transduced anti-CD3 Ab on the cell surface, as well as co-stimulatory molecules, such as CD80, CD83, CD86 and 4-1BBL. Expanded DN Tregs were harvested and used for functional and phenotypic studies on day 21. The phenotype of DN Tregs was assessed regularly to monitor for potential outgrowth of CD4⁺, CD8⁺, CD56⁺ or TCRγδ⁺ cells, which once found were depleted using magnetic beads sorting. We were able to expanded DN Tregs from 8 different donors and generated 1-10×10⁸ cells within 3 weeks, with the average fold expansion of 3422 ± 2341 (Figure 4b). By day 21, the average purity of DN Tregs was 95.48 ± 2.49% (n=8; Figure 4c).
Figure 3. Schematic representation of the method for *ex vivo* expansion of DN Tregs. PBMCs were isolated from peripheral blood of healthy donors by Ficoll-Hypaque gradient. DN Tregs were enriched for by depleting CD4+, CD8+, CD56+ and TCRγδ+ cells from PBMCs via magnetic beads sorting. On day 0, the sorted population was stimulated by plate-bound anti-CD3 mAb. On days 7, 12 and 17, DN Tregs were harvested, washed and co-cultured with irradiated (150 Gy) artificial APC cells (aAPCs), in the presence of the combination of cytokines. DN Tregs were collected on day 21 and used for phenotypic and functional studies.
Figure 4. Isolation and expansion of DN Tregs. (a) DN Tregs were enriched by staining PBMCs with CD4-FITC, CD8-FITC, CD56-FITC, TCRγδ-FITC Abs and depleting the stained cells using anti-FITC magnetic beads and MACS technology. Representative flow cytometry histograms of pre- and post-sort are shown. (b) Fold increase in DN Tregs cells after 21 days of ex vivo expansion of cells cultured in the presence of rhIL-7 (n=8). (c) The purity of DN Tregs was assessed by flow cytometry on day 0 and day 21. Numbers represent frequency in each gate or quadrant. Gating was based on FMO controls. A representative figure is shown.
3.3. Supplementation of IL-7 During Expansion Enhance Proliferation and Suppressive Function of DN Tregs

The homeostatic cytokines IL-7 and IL-15 have been shown to facilitate nTreg expansion and function (Asanuma et al., 2011; Ben Ahmed et al., 2009; Simonetta et al., 2014; Veerapathran et al., 2013). Since DN Tregs purified from peripheral blood express all components of IL-7 and IL-15-receptors, which are composed of IL-7Rα (CD127), IL-15Rα (CD215), as well as common beta and gamma subunit of IL-2R (CD122 and CD132, respectively) (Figure 1c & d), we wanted to evaluate if addition of these cytokines to the cultures could increase the yield of functional DN Tregs.

We found that supplementation to the culture media of either IL-7 or IL-15 in addition to IL-2 increased proliferation of DN Tregs, as demonstrated by the higher yield of DN Tregs on day 28 of co-culture, when compared to DN Tregs grown in the presence of IL-2 alone (Figure 5a). However, when the viability of DN Tregs was examined at the end point of expansion, 20% of DN Tregs cultured in the presence of IL-15 were dead, in comparison to <10% of DN Tregs grown in the presence of IL-2 with or without IL-7 (Figure 5b).

In a recent report, the addition of IL-7 directly to the suppression assay was found to abrogate the immunosuppressive function of human allo-Ag-specific DN Tregs (Allgauer et al., 2015). Consequently, we wanted to determine whether the different growth conditions affected the functionality of DN Tregs. Surprisingly, we found that DN Tregs expanded in the presence of IL-2 + IL-7 demonstrated three-fold increase in their suppressive activity on per cell basis as compared to the DN Tregs grown in the presence IL-2 + IL-15 (65.42 ± 17.56% vs 18.41 ± 9.31%, respectively, $p<0.05$) (Figure 5c). These data indicate that supplementation of either IL-7 or IL-15, in addition to IL-2, increases the total yield of DN Tregs, but only IL-2 + IL-7-grown DN Tregs have potent suppressive function. Therefore, DN Tregs that were grown in the presence of IL-2 and IL-7 were used in the subsequent functional studies.
Figure 5. The effect of supplementation of IL-7 and IL-15 during the expansion on DN Treg proliferation and function. (a) Supplementation of rhIL-7 (10 ng/ml) or rhIL-15 (10 ng/ml) to cell cultures on day 14 aided DN Tregs in proliferation in comparison to cells grown in rhIL-2 (250 U/ml) only. (b) The viability of DN Tregs grown in the presence of various cytokines as assessed by 7-AAD live/dead staining. (c) DN Tregs expanded in the presence of rhIL-7 exhibit amplified suppressive function against autologous CD4+ cells activated with αCD3/CD28 beads, as assed by CFSE suppression assay. Grouped data of percentage inhibition of suppression at 8:1 suppressor-to-responder ratio is shown. Each point on the graph represents a different donor. *p<0.05. **p<0.01. ***p<0.001. n.s., non-significant.
3.4. Ex Vivo Expanded DN Tregs are Potent Suppressors In Vitro

Previously, human DN Tregs activated by mature allogeneic DCs, or αCD3/CD28 beads, have been shown to suppress proliferation of CD4+ and CD8+ T cells (Allgauer et al., 2015; Fischer et al., 2005; Voelkl et al., 2011). However, whether polyclonally activated and ex vivo expanded DN Tregs also manifest suppressive function remained unknown. Consequently, we evaluated the ability of ex vivo expanded DN Tregs to inhibit proliferation of autologous CD4+ and CD8+ T cells, as well as CD19+ B cells. To this end, CFSE-labeled T cells or B cells were polyclonally stimulated with αCD3/CD28 coated beads, or F(ab’)2 fragment of IgM, respectively, and co-cultured in the presence or absence of DN Tregs at increasing ratios. At the end of the co-culture, proliferation of the responder cells was assessed by CFSE-dilution using flow cytometry. As shown in Figure 6a, CD4+ and CD8+ T cells failed to proliferate in the absence of stimulation, but when αCD3/CD28 beads were added to the co-culture, almost all of the responder cells proliferated within 5 days. Upon addition of DN Tregs at 8:1, suppressor-to-responder ratio, the proliferation of responder cells was reduced by half. The results from triplicate cultures are summarized in Figure 6b and show a dose-dependent suppression of CD4+ and CD8+ T cells. Interestingly, suppression of CD4+ T cells by DN Tregs was consistently stronger than suppression of CD8+ T cells for all the donors that we tested.

Additionally, DN Tregs are also potent suppressors of proliferation of CD19+ B cells (Figure 6c) in a dose-dependent manner. Together these data indicate that ex vivo expanded polyclonal DN Tregs are effective at suppressing proliferation of autologous B cells, as well as CD4+ and CD8+ T cells in vitro.
Figure 6. DN Tregs suppress proliferation of autologous T cells, and CD19+ B cells. Purified naïve CD4+, CD8+ or CD19+ cells were labeled with CFSE and co-cultured with αCD3/CD28 beads or F(ab’)2 fragment of goat anti-human IgM, respectively, in the presence of ex vivo expanded DN Tregs for 4-5 days. (a) The proliferation of responder cells was measured by CFSE dilution. Shown here is an example of gating strategy. Numbers represent the percentage of CD4+ proliferating cells. (b, c) The data are expressed as mean percentage inhibition of three replicate cultures. Error bars represent SD. The experiment was repeated 8 times (b) or 3 times (c) with cells obtained from different donors with similar results.
3.5. Phenotype of *Ex Vivo* Expanded DN Tregs

To define characteristics of expanded DN Tregs, we analyzed various markers associated with Treg phenotype and/or regulatory function with the findings summarized in Figure 7a. Expanded DN Tregs maintained high expression of CD3 and TCRαβ complexes, which were slightly downregulated upon strong non-specific stimulation with PMA/ionomycin - an observation that is consistent with the biology of T cells. CD25 expression was induced after expansion and retained upon stimulation. Since low concentration of IL-7 had been regularly supplemented to the expansion medium, DN Treg had little to no expression of CD127, an IL7Ra.

CD62L, also known as L-selectin, has been shown to be important for the function of Tregs, because it allows homing to lymphoid tissues necessary for induction of peripheral suppression (Ermann *et al.*, 2005). *Ex vivo* expanded DN Tregs retained high levels of CD62L, which was downregulated upon PMA/ionomycin stimulation. Surprisingly, CD69, an early activation marker upregulated only upon stimulation, whereas CD278 (ICOS), a late activation marker, was constitutively expressed on the cell surface of DN Tregs, which expression was further upregulated through stimulation. Expanded DN Tregs have T<sub>EM</sub> phenotype, as they are CCR7<sup>-</sup>CD45RO<sup>+</sup> (Figure 7b). Low intracellular expression of regulatory molecules CTLA-4 and PD-1 upon stimulation suggests that these molecules are unlikely facilitators of DN Treg-mediated immunosuppression (Figure 7c).
Figure 7. Phenotypic characteristics of ex vivo expanded DN Tregs. DN Tregs expanded for 21 days were harvested and stimulated for 4 h with PMA/Ionomycin. (a) Change in expression of cell surface markers upon stimulation as assessed by flow cytometry. (b) Expanded DN Tregs express effector memory phenotype. (c) DN Tregs express low intracellular levels of suppressive molecules CTLA-4 and PD-1. (a-c) Representative histograms from one donor are shown. Similar results were obtained from 4 different donors.
3.6. Cytokine Profile of Ex Vivo Expanded DN Tregs

Previous analysis of cytokine profile of human DN Tregs activated with allogeneic mature DCs revealed high production of IFN-γ, and some IL-4, IL-5 and IL-10, which is similar to what has been reported in murine DN Tregs (Fischer et al., 2005; Ford et al., 2002). We sought to investigate more thoroughly the cytokine profile of ex vivo expanded polyclonal DN Tregs. We tested supernatants of DN Tregs and CD8+ T cells, expanded by the same method, with Luminex™ platform that simultaneously measured concentration of 27 different cytokines and chemokines in a single sample. Despite the fact that expanded DN Tregs displayed an activated phenotype (Figure 7a), the only notable cytokine that they produced without additional stimulation, and that has not been produced by CD8+ T cells, was IL-5, as shown in Figure 8. However, upon stimulation with PMA/ionomycin, a robust non-specific pro-inflammatory response was detected. DN Tregs released pro-inflammatory Th1 cytokines IL-2, IFN-γ and TNFα, but to a lesser extent than CD8+ T cells. DN Tregs also secreted Th2 cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, as well as other pro-inflammatory cytokines such as IL-8, all to a much higher extent than CD8+ T cells. Moreover, upon stimulation secretion of chemokines MIP-1α, MIP-1β and RANTES was detected. A rather interesting finding was high production of an anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA) upon stimulation.
Figure 8. Cytokine profile of DN Tregs. Purified DN Tregs and CD8⁺ T cells obtained from the same donor were expanded in the same manner and harvested on day 21. DN Tregs and CD8⁺ T cells were cultured in media alone, or stimulated with PMA/Ionomycin. Supernatants were collected after 4 h of co-culture. Change in production of cytokines and chemokines upon stimulation measured by Luminex™ platform. Bars represent mean concentration ± s.e.m. of 2 different donors. All values are in pg/ml. ND, non-detectable values. OOR, values outside of detectable range.
3.7. DN Treg-mediated Suppression Is Not Facilitated by IFN-γ or IL-10 Cytokines and Requires Cell-to-Cell Contact

Since addition of extrinsic IL-2 or IL-7 to the in vitro suppression assay can abrogate suppressive function of nTregs or human allo-specific DN Tregs, we evaluated whether addition of IL-2 or IL-7 had any effect on in vitro function of DN Tregs expanded only in the presence of IL-2. As seen in Figure 9, the addition of either IL-2 or IL-7 did not impair regulatory effect of DN Tregs exerted on autologous CD4⁺ T cells.

IFN-γ has been shown to be important in the function of murine DN Tregs in order to upregulate surface FasL expression and ameliorate GVHD in vivo, and suppress and kill CD4⁺ T cells in vitro (Juvet et al., 2012). Moreover, both IFN-γ and IL-10 are produced by Ag- and allo-Ag-specific human DN Tregs (Fischer et al., 2005; Voelkl et al., 2011). Therefore, we investigated whether immunosuppression in polyclonal DN Tregs is mediated by IFN-γ, or IL-10. First, we confirmed by ELISA the Luminex assay results. As seen in Figure 10a, PMA/ionomycin stimulated DN Tregs produced large amounts of IFN-γ although significantly lower than stimulated CD8⁺ T cells. Interestingly, stimulated DN Tregs produce significantly larger quantities of IL-10 in comparison to stimulated CD8⁺ T cells (Figure 10b).

However, blocking IFN-γ and/or IL-10 by addition of neutralizing Abs to either cytokine during suppression assay did not inhibit proliferation of autologous CD4⁺ T cells (Figure 11a), while CD8⁺ T cells suppression was slightly reduced upon addition of either cytokine (Figure 11b), but the results were not statistically significant. However, when both cytokines were added, the effect was not synergistic. Therefore, we concluded that unlike Ag-specific mouse DN Tregs, IFN-γ and IL-10 are not important mediators of polyclonal activated human DN Treg suppression.
Figure 9. Addition of IL-2 and/or IL-7 directly to the suppression assay does not impair functionality of DN Tregs. CFSE-labeled CD4+ cells were cultured at increasing ratios with DN Tregs that were grown only in the presence of IL-2. The co-cultures were supplemented with either IL-2, or IL-2 and IL-7, or devoid of any cytokines. After 4 days, the proliferative response of CD4+ T cells stimulated with αCD3/CD28 beads was determined by CFSE dilution. Data is expressed as mean ± SD of three replicate co-cultures. Similar results were obtained with cells from another donor.
Figure 10. DN Tregs produce IL-10 and IFN-γ. (a,b) Change in the production of IFN-γ (a) and IL-10 (b) upon PMA/Ionomycin stimulation as assessed by ELISA. Similar results were obtained from 2 different donors. **p<0.01. ***p<0.001.
Figure 11. Role of IFN-γ and IL-10 in the mechanism of DN Treg suppression. (a, b) Anti-IFN-γ and anti-IL-10 blocking mAbs were added to the DN Treg-mediated suppression assay against CD4+ (a) and CD8+ (b) responder cells obtained from the same donor. After 4 days cells were harvested and responder cells were assessed for proliferation by CFSE dilution, and flow cytometry. Similar results were obtained from 3 different donors.
Next, we investigated whether cell contact or other soluble factors are important for DN Treg-mediated suppression. To this end, we have performed a transwell suppression assay that prevents cell-to-cell contact, but allows diffusion of soluble factors across the membrane. When both DN Tregs and CD4$^+$ (Figure 12a) or CD8$^+$ (Figure 12b) responders were cultured in the same chamber thus allowing direct cell-to-cell contact, a dose-dependent suppression was observed. However, separating DN Tregs in the upper compartment from the responder T-cells and αCD3/CD28 beads in the lower compartment completely prevented the suppression of responder T-cell proliferation, and it was not due to a lack of activation of DN Tregs, as αCD3/CD28 beads were also added to the upper chamber. Moreover, we tested whether any soluble factors that get released only upon interaction of DN Tregs with responder cells would promote suppression. To this end, we co-cultured responder cells with DN Tregs and αCD3/CD28 beads in the upper chamber, but no inhibition of proliferation of responder T-cells in the lower chamber was observed. These findings demonstrate that direct cell-to-cell contact is necessary for DN Treg function.

Lastly, we analyzed the supernatants from the suppression assay co-cultures to quantify the amount of IFN-γ present. Both CD4$^+$ and CD8$^+$ responder T cells produced high amounts of IFN-γ upon stimulation with αCD3/CD28 beads (Figure 13). However, upon addition of DN Tregs, the presence of IFN-γ in the supernatants was significantly reduced. These findings demonstrate that DN Tregs prevented CD4$^+$ T cells and CD8$^+$ T cells from releasing IFN-γ.
Figure 12. Mechanism of inhibition mediated by DN Tregs requires cell-to-cell contact. (a, b) CD4⁺ or CD8⁺ responder T cells were cultured in the bottom chamber of Transwell™ flat-bottom culture plate in media containing αCD3/CD28 beads. DN Tregs were placed in the top chamber with media containing αCD3/CD28 beads in the presence of absence of responder cells. Inhibition of autologous CD4⁺ T cells (a) and CD8⁺ T cells (b) was abrogated during co-culture suppression assays in a Transwell™ system. Similar results were obtained from three different donors.
Figure 13. DN Tregs suppress secretion of IFN-γ by CD4+ T cells and CD8+ T cells. The concentration of IFN-γ was measured in the supernatants obtained from a 4-day suppression assay of DN Tregs and CD4+ T cells, or CD8+ T cells, at 4:1 suppressor-to-target ratio. The values represent mean ± SD of 3 replicates. The amount secreted by DN Tregs in the co-cultures has been subtracted. Similar results were obtained from 3 different suppression assays, each executed with a different donor. * p<0.05. *** p<0.001. n.d., non-detected.
3.8. DN Tregs Do Not Suppress by Killing Responder Cells

There is a large body of evidence that murine DN Tregs suppress immune responses by direct cytotoxicity to responder cells through various mechanisms (Juvet and Zhang, 2012). Whether human DN Tregs function by directly killing responder cells remains controversial. To determine whether DN Tregs are also cytotoxic toward responder cells, we first directly measured the viability of CD4+ and CD8+ T cells isolated after 4 days of co-culture with DN Tregs (Figure 14a & b). Upon increasing the ratio of DN Tregs to responder cells, the viability of the responder cells did not change, as measured by the percentage of AnnexinV+ and 7-AAD+ cells, suggesting that DN Tregs do not suppress by killing the responder cells.

Since 4 days is a relatively long time, there was a chance that the time window when DN Tregs induced apoptosis in the responder cells might have been missed. Therefore, a standard chromium-release assay was used to validate the results. To this end, freshly isolated CD4+ or CD8+ T cells were activated with αCD3/CD28 beads in the absence of DN Tregs for 4 days. Activated CD4+ or CD8+ T cells were then isolated, labeled with 51Cr and co-cultured with ex vivo expanded DN Tregs for 4 or 22 hours. The death of CD4+ or CD8+ T cells was assessed by the amount of 51Cr released. Results indicate that DN Tregs are not cytotoxic towards activated autologous CD4+ or CD8+ T cells, regardless of the length of co-culture with DN Tregs (Figure 14c & d). Together, these data indicate that unlike murine DN Tregs, polyclonally expanded human DN Tregs do not suppress Tconv cells by direct cytotoxicity.
Figure 14. DN Tregs do not kill autologous CD4⁺ or CD8⁺ T cells. (a) After 4 days of suppression assay, CD4⁺ cells were assessed for viability (7-AAD) and apoptotic markers (AnnexinV) through flow cytometry. Bar graphs represent mean ± SD from 3 replicates. Similar results were obtained with DN Tregs from at least 4 different donors. (b) Naïve CD4⁺ cells were stimulated for 4 days with αCD3/CD28 beads. Cells were harvested, counted and co-cultured with ex vivo expanded DN Tregs at different ratios. After 24 h, apoptosis among targets was assessed by AnnexinV staining. The figure represents one replicate out of three. Similar results were obtained from two independent experiments. (c, d) The results were confirmed by ⁵¹Cr-release assay. Activated CD4⁺ T cells or CD8⁺ T cells were labeled with ⁵¹Cr and co-cultured with DN Tregs for 4 or 24 h. The amount of ⁵¹Cr released was quantified using scintillation counter. Similar results were obtained with cells from a different donor.
3.9. *Ex Vivo* Expanded DN Tregs Kill Human Cancer Cells

Previously, we demonstrated that both TCRαβ+ and TCRγδ+ populations sorted from DN T cells expanded from peripheral blood of AML patients in complete remission were cytotoxic against autologous and allogeneic leukemic blasts *in vitro* (Merims *et al.*, 2011). Therefore, we wanted to test whether DN Tregs expanded by the means of a novel protocol also demonstrate cytotoxic function against various human cancer lines. To assess DN Treg-induced killing of cancer cells, a flow cytometry-based killing assay was adapted in which cancer cells were stained with PKH-26 before co-culture with DN Tregs. The percentage of cytotoxicity in the PKH-26-gated cell population was calculated as described in Materials and Methods, and summarized in Figure 15. DN Tregs efficiently killed leukemic MV4-11 and K562 cell lines, but were less cytotoxic towards AML-3 and KG1a cells lines (Figure 15a). Furthermore, DN Tregs exerted a dose-dependent cytotoxicity against all human primary lung cancer cell lines that were tested: 186-144, 426-177 and H125 lines (Figure 15b). These findings demonstrate that DN Tregs exhibit not only immunoregulatory function, but are also cytotoxic to various cancer cells *in vitro*. 
**Figure 15. Ex vivo expanded DN Tregs can kill human cancer cells.** (a,b) Cytotoxicity against human leukemic (a) or lung cancer (b) cell lines by *ex vivo* expanded DN Tregs was determined by flow cytometry-based killing assay. In short, cancer cells were labeled with PKH and co-cultured for 2 hours (a) or 16 hours (b) with DN Tregs at the indicated ratios. Specific killing of cancer cells was determined by calculating the proportion of cells remaining alive after co-incubation with DN Tregs. Similar results were obtained from 3 different donors.
3.10. Spatial and Temporal Dynamics of Human DN Tregs In Vivo

Due to the limitation in obtaining large numbers of human DN Tregs, the survival, proliferative potential and distribution after adoptive transfer of DN Tregs have never been studied before. The expansion method presented here allows for large-scale production of DN Tregs, thus allowing for the first time to investigate the features of DN Tregs in vivo. For this purpose, ex vivo expanded DN Tregs were stained with CFSE and infused intravenously to sublethally irradiated NSG mice. The same numbers of freshly isolated PBMCs obtained from the same donor as DN Tregs, were labelled with CFSE and injected into a group of mice as controls. Every few days, 2 to 3 mice from each cluster were sacrificed and various organs were collected to determine proliferative capacity of the infused lymphocytes. As shown in Figure 16a & b, DN Tregs, just like Tconv cells, traveled to all hematopoietic and lymphoid tissues, including peripheral blood (PB), bone marrow (BM), spleen and lymph nodes (LNs), as well as other organs including lung, liver, and kidneys. However, the proliferative response of DN Tregs was lower when compared to that of Tconv cells, as determined by the CFSE dilution (Figure 16a). Over the span of 10 days, the frequency of DN Tregs in all of the studied tissues decreased, while the frequency of Tconv cells increased (Figure 16b). Together, these findings suggest that only small proportion of DN Tregs proliferate in vivo, which may indicate that DN Tregs are less responsive or even completely unresponsive to xeno-antigens in comparison to Tconv cells, which within 10 days proliferated to a high extent in all the tissues tested.
Figure 16. Tracking and proliferation of human lymphocytes adoptively transferred to NSG mice. Freshly isolated PBMCs or ex vivo expanded DN Tregs from the same healthy donor were stained with CFSE and injected into sublethally irradiated (250 cGy) NSG mice. On days 1, 3, 5, 7 and 10 post infusion, 2 to 3 mice per group were sacrificed, and peripheral blood (PB), bone marrow (BM) and following tissues were harvested: spleen, kidneys, liver, and lungs. (a) Analysis of proliferation of lymphocytes was assessed by CFSE dilution and gated on 7-AAD− CD45+ CD3+. (b) The percentage of human CD45+CD3+ cells within 7-AAD− fraction from each tissue was assessed by flow cytometry.
3.11. *Ex Vivo* Expanded DN Tregs Delayed Onset of Xenogeneic GVHD in NSG Mice

Since DN Tregs suppress autologous T cells and travel to the same organs as Tconv cells, the ability of *ex vivo* expanded human DN Tregs to suppress the immune responses *in vivo* was evaluated by using a xenogeneic GVHD mouse model (Figure 17). To induce xenogeneic GVHD, PBMCs were freshly isolated from peripheral blood and 5×10^6 cells were infused into sub-lethally irradiated NSG mice. As shown in Figure 18a, all mice injected with PBMC only died within 31 days with the median survival time (MST) of 19 days, as manifested by severe weight loss (Figure 18b). In contrast, infusion of the same number of *ex vivo* expanded DN Tregs from the same donor did not cause any weight loss, or induce xenogeneic GVHD in the recipient mice (Figure 18a & b), and all mice remained alive and healthy up to 100 days post injection.

To determine whether DN Tregs could ameliorate GVHD, PBMC-injected mice were treated with three doses of 10^7 DN Tregs on days 0, 3 and 7 after induction of GVHD (Figure 17). Infusion of DN Tregs protected mice from GVHD as it significantly delayed an onset of disease (MST=28, *p*<0.01, Figure 18a), with one of the mice surviving for 77 days. In addition to prolonged survival, three injections of DN Tregs also significantly protected mice from weight loss, as compared to PBMC only group (*p*<0.05, Figure 18b). These data indicate that infusion of *ex vivo* expanded polyclonal human DN Tregs does not cause xenogeneic GVHD in immunodeficient mice and can attenuate GVHD induced by human PBMCs.
Figure 17. **Schematic protocol of the xenogeneic GVHD experiment.** 6-8 week old NSG mice were sublethally irradiated (250 cGy) and i.v. injected with $5 \times 10^6$ PBMCs obtained from a healthy human donor or $10^7$ DN Tregs *ex vivo* expanded from the same donor. Treated mice were infused with PBMCs followed by injection of 3 doses of $10^7$ DN Tregs on day 0, 3 and 7. Mice were monitored daily for the signs of GVHD.
Figure 18. Treatment with DN Tregs delayed the onset of xenogeneic GVHD. (a) Survival curves of the mice injected with PBMC (n=23), DN Tregs (n=14) or PBMCs and 3 doses of DN Tregs (n=14). Statistical differences between the curves were compared using log-rank test. (b) Weight curves of the same animals as depicted in (a). Mice that lost >20% of the initial weight were euthanized. For consistency, the last weight measurement of each diseased animal was kept in the analysis until the last mouse in the group was sacrificed. Statistical differences between the curves were calculated with ANOVA. Survival and weight monitoring data were pooled from three separate experiments. *p<0.05. **p<0.01. ***p<0.001.
3.12. Rapamycin Augmented Immunosuppressive Function of DN Tregs \textit{In Vitro} and \textit{In Vivo}

Rapamycin (sirolimus, RAPA) is an mTOR inhibitor that facilitates expansion and promotes the function of nTregs and iTregs in mice and humans (Shan \textit{et al.}, 2014). In the recent report, the addition of IL-7 directly to the suppression assay abrogated the immunosuppressive function of human allo-Ag-specific DN Tregs via activation of Akt/mTOR pathway, whereas inhibition of this pathway reversed the IL-7 effect (Allgauer \textit{et al.}, 2015). We found that addition of IL-7 directly to the suppression assay did not affect the function of polyclonally activated DN Tregs (Figure 9). However, we investigated whether the inhibition of Akt/mTOR pathway by rapamycin could enhance the immunosuppressive function of DN Tregs.

For this purpose, \textit{ex vivo} expanded DN Tregs were pre-incubated with rapamycin for 2 hours, extensively washed and used in a 3-day suppression assay. Blockade of the Akt/mTOR pathway by rapamycin rendered DN Tregs more immunosuppressive, as they inhibited proliferation of autologous CD4$^+$ and CD8$^+$ T cells to a greater extent than non-treated DN Tregs (Figure 19a). Suppression of CD4$^+$ T cells increased significantly by almost $52 \pm 2\%$ at the 1:1 suppressor-to-responder ratio; while at the 5:1 ratio the difference was non-significant, likely due to saturation of the suppression by untreated DN Tregs (Figure 19b). Since DN Tregs always exerted a more modest suppression against CD8$^+$ cells as compared to CD4$^+$ cells, rapamycin-treated DN Tregs demonstrated significant improvements in their suppressive activity by $17 \pm 1\%$ at 1:1 suppressor-to-responder ratio and by $27 \pm 4\%$ at 5:1 ratio (Figure 19c). These data indicate that rapamycin treatment significantly augments the suppressive function of DN Tregs.

Whether rapamycin-treated DN Tregs can also exert enhanced immunosuppressive function \textit{in vivo} was evaluated. To this end, PBMCs were infused into irradiated NSG mice to instigate xenogeneic GVHD. Treatment groups received one injection of untreated DN Tregs or rapamycin-treated DN Tregs. As shown in Figure 20a,
one injection of rapamycin-treated DN Tregs significantly delayed the onset of xenogeneic GVHD (MST=32 days, \( p<0.001 \)) in comparison to PBMC-injected mice (MST=18 days), while infusion of untreated DN Tregs did not have any significant effect on survival (MST=20 days). In addition to prolonged survival, one injection of rapamycin-treated DN Tregs also significantly protected mice from weight loss as compared to PBMC only group \((p<0.05\), Figure 20b\), whereas one injection of untreated DN Tregs had no significant effect on weight loss. Together, these data indicate that treatment of DN Tregs with rapamycin renders them more immunosuppressive both \textit{in vitro} and \textit{in vivo}.
Figure 19. Rapamycin-treated DN Tregs manifest augmented regulatory function. *Ex vivo* expanded DN Tregs were pre-incubated with rapamycin for 2 h, washed extensively and used as suppressor cells in a suppression assay against autologous CD4+ and CD8+ cells stimulated with αCD3/CD28 beads. (a) On day 3, the proliferation of CD4+ and CD8+ responder cells was quantified by CFSE dilution. The histograms represent proliferation of responder cells at 1:1, DN Treg-to-responder ratio. The number represent percentage of proliferating responder cells. (b,c) The average suppression of proliferation of CD4+ (b) and CD8+ (c) responder cells from triplicates. Similar results were observed in 3 independent experiments. **p<0.01. n.s., non-significant.
Figure 20. Rapamycin-treated DN Tregs delayed the onset of xenogeneic GVHD. 6-8 week old NSG mice were sublethally irradiated (250 cGy) and i.v. injected with 5×10⁶ PBMCs (n=28) to induce GVHD. Treated mice were infused with PBMCs followed by one injection of 10⁷ untreated DN Tregs (n=19) or one injection of 10⁷ rapamycin-treated DN Tregs (n=9). (a) Survival curves of the recipient mice. Statistical differences between the curves were compared using log-rank test. (b) Weight curves of the same animals as shown in (a). The weight of mice was monitored daily to assess severity of GVHD. Mice that lost >20% of their initial weight were euthanized. For consistency, the last weight measurement of each diseased animal was kept in the analysis until the last mouse in the group expired. Statistical differences between curves were calculated with ANOVA. Survival and weight monitoring data were pooled from four separate experiments. ***p<0.001. *p<0.05. n.s., non-significant.
CHAPTER 4. DISCUSSION
Chapter 4

Discussion

4.1. General Discussion

DN Tregs comprise only 1 to 2% of lymphocytes in the human peripheral blood. The major obstacles in studying DN Tregs are their low frequency and the lack of effective expansion method. In this study, we presented a novel protocol for a large-scale ex vivo expansion of human DN Tregs. The methodologies in the previously published studies required a large starting population, generation of allogeneic mature DCs as a source of stimulation and activation, and extensive supplementation of growth factors, and cytokines (Fischer et al., 2005; Voelkl et al., 2011). We developed a less complicated and arguably cheaper protocol that allows for the generation of up to ~10^9 DN Tregs within 21 days. With the method here presented, a much smaller starting population can be used as opposed to leukophoresis product. On average we were able to expand DN Tregs ~3500-fold and generate clinically significant numbers with only 50 to 100 ml of fresh peripheral blood obtained from healthy donors, or 3-10×10^7 cryopreserved PBMCs.

DN Tregs do not express any specific markers that would allow for one-step isolation from PBMCs. Furthermore, DN Tregs being such a rare population of lymphocytes are unsuitable for FACS sorting, because it would have negative effect on the viability and the cell number of the starting population due to prolonged sorting times. To bypass these obstacles and to reduce the complexity of the process of selection from PBMCs, we decided to deplete other T cells and NK cells by magnetic beads sorting, as these are the cells would proliferate extensively under the DN Treg expansion conditions. Therefore, the initial DN Treg population contained other mononuclear cells such as monocytes and B cells, which briefly played a role of supporting cells until they died off due to lack of specific activation. Currently, the most common T cell expansion methods include supplementation of IL-2 and stimulation of cells with anti-CD3 mAb,
αCD3/CD28 beads, allogeneic APCs in the form of B cells or DCs, or artificial APCs (aAPCs). Since autologous APCs were present in the initial co-culture, the stimulatory signal was provided by plate bound anti-CD3 mAb to ensure DN Treg activation. Efforts in using soluble anti-CD3 mAb to facilitate further expansion of DN Tregs were undertaken, but were fruitless suggesting that DN Tregs need other co-stimulatory signals. This may be due to the fact that anti-CD3 mAb delivers a moderate proliferative signal through the T cell receptor complex (signal 1), but in the absence of additional co-stimulatory signals (signal 2) the resulting proliferation is often followed by premature T cell apoptosis or anergy (Schwartz, 1990). However, too strong of stimuli can also lead to the exhaustion of the cell colony. Thus every round of stimulation is a balancing act between stimuli strong enough to drive proliferation, but weak enough to not cause activation induced cell death (AICD).

Previously reported was that human DN Tregs exerted their suppressive activity exclusively after pre-activation (Voelkl et al., 2011). Thereby in preceding studies DN Tregs were activated with allogeneic mDCs (Allgauer et al., 2015; Fischer et al., 2005; Voelkl et al., 2011). However, the proliferation of DN Tregs was modest even though mDCs would provide signals 1 and 2. Furthermore, using mDCs for the expansion purposes is challenging, as they have to be differentiated in vitro from mononuclear cells isolated from the donors’ blood prior to DN Treg expansion. Since mDCs are unsuitable for cryopreservation, the number of generated mDCs would be a limiting factor if used in a large-scale expansion. Since mDCs would have to be constantly in culture, it would impose additional materials and labour costs. aAPCs, a K562 cells line that expresses transduced anti-CD3 Ab and co-stimulatory molecules 4-11BL, CD80 and CD86 among other things, provides co-stimulatory signals similar to mDCs, and may offer an alternative method of the expansion of DN Tregs with the exception in TCR-specificity, as mDC-stimulated DN Tregs would have allogeneic specificity, whereas aAPC-stimulated DN Tregs would be polyclonal. There are many advantages in using aAPCs as opposed to mDCs: aAPCs being an immortalized cell line are easy to grow, suitable for cryopreservation for later use and represent a readily available off-the-shelf product appropriate for the expansion of DN Tregs from virtually any donor. The final protocol
that yields up to $1\times10^8$ DN Tregs calls for 1 round of stimulation with anti-CD3 mAb to specifically activate DN Tregs and 3 rounds of stimulation with irradiated aAPCs to provide a broad range of co-stimulatory signals, for the total expansion time of 21 days. We were able to expand cells from all donors that we tested (n=8) and using this protocol we achieved ~3500 fold expansion of DN Tregs. DN Tregs continue to proliferate after 21 days upon addition of irradiated aAPCs every 5-7 days. However, the viability of the cells progressively decreases, along with their function, most likely due to AICD. After 42 days of expansion, DN Tregs manifested severely diminished function (data not shown).

One of the most surprising findings of this study was the difference in the magnitude of suppressive function of DN Tregs that were grown in the presence of IL-7 or IL-15, in addition to IL-2. Although the scientific community is still uncertain about the requirement and the role of these two cytokines on the subject of homeostasis and function of Tregs, both IL-7 and IL-15 have been used as growth factors in Treg expansion protocols. The fact that freshly isolated DN Tregs expressed all the components of IL-7 and IL-15 receptors indicated that DN Treg may be receptive to these cytokines. The addition of IL-15 apart from IL-2 resulted in more robust proliferation in comparison to DN Tregs grown solely in IL-2, or IL-2 + IL-7. However, the viability of IL-2 + IL-15-grown DN Tregs was significantly reduced as compared to either IL-2 or IL-2 + IL-7-grown DN Tregs, which may be a sign of AICD. Also, the addition of IL-15 resulted in the outgrowth of residual NK cells that had to be depleted on a regular basis, a phenomenon absent in the other culture conditions. Looking at the viability and the proliferative potential, supplementing expansion culture with IL-2 + IL-7 seemed to generate most promising candidates. When we compared the ability of differently grown DN Tregs to suppress autologous CD4$^+$ T cells, DN Tregs that were expanded in the presence of IL-2 + IL-7 significantly outperformed other contenders. Therefore, DN Treg expansion culture was supplemented with IL-2 and IL-7.

Interestingly, when the phenotype of IL-7 and IL-15-grown DN Tregs was compared it did not reveal any significant differences with regards to the expression of cell surface markers that were tested (data not shown). However, detailed analysis of the
activation, as well as inhibitory molecules, may give guidance to the mechanisms of action of DN Tregs.

The phenotype of \textit{ex vivo} expanded DN Tregs using the proposed method is comparable to what was previously observed with allo-Ag-specific DN Tregs (Fischer \textit{et al.}, 2005; Voelkl \textit{et al.}, 2011). Expanded DN Tregs have higher expression of CD25 and CD122, the components of IL-2 receptor, which is on par with what is generally observed in any T cell expansion protocol due to supplementation of exogenous IL-2. CD25 is a well-known activation marker, thus multiple rounds of activation with aAPCs would further induce its expression. Furthermore, expanded DN Tregs have somewhat surprising expression of other activation markers. CD278, known as inducible T cell co-stimulator (ICOS), is a late activation marker that is expressed on the expanded DN Tregs. An early activation marker CD69 is not expressed on the expanded DN Tregs and only gets upregulated upon very strong stimulation with PMA/ionomycin. This property of DN Tregs differs from that of conventional T cells. However, we are unable to propose explanation of this phenomenon at this moment. Kinetic studies involving induced activation and changes in the receptor expression need to be conducted. Lastly, DN Tregs express high levels of L-selectin (CD62L), a homing receptor to secondary lymphoid organs, even after a three-week expansion. L-selectin has been shown to be critical in nTreg function, as its expression indicated highly suppressive Tregs within the CD4^+CD25^+Foxp3^+ cohort. Upon further stimulation, L-selectin gets downregulated and thus would render DN Treg unable to enter secondary lymphoid tissues. In this study we were unable to determine the importance of L-selectin expression on the DN Treg function. However, using DN Tregs activated with PMA/ionomycin in the suppression assay could reveal the role of L-selectin as it may be important in \textit{in vivo} studies and clinical applications.

A potential problem with the expansion that has not been addressed could be a reduction in DN Treg TCR repertoire and presence of non-regulatory cells in the \textit{ex vivo} expanded population. Data from multiple studies regarding V\(\beta\) and V\(\alpha\) gene usage of DN Tregs is conflicting (Bristeau-Leprince \textit{et al.}, 2008; Brooks \textit{et al.}, 1993; Fischer \textit{et al.}, 2005; Niehues \textit{et al.}, 1994) and most likely reflects the heterogeneity of this population.
We have shown that DN Tregs are composed of naïve and effector memory phenotypes, and it has been reported that different expansion methods may preferentially target one cell type over the other, as it has been observed apropos expansion of Tconv cells. Higher L-selectin expression in the ex vivo expanded DN Treg population than on freshly isolated DN Tregs suggests that DN Tregs expanded mostly likely from the naïve cohort. If our suspicion is correct these cells should have highly diverse TCR, which would not be the case with memory or effector phenotypes. However, only comparative analysis of the TCR Vβ repertoire of freshly isolated and ex vivo expanded DN Tregs would reveal whether all, or only specific DN Tregs clones were expanded further confirming the polyclonal status of DN Tregs.

Previous studies demonstrated that DN Tregs can induce Ag-specific immune tolerance in various murine models (Chen et al., 2003a; Chen et al., 2005; Ford et al., 2002; Minagawa et al., 2004; Thomson et al., 2007; Zhang et al., 2007) and human DN Tregs have been shown to supress in vitro in Ag- or allo-Ag-specific manner (Allgauer et al., 2015; Fischer et al., 2005; Voelkl et al., 2011). For the first time, we show that DN Treg-mediated suppression can also be non-specific, as ex vivo expanded DN Tregs with polyclonal specificity successfully suppressed proliferation of autologous CD4+ T cells and CD8+ T cells stimulated with αCD3/CD28 beads. In all the donors that we tested, CD4+ T cells were consistently suppressed more than CD8+ T cells. One possibility for this discrepancy may come from the fact that CD4+ T cells were positively selected using solely CD4 mAb, and thus 5-10% of total CD4+ T population may represent CD4+CD25+ T cells, the majority of which would be nTregs that might add to the overall suppression. However, no suppression of responder CD4+ T cells has been observed in the absence of DN Tregs. Thus nTregs, although potent, had minimal effect on the suppression of CD4+ T cells, likely due to insufficient stimulation. However, it is possible although unlikely that DN Tregs may directly interact with nTregs or induce naïve CD4+ T cells to differentiate into iTregs, either directly or via soluble factors such as IL-10, thus contributing to the overall suppression. Studies need to be conducted that would evaluate the relationship of DN Tregs with nTregs and the ability of DN Tregs to aid in differentiation of iTregs.
DN Tregs exhibit a unique cytokine production profile that differs from that of CD4+ T cells and CD8+ T cells. Since a proportion of DN Tregs may be CD8+ T cells that downregulated CD8 co-receptor, we compared the cytokine production of DN Tregs to CD8+ T cells. Similarly to CD8+ T cells, minimal cytokine production by the ex vivo expanded DN Tregs was detected in the absence of stimulation. However, upon potent non-specific stimulation with PMA and ionomycin, DN Tregs secreted a robust array of different cytokines, which differed from those secreted by CD8+ T cells in the amount and type of cytokine secreted. Stimulated DN Tregs were skewed towards the production of Th2 cytokines, which in nTregs were found to promote immune tolerance (Tran et al., 2012), and thus may act as an important regulatory factors in DN Treg suppression. Furthermore, a lot of cytokines have pro- and anti-inflammatory properties depending on the context of stimulation. The ability to robustly produce such a wide array of different cytokines may also indicate heterogeneity of DN Tregs. This could mean two different things: 1) there could be many subtypes of DN Tregs and each subtype may produce different set of cytokines; or 2) a single DN Treg cell may be able to produce different set of cytokines depending on the milieu, site, Ag recognition or interactions with different immune cells.

Studies on nTregs, as well as different types of iTreg subsets, revealed that Tregs regulate immune responses via production of immune cytokines such as IL-10 and TGF-β. Human DN Tregs are potent producers of IL-10 and IFN-γ upon stimulation, and these cytokines played a significant role in the murine DN Treg-mediated suppression (Dugas et al., 2010; Ford et al., 2002; Juvet et al., 2012). However, neutralization of IL-10 or IFN-γ during the inhibition of proliferation assays of CD4+ T cells and CD8+ T cells had insignificant effect on the DN Treg-mediated suppression. Furthermore, we showed that polyclonal DN Tregs require cell-to-cell contact to mediate suppression, since the presence of the membrane in transwell assay prevented suppression of the responder cells, indicating that immunosuppression is not dependent on IL-10, IFN-γ, or other soluble factors. Furthermore, the addition of exogenous IL-2 or IL-7 to the suppression assay failed to abolish DN Treg regulatory function, indicating that unlike nTregs, they do not suppress through consumption of IL-2.
Multiple studies have reported that murine DN Tregs mediate suppression by eliminating T cells through Fas/FasL interaction or via perforin/granzyme, which would ultimately induce apoptosis in the responder cells (Chen et al., 2003a; Ford et al., 2002; Young et al., 2002; Zhang et al., 2007; Zhang et al., 2006; Zhang et al., 2000). However, there are conflicting studies regarding human DN Tregs mediation of suppression by elimination of responder T cells (Fischer et al., 2005; Voelkl et al., 2011). AnnexinV/7-AAD staining did not reveal a decrease in the viability of CD4$^+$ T cells or CD8$^+$ T cells upon addition of DN Tregs, suggesting that DN Tregs do not induce apoptosis in the responder T cells. Further study was conducted to measure directly the ability of DN Tregs to kill activated responder T cells and revealed that DN Tregs are not cytotoxic towards responder T cells. Interestingly, DN Tregs are capable of cytotoxic activity, as they successfully killed various leukemic and lung cancer cell lines.

We have shown that DN Tregs exhibit dual function; they can successfully kill some human cancer lines, as well as suppress the proliferation of lymphocytes. This dual function is somewhat unusual. However, it is yet to be determined if the suppressive and cytotoxic functions are performed by the same cells. If one cell is capable of both functions, then depending on the milieu cytotoxic function may override regulatory function and vice versa. However, since both regulatory and cytotoxic capacity of DN Tregs is lower in comparison to nTregs and TCR$\gamma$$\delta$$^+$CD4$^+$CD8$^-$ T cells, respectively, it is also possible that certain subsets of DN Tregs are specialized to carry out different effector functions. Finding a definitive phenotypic marker that would distinguish a truly regulatory population would certainly magnify appeal of these cells as potential candidates for adoptive cellular therapy.

Furthermore, for the first time we show that human DN Tregs are capable of suppression of proliferation of autologous CD19$^+$ B cells, just as murine DN Tregs do so but in an Ag-specific manner (Hillhouse et al., 2010; Ma et al., 2008; Zhang et al., 2006). This dose-dependent suppression of B cells opens doors for wider applications of DN Tregs in the clinic, as autoreactive B cells mediate many autoimmune diseases (Browning, 2006). The in vitro results suggest that DN Tregs participate not only in the regulation of cellular immunity, but also humoral immunity. Due to time constraints, in this study we
were unable to determine whether DN Tregs also hamper B cell activation, class-switching or antibody production. However, all these questions should be addressed in the future.

The major caveat of the proposed expansion protocol is that expanded DN Tregs have polyclonal reactivity and thus exhibit reduced suppressive function in comparison to Ag- or allo-Ag-specific DN Tregs, i.e. more DN Tregs are needed to generate similar levels of suppression. However, a reduction in polyclonal nTreg potency following ex vivo expansion has been observed previously. Therefore, we are working towards production of allo-Ag-specific DN Tregs by using allogeneic B cells as activators instead of mDCs. Working with B cells is arguably less challenging than working with mDCs, as large quantity of B cells may be produced in relatively short period of time using CD40L-transduced fibroblasts. B cells are not temperature sensitive and can be cryopreserved for immediate, off-the-shelf use. However, this expansion approach requires further optimization, as we could only produce limited amount of cells thus far. Nevertheless, we believe that stimulation with aAPCs post allogeneic B cell activation would increase the yield of allo-Ag-specific DN Tregs, which would essentially increase DN Treg potency and reduce the number of DN Tregs needed.

To increase the potency of polyclonal DN Tregs we further evaluated whether treatment of DN Tregs with rapamycin, an mTOR inhibitor, would have any effect on the suppressive function of DN Tregs. In a recently published study, human allo-Ag-specific DN Tregs were found to be sensitive to IL-7 and addition of IL-7 to the suppression assay severely hampered the suppressive function of DN Tregs (Allgauer et al., 2015). The authors found that IL-7 hyperactivated Akt/mTOR pathway, however inhibition of this pathway by Akt or mTOR inhibitors restored the functionality of DN Tregs. Furthermore, it has been found that rapamycin increased suppressive capacity of nTregs and iTregs, as well as enhanced generation of iTregs (Lu et al., 2014). In this study, we showed that DN Tregs were most immunosuppressive, when they were grown in the presence of IL-7. Additionally, supplementation of exogenous IL-7 to the suppression assay did not have any effect on DN Treg function. We found that a 2-hour pre-incubation with rapamycin prior to the suppression assay significantly increased the DN Treg-mediated suppressive
function on par with what has been observed in other Tregs (Lu et al., 2014). However, the molecular mechanisms behind this suppression are unknown. We hypothesize that rapamycin may induce T cell anergy by blocking the Akt/mTOR pathway that is responsible for survival and proliferation, and switching to STAT5 signalling pathway that drives anergy associated molecules to be upregulated on the cell surface of these cells. Further studies are needed to evaluate changes in any cell surface markers upon mTOR inhibition. It is yet to be determined what impact the mTOR inhibition has on the proliferation of DN Tregs, as rapamycin drives proliferation in nTregs and iTregs but not conventional T cells (Lu et al., 2014). Whether there are any alterations in the phenotype or expression of any suppressive molecules in rapamycin treated-DN Tregs should also be evaluated in the future. Comparison of the phenotypes of the rapamycin-treated and untreated DN Tregs could be useful in revealing markers that partake in the immunosuppressive function of DN Tregs.

We demonstrate for the first time that DN Treg infusion attenuated xenogeneic GVHD. However, DN Tregs only provided a temporary protection from GVHD since all mice that received DN Treg infusion eventually developed signs of GVHD, such as weight loss, hunched posture and apathy. This could be due to lack of persistence, or survival of the infused human DN Tregs in the mouse environment. Since the in vitro studies revealed that DN Treg-mediated suppression is cell-contact dependent and does not involve elimination of the responder T cells, the ability of DN Tregs to protect mice for GVHD would continue as long as the DN Tregs survive inside the host. Furthermore, we propose that the presence of DN Tregs may increase the activation threshold of the responder cells, thus the magnitude of suppression mediated by DN Tregs in vivo may directly depend on the number of injected DN Tregs. One injection of DN Tregs was insufficient to significantly delay the onset of GVHD. Three injections over the span of one week, however, significantly delayed onset GVHD by ~10 days and reduced the severity of GVHD based on the weight loss data. We have also evaluated whether injection of rapamycin treated-DN Tregs would minimize the amount of DN Tregs required to have protective effect from GVHD, as rapamycin treatment rendered DN Tregs more immunosuppressive than untreated DN Tregs in vitro. Intrestingly, one
injection of rapamycin treated-DN Tregs protected mice to similar levels as treatment with three doses of untreated DN Tregs, when we compare the median survival time between the two groups. Further studies are needed to assess whether infusion of more rapamycin treated-DN Tregs, or injection of higher doses of DN Treg would halt GVHD progression entirely. Also, it is yet to be determined whether approaches combining DN Treg infusion together with rapamycin, or low-dose IL-7 would be more effective than infusion DN Tregs alone.

Importantly, unlike the infusion of PBMCs, the infusion of DN Tregs did not induce xenogeneic GVHD. DN Tregs migrate to lymphatic organs and other tissues in a similar fashion to PBMCs. However, DN Tregs only expanded marginally in vivo, as evident by the decreasing numbers of DN Tregs infiltrating the organs of NSG mice. This unresponsiveness of DN Tregs to xeno-antigens may be attributable to the their lack of expression of TCR co-receptors, CD4 and CD8, on their cell surface, which is a very attractive and important feature in the clinical applications of DN Tregs.

DN Tregs appear to have an interesting property of being able to inhibit GVHD, while mediating cytotoxicity towards cancer cells. Harnessing their potential in the clinic as candidates for adoptive cellular therapy would have enormous implications in the prevention of GVHD in leukemia patients undergoing allogeneic HSCT, as well as aid solid organ transplant recipients with pre-existing malignancies in remission. Further understanding of DN Treg mechanisms of action may help in development of novel immunotherapies.
4.2. Future Directions

Understanding of the DN Treg development and biology is severely lagging behind other immune cells due to their low frequency, the lack of markers to identify/isolate them, and the lack of effective expansion methods. Over the past years many studies of murine DN Tregs have surfaced and there is no doubt that they are strong suppressors of the immune system. However, there are still a lot of unanswered questions with regards to human DN Tregs. Herein, we presented a method for a large-scale expansion of human DN Tregs that will hopefully invite more researchers to explore in-depth the DN Treg biology and function.

The presence DN Tregs have been found in many organs, but their niche has not been completely identified. Many questions remain, for instance what activates DN Tregs? What drives their proliferation? Since they are capable of producing many different cytokines upon non-specific stimulation, what cytokines get released under what pathophysiological settings? How do DN Tregs interact and communicate with other cells in the body? The answers to the stated questions may not only advance our understanding of the mechanisms of suppression mediated by DN Tregs, but may also facilitate the clinical application of DN Tregs. In the future, infusion of DN Tregs into human patients may provide necessary means to induce transplantation tolerance along with the reduced risk of developing GVHD. In turn this would lead to improved patient survival and decreased likelihood of graft rejection, ultimately reducing health care costs and enhancing the well being of transplant patients. However, before this could be possible the studies outlined below will aid in understanding of these cells bringing them one step closer to the clinic.
4.2.1. To Identify Markers That Are Critical for DN Treg Function

A major challenge that is yet to be addressed is identifying markers that are associated with truly regulatory, or truly cytotoxic DN Tregs, as well as markers that would set ‘functional’ DN Tregs apart from the ‘pathological loss of function’ DN Tregs in ALPS, or CD4$^+$ and CD8$^+$ T cells that have downregulated their co-receptors. Furthermore, the origin and thymic development of DN Tregs are still largely unknown. Although multiple mechanisms of suppression have been described from murine models to date no specific mechanisms have been proposed for human DN Tregs. We and others have demonstrated that DN Treg suppression is cell contact-dependent, but does not involve killing of the responder T cells (Voelkl et al., 2011). Furthermore, DN Tregs require TCR signalling and de novo protein synthesis (Fischer et al., 2005; Voelkl et al., 2011) implying that certain protein molecules are produced. These may include cell surface proteins and/or soluble factors. Using flow cytometry, DN Tregs were examined for the multitude of surface proteins found to be involved in inhibitory function in their murine counterparts, as well as other Treg subsets, such as Fas/FasL, CTLA-4, perforin/granzyme and PD-1. Although DN Tregs are capable of producing perforin/granzyme upon TCR stimulation, DN Treg did not induce cell death in the responder cells, thus eliminating the possibility of perforin/granzyme involvement. Moreover, we have found that IL-2 + IL-7-grown DN Tregs had lower expression of CTLA-4 and PD-1 as compared to IL-2 + IL-15-grown DN Tregs (data not shown), suggesting other molecules are involved in the suppression mechanisms. Lastly, DN Tregs did not express Fas on their cell surface.

To identify new molecules that may be associated with DN Treg-mediated suppression, we can take advantage of high-throughput screening (HTS) technology that allows for screening of over 370 cell surface molecules including a majority of functionally known CD molecules, as well as non-CD molecules including a variety of TCR and HLA molecules. Experiments can be performed on any flow cytometer with a high-throughput sampler. Since DN Tregs grown in the presence of IL-2 + IL-7 or IL-2 + IL-15 manifest different inhibitory abilities, we hypothesize that the differences in the
receptor expression between the two groups may pinpoint to the molecules involved in
the mechanism of suppression of DN Tregs, as well the expression of markers unique to
DN Tregs if compared to other subtypes of T cells from the same donor. An even better
comparison of the cell surface molecules may be between rapamycin-treated and
untreated DN Tregs. However, this comparison may be proven problematic due to the
differences in the receptor expression on the cell surface rather than presence or absence
of individual markers. An alternative method could be an exploitation of microarray-
based gene expression profiling, which can be used to identify genes whose expression
are changed in response to either IL-2 + IL-7 or IL-2 + IL-15 cytokine treatment during
the expansion, or rapamycin-treatment post expansion. The comparison of gene
expression in these two conditions may pinpoint to the molecules involved in the
suppression.

4.2.2. To Determine the Effects of DN Treg-Immunosuppressive
Agents Combination Therapy on the Treatment of
Xenogeneic GVHD

Another obstacle that would hinder clinical application of DN Tregs is the fact
that DN Tregs do not prevent GVHD from occurring in a xenogeneic mouse model, but
rather the infusion of DN Tregs delays its onset and severity. Therefore, modified
treatment regimens should be explored that would ideally prevent GVHD from occurring
all together. Since rapamycin inhibits activation of B cells and T cells, and we have
shown that the pre-treatment of DN Tregs with rapamycin prior to the infusion augments
their suppressive function, it would be worthwhile to evaluate the effects of the co-
 injection of rapamycin with DN Tregs in the xenogeneic GVHD model. We suspect that
the rapamycin would hinder the proliferation of conventional T cells, while at the same
time promote the regulatory function of DN Tregs, rendering them more
immunosuppressive.

Furthermore, the data from the tracking study revealed that DN Tregs proliferate
poorly in vivo likely due to the lack of human cytokines that would support their survival
and proliferation. Therefore, the next step should involve evaluation of injection of exogenous IL-2 and/or IL-7 on the suppressive function, proliferation and survival of human DN Tregs in mice. Furthermore, the \textit{in vitro} mechanisms of suppression may be different from the mechanisms engaged during suppression \textit{in vivo}, as it has been exemplified by nTregs and their effect of IL-10 production. Since we have showed that DN Tregs are capable of producing copious amounts of IL-10, yet IL-10 does not seem to play a role in DN Treg-mediated immunosuppression \textit{in vitro}, it is important to evaluate whether IL-10 plays any role \textit{in vivo} as it may for example induce generation of iTregs from conventional cells. Additionally, DN Tregs seem to reduce the ability of responder T cells to secrete IFN-\(\gamma\). Therefore, it would be worthwhile to measure the differences in the levels of pro- and anti-inflammatory cytokines in the mouse sera between the DN Treg-treated and untreated groups. This may be achieved by using a simple ELISA method or with the help of Luminex platform that allows for simultaneous measurement of more than 27 different cytokines. These studies will shed more light on the function of DN Tregs \textit{in vivo}.

### 4.2.3. To Determine Whether Human DN Tregs Suppress DCs

Both donor and recipient DCs play a critical role in mounting allogeneic immune responses through direct and indirect antigen presentation. Moreover, both human and mouse nTregs and iTregs can suppress allo-reactive CD4\(^+\) and CD8\(^+\) T cell proliferation by down regulating CD80/CD86 on mDCs through CTLA-4. Similarly, murine DN Tregs also express a high level of CTLA-4 that is critical for down regulation of CD80 and CD86 expression on DCs, as this capacity is lost in murine DN Tregs from CTLA-4 deficient mice (Kowalczyk \textit{et al.}, 2014). Since human DN Tregs do not express high levels of CTLA-4, whether human DN Tregs can alter the function or kill allo-Ag-expressing DCs is still unknown. To see if human DN Tregs are cytotoxic to allogeneic DCs, allo-Ag-specific DCs need to be generated first and co-cultured with DN Tregs in an \textit{in vitro} suppression assay. After 4 days, DCs can be stained with AnnexinV and 7-AAD to determine their viability and whether they are susceptible to DN Tregs. If DCs
would be found to be alive, to study the function of the DCs after the co-culture human DN Tregs will be removed using CD3-coated beads and the ability of remaining DCs to stimulate naïve CD4⁺ and CD8⁺ T cell proliferation will be assessed by CFSE dilution. These studies will determine if human DN Tregs are cytotoxic towards DCs and/or whether DN Tregs can impair their function despite the negligible expression of CTLA-4.

4.2.4. To Determine Whether Trogocytosis is Critical for Human DN Treg Function

In this study, we presented for the first time the ability of polyclonally stimulated ex vivo expanded human DN Tregs to suppress the responder cells in vitro. However, it is a well-known phenomenon that Ag-specific Tregs are more effective at suppressing allograft rejection than polyclonally activated Tregs (Putnam et al., 2013; Sagoo et al., 2011). Furthermore, CD40 ligand-stimulated human B cells are more potent than DCs at inducing proliferation of donor-specific human Tregs (Tang et al., 2012; Zheng et al., 2010). However, why activated B cells are better than DCs at generating donor-specific human Tregs is not known. Since we had some success in generating (but not expanding) allo-Ag-specific DN Tregs using allogeneic B cells, it would be important to determine whether differences in the acquisition of allo-Ag from donor APC alter human DN Treg ability to mount Ag-specific suppression. To this end, human DN Tregs can be isolated from HLA-A2⁻ donors and co-cultured with either CD40L-activated HLA-A2⁺ donor B cells or mDCs. The expression of HLA-A2 on human DN Tregs can be measured using anti-HLA-A2 Ab at varying time points after co-culture. To determine the importance of acquired allo-Ag in human DN Tregs-mediated suppression, A2⁺ APC-primed HLA-A2⁺ and A2⁻ DN Tregs can be sorted by FACS and used to suppress autologous CD4⁺ and CD8⁺ T cells in vitro. DN Tregs from the same donor activated by the anti-CD3 and aAPC that do not express HLA (Butler et al., 2012) will be used as a control. The specificity of acquisition of allo-Ag can be determined by blocking TCR-HLA interactions with anti-HLA-A2 Ab. Together, these studies will establish whether CD40L-activated B cells are better than mDCs at donating allo-Ag to human DN Tregs.
and enhancing their suppressive function and whether acquisition of allo-Ag by human DN Tregs is critical for their ability to suppress allo-reactive T cell responses *in vitro*.

### 4.2.5. To Determine Signalling Pathways That Govern DN Tregs

It is unknown, which nutrients and growth factors are necessary for DN Treg function. DN Treg preference for metabolic pathways is also unknown. Since DN Tregs behave differently when grown in the presence of IL-7 versus IL-15, it strongly suggests that common gamma chain receptors are important modulators. To determine the signalling pathways that govern DN Treg function, a series of experiments measuring the downstream signalling molecules should be performed. Developed in the last decade, the phosphoflow technique could be used to track and measure the phosphorylation of the key signalling molecules such as “STATs, members of the MAPK and stress-activated protein kinase families, other cell survival kinases and adaptor molecules” (Wu *et al.*, 2010). This technique would determine which pathways are involved when different growth factors or antigen stimuli are present, together with the metabolic pathway experiments that would define the preference for nutrients and measure metabolic waste, and respiration, would shine the light on signalling pathways and metabolic requirements of DN Tregs. Furthermore, qPCR could be used to determine which transcription factors play a role in DN Treg function.
4.3. Conclusion

Presented here is a novel method that allows for large-scale *ex vivo* expansion of human DN Tregs. Using this protocol, up to ~$10^9$ DN Tregs of very high purity can be obtained from 50 to 100 ml of blood within 3 weeks. Importantly, these *ex vivo* expanded DN Tregs are functional *in vitro* as they suppress the proliferation of autologous T cells and B cells.

For the first time, we show that DN Treg suppressive function is modulated by the presence of cytokines during the expansion. Addition of IL-7 to the expansion co-culture was found to increase suppressive function of DN Tregs, whereas addition of IL-15 promoted expansion of DN Tregs, but resulted in hampered suppressive function. The mechanism of DN Treg suppression is cell-contact-dependent and results in reduced production of IFN-γ by CD4$^+$ and CD8$^+$ T cells. However, DN Tregs do not supress by inducing apoptosis in the responder cells, even though they are capable of cytotoxic function as they successfully eliminated various leukemic and lung cancer cells.

The function of *ex vivo* expanded human DN Tregs *in vivo* has been investigated for the first time by employing xenogeneic GVHD mouse model. The infusion of human PBMC into immunodeficient NSG mice induced severe acute xenogeneic GVHD. However, injection of 3 doses of DN Tregs within a week of initiation of GVHD resulted in significantly delayed onset of GVHD and significant reduction in weight loss. Furthermore, a single infusion of *ex vivo* expanded DN Tregs to NSG mice did not cause GVHD or tissue damage in the recipient, highlighting the safety of DN Tregs.

Lastly, pre-treatment of *ex vivo* expanded DN Tregs with an mTOR inhibitor rapamycin significantly improved regulatory function of DN Tregs both *in vitro* and *in vivo*. These finding hold important implications for the future use of DN Tregs in the clinic, since it can significantly reduce the amount of DN Tregs required for infusion, ultimately decreasing the need to expand large amount of cells, which would widen the feasibility of DN Treg application in adaptive cellular therapy.
The ability to obtain large quantity of pure and functional DN Tregs not only makes it possible to study their function and mechanisms of action *in vivo*, but also highlights the possibility of using *ex vivo* expanded human DN Tregs in immunotherapy. DN Tregs have enormous potential of acting as double-edge swords in the treatment and prophylaxis of GVHD, especially after BMT for leukemia, as DN Tregs would not only inhibit GVHD, but also promote anti-cancer effect in these patients.
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