

REGULATION OF AUTOIMMUNITY AND INTEGRIN  
SIGNALING BY ADAPTOR PROTEINS  
ADAP, PRAM-1 AND C-CBL

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## **ABSTRACT**

### **Regulation of Autoimmunity and Integrin Signaling by Adaptor Proteins ADAP, PRAM-1 and C-Cbl**

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To increase our understanding of adaptor proteins in immune responses, we investigated the roles of adaptor protein ADAP in autoimmune diabetes, and PRAM-1 and c-Cbl in integrin signaling in neutrophils. ADAP deficiency introduced into BDC2.5 transgenic background enhances lymphopenia-stimulated proliferation of autoreactive T cells and autoimmune diabetes, which can be relieved by syngeneic transferred T cells. Further studies suggest that impaired thymic selection, but not defective survival of peripheral T cells, contributes to enhanced lymphopenic stimulation in ADAP-deficient BDC2.5 mice. Therefore, we conclude that ADAP suppresses lymphopenia-dependent autoimmune diabetes through the promotion of thymic output. These data reveal a novel mechanism of autoimmunity regulated by adaptor protein ADAP. When we started to explore the molecular mechanisms regulated by PRAM-1 of integrin signaling in neutrophils, we detected coimmunoprecipitation of c-Cbl and PRAM-1 in NB4 cells. Further, we discovered that c-Cbl positively regulates integrin-dependent oxygen burst but negatively regulates Fc receptor-dependent Ca<sup>2+</sup> flux in neutrophils. The underlying molecular mechanisms are currently under investigation. Collectively, data presented in this thesis emphasize the essential roles of adaptor proteins in signal transduction and autoimmunity, and will increase our understanding of adaptor proteins in immune responses.

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## ABBREVIATIONS USED IN THIS THESIS

ADAP	Adhesion and Degranulation-promoting Adapter Protein
AICD	Activation Induced Cell Death
AIRE/aire	Autoimmune Regulator
APC	Antigen Presenting Cell
APECED	Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy
ATRA	All-Trans-Retinoic Acid
Bax	Bcl-2-Associated X protein
BCL2	B-cell Lymphoma/leukemia-2 Gene
Bcl-xL	Basal Cell Lymphoma-Extra Large
BDC2.5 g7b I-A <sup>g7/b</sup>	BDC2.5 TCR transgenic mice, C57Bl/6 background, bearing I-A <sup>g7/b</sup>
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
C3bi	C3bINA-cleaved C3b
C3b	181,000 Mr fragment of C3
cAMP	Cyclic Adenosine Monophosphate
Card9	Caspase Recruitment Domain-containing Protein 9
CARMA1	Caspase Recruitment Domain, CARD, Membrane-Associated Guanylate Kinase, MAGUK, Protein 1
Cbl	Casitas B-lineage Lymphoma
CDC42	Cell Division Cycle 42
CD	Cluster Determinant
CGD	Chronic Granulomatous Disease
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFA	Complete (heat-killed mycobacterium-containing) Freund's Adjuvant
CrkL	Crk-like (CrkL) Adaptor Proteins
Ctl	Control
CTLA-4	cytotoxic T-lymphocyte antigen-4
DAG	Diacylglycerol
DAP12	DNAX Activation Protein of 12kDa
DP	Double Positive
DOCK2	Dedicator of Cyto-Kinesis 2
Dx5 <sup>+</sup>	CD49b
EAE	Experimental Autoimmune Encephalomyelitis
ER	Endoplasmic Reticulum
EVH1	Ena/VASP Homology-1
FACS	Fluorescence Activated Cell Sorting
FasL	Fas ligand
FcR $\gamma$	Fc receptor $\gamma$
Fgr	Feline Sarcoma Viral
FMLP	N-Formyl-Methionyl-Leucyl-Phenylalanine
Foxp3	Forkhead Box P3
Fyb	Fyn-T-binding Protein
GAD	Glutamic Acid Decarboxylase
Gads	Grb2-related Adaptor Downstream of Shc
GEF	Guanine nucleotide-exchange factors

Grb2	Growth Factor Receptor Binding Protein 2
HIP55	HPK1-Interacting Protein of 55 kDa
Hck	Hematopoietic Cell Kinase
ICAM	Intercellular Adhesion Molecules
IDDM	Insulin-Dependent Diabetes Mellitus
IL	Interleukin
IFN	Interferon
IP	Intra Peritoneal
ITAM	Immunoreceptor Tyrosine-based Activation Motif LAD leukocyte Adhesion Deficiency
IV	Intravenous
JAK3	Janus Kinase 3
JNK	c-jun N-terminal kinase
LAT	Linker for Activation of T cells
LFA-1	Lymphocyte Function-associated Antigen 1
LPS	Lipopolysaccharide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NB4	A Human Promyelocytic Leukemia Cell Line
NF $\kappa$ B	Nuclear Factor- $\kappa$ B
NK	Natural Killer
MALT-1	Mucosa-Associated Lymphoid Tissue Lymphoma Translocation Protein 1
MAP kinase	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex;
MFG-E8	Milk Fat Globule-EGF Factor 8 Protein
MRL/lpr	MRL mice carry lymphoproliferation spontaneous mutation ( <i>Fas<sup>lpr</sup></i> )
NOD	Non-Obese Diabetic
NKT:	Natural Killer T cells;
N-LN	Non-draining Lymph Node;
PD-1	Programmed Death 1
PDZ domain	Domain shared by synaptic density protein (PSD95), Drosophila disc large Tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)
PI3 kinase	Phosphatidylinositol 3' kinase
PIP2	Phosphatidylinositol Bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
P-LN	Pancreatic Lymph Node;
pTen	Phosphatase and Tensin Homolog
PRAM-1	PML-RARa target gene encoding an Adaptor Molecule-1
PTB domain	Phosphotyrosine-Binding Domain
Pyk2	Proline-rich Tyrosine Kinase 2
RAIM	Rap1-GTP-Interacting Adapter Molecule
RAG	Recombination Activating Genes
Rap1A	RAS-Related Protein-1a
RHOA	Ras Homolog Gene Family, Member A
ROI	Reactive Oxygen Intermediates
SAKP55	55kD Src Kinase-Associated Phosphoprotein
Scid	Severe Combined Immunodeficiency
S.D.	Standard Deviation

SH2/3	Src Homology Domain
Shc	Src Homology 2 Domain Containing Protein
SKAP-HOM	55kD Src Kinase-Associated Phosphoprotein (Homolog)
SLP-76	SH2 Domain Containing Leukocyte Phosphoprotein of 76 kD
SLAP-130	SLP-76-Associated Protein of 130 Kilodaltons
SOS	Son of Sevenless
SP	Single Positive;
Syk	Spleen Tyrosine Kinase
T1D	Type 1 Diabetes
TCR	T cell Receptor
TGF $\beta$	Transforming Growth Factor $\beta$
Th	T cell Helper
TLR	Toll Like Receptor
TNF $\alpha$	Tumour Necrosis Factor $\alpha$
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
Tregs	Regulatory T cells
VCAM-1	Vascular Cell Adhesion Molecule
WASP	Wiskott-Aldrich Syndrome Protein
Zap70	$\zeta$ -associated protein of 70 kDa

## **INTRODUCTION TO ADAPTOR PROTEINS IN IMMUNE RESPONSES**

Immune responses are regulated by different types of cells whose activities rely on signaling transductions by numerous proteins as a network. Among regulators of signal transduction, adaptor proteins play a critical role in assembling different modules as a complex in response to different stimuli. Adaptor proteins represent a group of proteins, which lack enzymatic activities but possess different protein-protein interacting domains and specific motifs. (Some proteins have enzymatic activity but also function as adaptor proteins.) These domains include Src homology (SH2), phosphotyrosine-binding (PTB), Src homology 3 (SH3), pleckstrin homology, WW and PDZ domains. Different domains and motifs show specific interaction with each other. For example, SH2 and PTB domains recognize phosphorylated tyrosine residues, while the SH3 domain binds to proline-rich sequences. The PH domain interacts with phospholipids, and the PDZ domain binds to carboxy-terminal hydrophobic residues. Many adaptor proteins containing proline rich sequences are able to bind to SH3 domain of other proteins <sup>1</sup>. In leukocytes, a large number of adaptor proteins have been identified in signal transduction downstream of different receptors, such as TCR on T cells, Fc receptor, integrins, Toll like receptors on neutrophils. To increase our understanding of the function of adaptor proteins in immune responses, we focused on adaptor proteins ADAP in autoimmunity, and PRAM-1 and c-Cbl in integrin signal transduction in neutrophils.

## **CHAPTER 1**

### **REGULATION OF AUTOIMMUNITY: AN OVERVIEW**

The immune system has the capacity to purge foreign antigens such as bacteria, virus, fungi, and parasites while ignoring self-antigens. Ignorance of self-antigens by the immune system is called self-tolerance. Breakdown of self-tolerance results in autoimmunity. There are multiple mechanisms as a network to secure immune system tolerance to self-antigens. Self-tolerance can generally be divided into central and peripheral tolerance (in this thesis, only T cell tolerance will be discussed). Central tolerance describes the process of elimination of autoreactive T cells during T cell development in the thymus, while peripheral tolerance defines the suppression of autoreactive T cells in the peripheral lymph organs, including T cell intrinsic and extrinsic mechanisms. In contrast, suppression of lymphopenia is another distinct mechanism in self-tolerance, since lymphopenia mediated autoimmunity is only due to low lymphocyte (more precisely, autoreactive cells) number in spite of normal function of effector lymphocytes and normal function and development of other individual regulatory cell populations.

#### **Regulation of Autoimmunity by Lymphopenic Stimulation**

##### **Introduction to homeostatic proliferation**

The immune system is predisposed to keep a consistent size during one's lifespan. It has been noticed for a long time that significant loss of lymphocytes is associated with aggressive proliferation of

remaining lymphocytes and that transferred lymphocytes proliferate rapidly in irradiated or immune deficient (like RAG deficient) recipients <sup>2</sup>. Even in healthy hosts, chronic proliferation of mature lymphocytes in the periphery occurs to replace the loss of apoptotic cells. This chronic proliferation is more obvious and meaningful after thymic output of lymphocytes is decreased and stopped. The process whereby lymphocytes proliferate and fill out the “free lymph space” is called homeostatic proliferation <sup>3</sup>. Two types of homeostatic proliferation have been recently demonstrated: spontaneous and nonspontaneous (note: “spontaneous” and “homeostatic” were first proposed by Min et al in 2005 to refer the two types of lymphopenia stimulation proliferation. To avoid confusion, “spontaneous” and “nonspontaneous” are used in this thesis) <sup>2</sup>. These two different types of homeostatic proliferation differ in several aspects. Spontaneous proliferation is characterized by a burst-like cell cycle, while nonspontaneous homeostatic proliferation has a slow and stable cell cycle. Only spontaneously proliferating cells show memory like phenotype. Spontaneous proliferation depends on the interaction between TCR and self-ligand-MHC complex, but not the IL7 signal. In contrast, the IL7 signal is required for nonspontaneous proliferation <sup>2</sup>. Tregs can selectively suppress spontaneous proliferation <sup>4</sup>. Whether spontaneous and nonspontaneous proliferations differ in autoimmune pathogenesis remains unknown.

### **Regulation of T cell homeostatic proliferation**

How the immune system senses “free space” is one of the essential and interesting questions in immunology. One model holds that,

in a lymphopenic environment, remaining lymphocytes proliferate with access to free growth factors like cytokines and self-antigen MHC complex. Studies showed that, when transferred into MHC deficient hosts, naïve T cells, including CD4 and CD8 T cells, were unable to go homeostatic proliferation and failed to survive for the long term, emphasizing the requirement of antigen/MHC complex for T cell survival and homeostatic proliferation<sup>5-9</sup>. The importance of antigen/MHC complex is also illustrated with differential requirement of secondary lymph organs in proliferation of CD4 and CD8 T cells. In animals lacking secondary lymph organs, CD8 but not CD4 T cells were able to proliferate<sup>10</sup>. A plausible explanation is that non-professional antigen presenting cells outside of secondary lymph organs express MHC class I but not MHC class II so that CD8 T cells can still contact with MHC complex without secondary lymph organs but CD4 T cells do not<sup>10</sup>. Partial tyrosine phosphorylation of the TCR $\xi$  chain<sup>11</sup> and constitutive association of Zap70 and TCR $\xi$  chain<sup>12</sup> were detected in peripheral T cells but not in cell lines, indicating that self-antigen/MHC complex may provide persistent and weak signal important for T cell survival.

Besides self-antigen/MHC complex, a number of studies showed that cytokines are critical in T cell survival and homeostatic proliferation. Among cytokines studied, IL7 plays a pivotal role in both naïve and memory T cell homeostatic proliferation. In contrast to IL7 sufficient hosts, wild-type T cells could not proliferate after being transferred into IL7 deficient recipients (irradiated or Rag deficient) and IL7R deficient T cells could not survive in vivo, underscoring absolute requirement of IL7 signaling in T cell survival and homeostatic proliferation<sup>13,14</sup>. As a

downstream effector of IL7 receptor signaling, JAK3 was also demonstrated to be indispensable for T cell homeostatic proliferation<sup>15</sup>. It was shown that IL7 promotes T cell survival through regulation of pro- and anti-apoptotic genes like BCL2 and Bax<sup>16</sup>. However, overexpression of BCL2 did not rescue the defective homeostatic proliferation of IL7 deficient T cells, suggesting additional contribution by IL7 signal to homeostatic expansion<sup>14</sup>. Moreover, overexpression of IL7 increased the size of the lymphocyte pool, indicating that IL7 functions as a limiting factor for T cell homeostatic proliferation<sup>17</sup>. The IL7 receptor shares a common  $\gamma$  chain with other cytokine receptors, including IL2, IL4, IL7, IL9, IL15 and IL21 receptors. Although cytokines like IL2, IL4, and IL15 could promote T cell proliferation in vitro, they are not required for homeostatic expansion of naive T cells in vivo<sup>14</sup>, highlighting a unique and nonredundant role of IL7 in T cell homeostatic proliferation.

### **Unique features of homeostatic proliferation**

Although homeostatic proliferation is also driven by the MHC complex, it shows several features different from foreign antigen-mediated proliferation. First, homeostatic proliferation can occur in the absence of co-stimulatory molecules like CD28 on T cells and CD40 on APCs<sup>18</sup>. Second, homeostatic proliferating T cells manifest memory phenotypes. For example, they show CD44<sup>hi</sup> without upregulation of CD25 and CD69, the latter two markers are usually transiently upregulated on antigen activated T cells<sup>3</sup>. Moreover, CD8 T cells taking homeostatic proliferation acquire cytotoxic T-lymphocyte function to

foreign antigen and display similar magnitude and kinetics of immune responses as memory cells<sup>3</sup>.

### **Regulation of autoimmunity by lymphopenic stimulation**

Clinically, a strong correlation between lymphopenia and autoimmune diseases has been documented for a long time. Patients with autoimmune diseases, including rheumatoid arthritis, insulin-dependent diabetes mellitus, Crohn's disease, systemic lupus erythematosus, and primary vasculitides often show low lymphocyte counts<sup>19</sup>. Chemo- and radio-therapies that wipe out transiently or permanently lymphocytes usually induce autoimmune syndromes<sup>20</sup>. Coincidence of autoimmune problems and lymphopenia can be often observed after viral infections<sup>21,22</sup>. A typical example is human immunodeficiency virus infection, which profoundly depletes CD4 T cells and induces autoimmune syndromes<sup>23</sup>.

Lymphopenia has been also studied in animal models for a long time. Decades ago, one study showed that thymectomy of neonatal mice induced multiple organ specific autoimmune diseases<sup>24</sup>, having sparked immunologists' interest in lymphopenia and autoimmunity. Although lymphopenia has been studied for a long time, whether lymphopenia itself is a causative factor or just a bystander effect from autoimmune responses remains in debate. The major reason for this debate is involvement of Tregs (CD4<sup>+</sup> Foxp3<sup>+</sup>) in regulation of both homeostatic proliferation and autoimmunity<sup>25</sup>. Many previous studies on lymphopenia in autoimmunity did not exclude Tregs' contribution. For instance, thymectomy at neonatal day 3 induced lymphopenia and autoimmune

disorders<sup>26,27</sup>. However, thymectomy at neonatal day 3 also specifically removes Tregs, because Tregs start to migrate out of the thymus after day 3 of birth<sup>26,27</sup>. As a result, it is difficult to distinguish the contribution of removed Tregs from that of lymphopenia to induction of autoimmunity in thymectomized mice<sup>26,27</sup>. In spite of the difficulty in discriminating between effects of Tregs and lymphopenia in autoimmunity studies, there are increasing evidences suggesting a causative role for lymphopenia in autoimmunity. For example, thymectomy of NOD female at weaning age accelerated autoimmunity<sup>28</sup> and transferred total spleen cells from healthy mice can cause autoimmune diabetes in immunodeficient NODscid mice with intact ratio of effectors/Tregs and normal function of Tregs. These observations suggest that lymphopenia can promote autoimmunity when Tregs are normal in function and number (ratio to autoreactive T cells). Recently, King and his colleagues observed a decreased lymphocyte number and enhanced autoimmune diabetes in NOD mice with age. When they transferred syngeneic T cells into NOD mice, autoimmune diabetes was suppressed. Since syngeneic transfer does not change the ratio of Tregs versus effectors, these findings suggest that lymphopenia can cause autoimmunity, although Tregs have not been evaluated directly in these mice<sup>29</sup>.

### **A model of how lymphopenia mediates autoimmunity**

How does lymphopenia cause autoimmunity? It was proposed that in a lymphopenic scenario, free self-ligand MHC complexes and growth factors such cytokine IL7 are accessible. As a result, free self-ligand MHC complex can preferentially drive autoreactive cells to

proliferate, since TCR on these cells has higher avidity to self-ligand MHC complex. Although Tregs with TCR specific to self antigen could also expand in a lymphopenic environment, Tregs and autoreactive T cells may have different thresholds or kinetics to proliferate in response to lymphopenic stimuli. The balance between autoimmunity and self-tolerance depends on the race between Tregs and effectors.

### **Thymic selection regulated by adaptor proteins in lymphopenia and autoimmunity**

A number of adaptor proteins have been characterized as key regulators in TCR signaling, including Grb2, Gads, LAT, CrkL, SAKP55, Shc, SLP-76, and ADAP<sup>30</sup>. Genetic and biochemical approaches have been revealing a fundamental role for adaptor proteins in thymocyte development and regulation of lymphocyte homeostasis. For instance, genetic depletion of LAT resulted in defective thymocyte development and severe lymphopenia<sup>31 32,33</sup>. SLP-76 deficient mice lacked lymph nodes and peripheral lymphocytes due to a block in thymocyte development at CD4 /CD8 double negative stage<sup>34</sup>. Other adaptors, including SKAP55, Grb2, CrkL, and Shc, as well as ADAP, all have been implicated in T cell activity and thymocyte development<sup>35</sup>. Given the importance of adaptor proteins on thymic selection, adaptor proteins may regulate autoimmunity through selection of autoreactive/regulatory T cells or thymus-dependent lymphopenic stimulation. Several studies indicate that adaptor proteins are critical in regulation of autoreactive T cells. For instance, it was documented that LAT regulated T cell anergy<sup>36</sup> and that a mutation of LAT (tyrosine 136 was replaced with

phenylalanine) skewed T cell differentiation towards Th2 lineage and caused a fast-onset lymphoproliferative disorder<sup>37</sup>. However, whether adaptor proteins downstream of TCR regulate autoimmunity through lymphopenic stimulation is still poorly understood.

### **Regulation of TCR signal by adaptor protein ADAP**

ADAP (also known as SLAP-130 or Fyb) was identified in a screening of SLP-76 and fyn binding proteins<sup>38,39</sup>. ADAP is expressed in hematopoietic cells including  $\alpha\beta$  T cells,  $\gamma\delta$  T cells, NK cells, and myeloid cells but not in B cells<sup>40</sup>. There are two isoforms of ADAP proteins: p120 and p130, with an insertion of additional 46 amino acid domain in the latter. The p120 isoform is predominantly expressed in thymocytes while p130 is dominant in peripheral T cells<sup>41</sup>. Evidence from bone marrow chimera demonstrated that both p120 and p130 could rescue the thymocyte development (Joanna Dluzniewska's unpublished data).

As an adaptor protein, ADAP contains multiple protein-protein interaction domains. From n-terminal to c-terminal, ADAP contains a central proline rich region, E/K-rich region, a first SH3-like domain, an EVH1 binding domain, and a second SH3-like domain, plus multiple tyrosine residues distributed<sup>42</sup>. The proline-rich domain of ADAP mediates its binding to the SH3 domains of SKAP55 and SKAP55-hom, which have been implicated in cell cytoskeleton rearrangement<sup>43</sup>. A second SKAP55 binding site in ADAP locates in the second SH3-like domain. The EVH1 binding domain is essential for the association with Ena/VASP family of actin regulatory proteins<sup>44</sup>. The first SH3 like domain and E/K-rich region both are required and sufficient for the association

with CARMA1/BCL10/MALT-1 complex<sup>44,45</sup>. ADAP also has multiple tyrosine residues, which can be phosphorylated by FynT kinase and which mediate inducible binding of SLP-76 after TCR engagement<sup>38,39</sup>.

Genetic approach revealed a positive function for ADAP in TCR signaling<sup>46,47</sup>. With CD3 stimulation, ADAP-deficient T cells are defective in CD69, CD25 upregulation, cytokine IL2 secretion, and proliferation in vitro<sup>46,47</sup>. Further studies demonstrated that ADAP controls optimal T cell proliferation, cytokine production, and expression of the prosurvival protein Bcl-xL in a dosage-dependent manner<sup>48</sup>. Moreover, ADAP-deficient T cells showed impaired integrin clustering and conjugate formation after TCR engagement<sup>46,47</sup>. Recently, thymocyte development has been examined in ADAP-deficient mice<sup>48,49</sup>. Nontransgenic ADAP-deficient mice showed moderate reduction of CD4 and CD8 single positive cells, and a more dramatic defect has been observed in TCR transgenic mouse models, suggesting that ADAP is required for thymocyte development<sup>48,49</sup>.

The molecular mechanisms of regulation of TCR signaling by ADAP have been understood only recently. Initial studies showed that ADAP is dispensable for the TCR proximal signaling events, including activation of PLC $\gamma$ , SLP76, Vav, Ca<sup>2+</sup> flux, and MAPK pathway<sup>46,47</sup>. One recent study showed that ADAP regulates T cell activation through activation of NF $\kappa$ B, since defective activation of NF $\kappa$ B is impaired in ADAP-deficient T cells after TCR stimulation<sup>45</sup>. Further, ADAP-deficient T cells showed defective assembly and membrane translocation of CARMA-1/ BCL-10/MALT-1 complex, which is required for NF $\kappa$ B nuclear translocation, suggesting that ADAP regulates NF $\kappa$ B nuclear translocation through CARMA-1/ BCL-

10/MALT-1 complex in T cell activation <sup>45,45,50,51</sup>. ADAP also regulates integrin activation on T cells through SKAP55, which is required for T cell/APC conjugate formation <sup>52,53</sup>. Based on these findings, it was proposed that ADAP may regulate T cell activation and integrin activation through two different mechanisms: CARMA-1/ BCL-10/MALT-1/NF $\kappa$ B and SKAP55 <sup>54,55</sup>.

## **Central Tolerance in Autoimmunity**

### **Introduction to central tolerance**

During development, T cells randomly express a TCR repertoire, recognizing a broad range of self and nonself antigens. To generate a diverse and mature repertoire of TCRs with appropriate affinity to numerous pathogens, but not active to self-antigens, T cells with newly expressed TCRs need to undergo positive and negative selections. The fate of thymocytes depends on TCR affinity to self-antigen/MHC complexes. Only thymocytes with intermediate affinity to self-antigen MHC complexes receive survival signals and go through positive selection. In contrast, thymocytes expressing TCRs with strong affinity to self-antigens go through negative selection. The process of elimination of self-reactive T cell clones is called central tolerance. T cells bearing TCRs with low affinity also die by ignorance. As a conclusion, TCR affinity is critical for thymic selection. Alteration of TCR signal strength may disrupt central tolerance, resulting in the escape of autoreactive T cells into periphery and autoimmune disorders. For example, a point mutation identified in Zap70, a TCR signaling module, was able to cause spontaneous rheumatoid arthritis due to impaired negative selection of autoreactive T cells <sup>56</sup>. Defective apoptosis is another major mechanism to break down central tolerance, emphasized by studies of pro-apoptotic genes such as BIM <sup>57</sup>, TRAIL <sup>58</sup>, BCL-3 <sup>59</sup> and pTen <sup>60</sup> in autoimmunity.

### **Mechanisms of central tolerance**

Medullar epithelial cells have been shown critical in thymocyte negative selection <sup>61</sup>. To eliminate self-reactive T cells, medullar epithelial cells can ectopically express organ specific antigens by AIRE/aire, a transcription factor, in the thymus <sup>62</sup>. This process is called promiscuous gene expression of peripheral antigens <sup>63,64</sup>. Micro array technique revealed that 5-10% of total genes can be transcribed in medullar epithelial cells, including insulin, acetylcholine receptor and myelin basic protein <sup>62,64,65</sup>. The significance of central tolerance in autoimmunity is underscored with identification of mutations in the AIRE gene in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. APECED is a spontaneous, polyglandular autoimmune disease characterized by damaged parathyroid and/or adrenal glands, plus other autoimmune syndromes, including premature ovarian failure and diabetes <sup>62</sup>. The function of aire in autoimmunity has been also proved in animal models <sup>62</sup>.

## **Peripheral Tolerance in Autoimmunity**

### **Introduction to peripheral tolerance**

Although central tolerance functions as the first guard line to eliminate self-reactive T cells, peripheral tolerance is also required to prevent autoimmune diseases. Due to ineffective thymic selection, there are always some autoreactive T cells capable of escaping from thymic selection even in healthy hosts. This is reflected by autoimmunity in immune-deficient hosts induced by transferred spleen cells from healthy mice <sup>66</sup>. There are multiple mechanisms to secure peripheral tolerance,

including T cell intrinsic and extrinsic tolerance. Mechanisms of induction of T cell intrinsic tolerance include ignorance, anergy, cytokines skewing, and apoptosis, while extrinsic mechanisms are mediated by regulatory cells and tolerogenic dendritic cells <sup>67</sup>.

### **T cell intrinsic mechanisms of peripheral tolerance**

Ignorance describes a scenario where autoreactive T cells are sequestered from self-antigen or concentration of self-antigen is too low to activate these cells <sup>68-70</sup>. Co-stimulation and cytokine IL12 are required to fully activate T cells<sup>71,72</sup>. Without infection and tissue damage, a low level of co-stimulatory receptor on APCs and lack of cytokine may cause T cell anergy and avoid autoimmunity. This notion has been supported with observation of infection mediated autoimmunity <sup>73</sup>. Moreover, CTLA4 and PD-1 can attenuate co-stimulatory receptor signaling, resulting in T cell anergy <sup>74,75</sup>, and disruptions of either of these two proteins have been demonstrated to cause autoimmune diseases <sup>76-79</sup>, emphasizing the importance of T cell anergy in self-tolerance. Endogenous antigen can last for the long term and repeatedly stimulate autoreactive T cells, causing cell death (activation induced cell death, AICD). Fas/FasL mediated AICD has been implicated as another essential mechanism of self-tolerance in both humans and mice <sup>67,80,81</sup>.

### **T cell extrinsic mechanisms of peripheral tolerance**

Cell extrinsic mechanisms of self-tolerance include non T cell regulatory populations, such as Tregs, APCs, NKT cells, and others. Initially Tregs have been defined with CD4<sup>+</sup>, CD25<sup>+</sup> and CD45RC<sup>low</sup> <sup>82,83</sup>.

Recent studies showed that Tregs express the functional marker FoxP3, a member of the forkhead/winged-helix family of transcription factors. Mutations of the FoxP3 gene have been identified in a spontaneous X-linked mutation mouse strain scurf<sup>84</sup>, revealing a critical role of Tregs in self-tolerance<sup>85</sup>. Tregs may suppress autoreactive T cells in more than one way. Tregs can suppress autoreactive T cells by direct contact<sup>86</sup> or through inhibitory factors like TGF $\beta$ <sup>87</sup>, IL10<sup>88</sup>, or cAMP<sup>89</sup>. Moreover, Tregs can also directly kill target cells with granzyme and perforin<sup>90</sup> or suppress autoreactive T cells through APCs<sup>91,92</sup>. Another mechanism for inhibitory activity of Tregs is to compete for IL2 with effector T cells, since Tregs express a higher level of IL2 receptor (CD25)<sup>93</sup>.

Two major antigen presenting cells, dendritic cells and macrophages, play essential roles in autoimmunity. Dendritic cells can regulate self-tolerance through multiple ways, including T cell anergy, cytokine mediated cell differentiation, and prolonged antigen presentation<sup>94-100</sup>. Depletion of plasmacytoid dendritic cells enhanced experimental autoimmune encephalomyelitis<sup>101</sup>, suggesting that a specific subpopulation of dendritic cells negatively regulates autoimmune diseases. Clearance of apoptotic cells by macrophage has a critical influence on the development of autoimmunity. Genetic deficiency of MFG-E8 (a receptor for phosphatidylserine exposed on apoptotic cells) on macrophages resulted in lupus-like diseases<sup>102</sup>. Auto-antigens like chromatin and small nuclear ribonucleoproteins, when they are cleared ineffectively, can engage TLR receptors on dendritic cells and cause autoimmune problems<sup>103-109</sup>. Macrophages also play a role in the

differentiation of autoreactive T cells in regulation of autoimmune responses <sup>110</sup>.

Besides Tregs and APCs, many other regulatory cell populations, including NKT cells, Dx5<sup>+</sup>CD4<sup>+</sup> T cells, CD8 $\alpha\alpha$  T cells and NK cells, are also implicated in regulation of autoimmune responses. NKT cells are characterized by expressions of NK receptors (NK1.1) and T cell receptors (TCR). The TCR on NKT cells recognizes an antigen, glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), presented by a nonclassic antigen-presenting molecule CD1d <sup>111</sup>. Many studies showed that NKT cells negatively regulate autoimmune diabetes <sup>112-114</sup>. Cytokines may be the key mediators for NTK cells to regulate autoimmune diseases <sup>115,116</sup>. CD4<sup>+</sup>Dx5<sup>+</sup> conventional  $\alpha\beta$  T cells are another regulatory population implicated in autoimmune diabetes in BDC2.5 transgenic mouse model in Rag deficient background <sup>117</sup>. Unlike conventional CD8 $\alpha\beta$  T cells, CD8 $\alpha\alpha$  T cells express two alpha chains without a beta chain. CD8 $\alpha\alpha$  cells can directly kill activated target cells with TCR specificity and suppress autoimmunity <sup>118</sup>. Although NK cells are well known in viral clearance and tumor suppression, they are also implicated in autoimmune response <sup>119-121</sup>.

## **Autoimmune Diabetes as an Organ-Specific Autoimmune Disease**

### **Introduction to autoimmune diabetes**

Based on the targets of immune responses, autoimmune disorders can be categorized into systemic or organ-specific autoimmunity. Systemic Lupus Erythematosus is a typical example of systemic

autoimmune disorders. This systemic disease is characterized by autoreactive antibodies against universal nuclear antigens, such as dsDNA, histone, and nucleosomes. In contrast, organ-specific autoimmune responses, including EAE, and autoimmune diabetes, manifest syndromes in individual organs. For example, Experimental Autoimmune Encephalomyelitis (EAE), a model for multiple sclerosis study in humans, can be induced with injection of myelin antigen, resulting in inflammation in the central neutral system <sup>122</sup>.

Autoimmune diabetes (also named as Insulin-dependent diabetes mellitus, IDDM, Type 1 Diabetes, T1D), as an organ-specific autoimmune disease, is characterized by destruction of insulin producing  $\beta$  cells in pancreas by autoreactive T cells recognizing the  $\beta$  cell-derived antigens. Although either CD4 or CD8 T cells alone are sufficient to induce autoimmune diabetes in different models, cooperation of CD4 and CD8 T cells is more efficient than either of these populations in pathogenesis of diabetes <sup>123,124</sup>. More than one antigen can trigger autoimmune diabetes, including insulin <sup>125</sup> and GAD <sup>126</sup>. A wave of physical apoptosis of  $\beta$  cells at juvenile stage was observed in correspondence with high frequency of diabetes, suggesting that self antigen released from natural apoptic islet cells causes autoimmune diabetes <sup>127</sup>.

### **Central and peripheral tolerance in autoimmune diabetes**

Both environmental (like gluten and milk exposure) and genetic risk factors contribute to the development of autoimmune diabetes. Genetic contribution has been emphasized with study of monozygotic twins with concordance rate of 30-50%, compared to sibling rate of 6%

incidence<sup>128</sup>. As a typical example of autoimmune diseases, autoimmune diabetes is regulated by both central and peripheral tolerance. Among the genetic susceptibility loci, the MHC locus shows the strongest linkage with diabetes incidence. Ser $\beta$ 57 residues harbored in I-A<sup>g7</sup> render the MHC susceptibility of diabetes, while Asp $\beta$ 57 residue in I-A<sup>b</sup> is protective<sup>129</sup>. MHC may regulate autoimmunity through both central and peripheral tolerance (selection of regulatory cells)<sup>130</sup>. For example, one I-A<sup>b</sup> allele induced into BDC2.5 transgenic mice preferentially selected V $\alpha$ 2<sup>+</sup> ( $\alpha$  chain for TCR) thymocytes and suppressed autoimmune diabetes. T cells expressing endogenous V $\alpha$ 2<sup>+</sup> chain were demonstrated to be protective in autoimmunity, suggesting selection of regulatory T cells by MHC in autoimmunity<sup>131</sup>. Demonstration of expression of  $\beta$  islet cell-derived antigens like insulin and GAD in thymus by transcription factor aire<sup>62</sup> suggests the importance of thymic selection in autoimmune diabetes.

Antigen Presenting Cells (APCs) have been implicated in the regulation of autoimmune diabetes, including dendritic cells, macrophages, and B cells. Dendritic cells may present antigen from apoptotic  $\beta$  islet cells to activate autoreactive T cells<sup>127</sup>. Depletion of macrophages revealed an essential role for macrophages in the development of diabetes in NOD mice through mediation of cytotoxic T cell development<sup>132</sup>. Evidence has also demonstrated contribution by B cells to the development of autoimmune diabetes<sup>133</sup>. Similar to other autoimmune diseases, autoimmune diabetes is subject to regulation by Tregs<sup>134</sup> as well as by Dx5<sup>+</sup>CD4 T cells<sup>135</sup>.

### **BDC2.5 transgenic mouse model in autoimmune diabetes study**

There are multiple animal models useful in the study of autoimmune diabetes, including BB rat, NOD mice, transgenic mice like BDC2.5 mice, and others. BDC2.5 transgenic animals possess T cells bearing a T cell receptor (TCR) that is reactive with an unknown pancreatic islet cell antigen and that is restricted by the NOD-derived MHC Class II molecule I-A<sup>g7</sup>. Infiltration of the pancreatic islets by the autoreactive T cells reproducibly occurs within weeks of birth <sup>136</sup>. Histological analysis of the pancreas reveals mixed lymphocytic infiltrates similar in composition to those seen in human autoimmune diabetes. When the BDC2.5 transgene is expressed on a C57Bl/6 background, a high percentage of transgene positive mice (60% of animals homozygous for I-A<sup>g7</sup> <sup>137</sup> and 25% of animals bearing a protective I-A<sup>b</sup> allele <sup>138</sup>) develop hyperglycemia by 10 weeks of age. BDC2.5 TCR transgenic mice have been used to define activation characteristics of diabetogenic T cells <sup>139</sup>, to explore the role of suppressor cell populations <sup>117,140</sup>, and to identify background genes that modify the autoimmune process <sup>138</sup>. Thus, the BDC2.5 line forms a robust, highly-penetrated model of spontaneous autoimmune diabetes.

## CHAPTER 2

### REGULATION OF AUTOIMMUNE DIABETES IN BDC2.5 MICE BY ADAPTOR PROTEIN ADAP

#### **Introduction**

Autoimmune diabetes develops due to an organ-specific autoimmune reaction mediated by T lymphocytes responsive to  $\beta$ -islet cell self-antigens<sup>141,142</sup>. In part, failure to maintain self-tolerance in autoimmune diabetes may be ascribed to dysregulation of peripheral T cell homeostasis. Alterations in both the quality (reduced repertoire diversity) and the quantity (T cell lymphopenia) of the T cell compartment have been causally linked with increased diabetes incidence in animals<sup>117,138,143</sup>. In NOD mice, T cell lymphopenia has been strongly implicated in diabetes development<sup>29</sup>. A prevailing model of T lymphocyte homeostasis holds that in a lymphopenic animal, decreased competition for growth-regulating resources and for antigen/MHC<sup>144</sup> results in enhanced proliferation of the remaining cells. Selective expansion and activation of autoreactive clones induced by T cell lymphopenia may result in augmented destructive T cell potential at the inflammation site<sup>145</sup>.

Causes of lymphopenia may include the actions of environmental toxins (e.g., drugs, irradiation) and impaired regulation of T cell homeostasis determined by either decreased T cell survival or production<sup>145</sup>. A causal link between increased turnover and susceptibility of autoreactive NOD T cells to apoptosis and lymphopenia-induced diabetes was recently established<sup>29</sup>. Ablation of thymic output by thymectomy

results in lymphopenia associated with exaggerated diabetes incidence<sup>28,146</sup>, suggesting the essential contribution of thymic output to lymphopenia-mediated autoimmunity. A large number of TCR modules, including tyrosine kinases and adaptor proteins, have been implicated in thymic selection. However, a causal relationship between abnormalities in T cell receptor-dependent positive selection of autoreactive T cells and lymphopenia leading to disease has not been demonstrated.

Adaptor proteins as a group have been appreciated in signal transduction downstream of different surface receptors, including TCR on T cells. Adaptor proteins identified in TCR signaling include LAT, SLP76, Gads, SKAP55, ADAP, and others. ADAP is a recently characterized positive regulator of both thymocyte development and TCR signaling<sup>38,147</sup>. ADAP is a cytoplasmic adapter protein with expression limited to hematopoietic cells<sup>39,49</sup>. Loss of ADAP results in impaired positive and negative thymocyte selection, as well as reduced peripheral T cell population<sup>46,47,148</sup>. However, the role of ADAP in regulating autoimmune responses in vivo has not been characterized. To increase our understanding of the role of adaptor proteins in autoimmunity, we set out to determine whether defects in thymic selection and/or T cell homeostasis engendered by ADAP deficiency could modulate the course of autoimmune diabetes.

BDC2.5 mice are a well-characterized model for autoimmune diabetes<sup>137</sup>. Disease features in BDC2.5 mice closely mimic those observed in humans and NOD mice. TCR transgenic, CD4<sup>+</sup> T cells bearing a rearranged islet-antigen specific TCR (V $\beta$ 4<sup>+</sup>) are both required and sufficient to cause highly penetrant and synchronized disease in BDC2.5

mice. However, development of frank hyperglycemia in BDC2.5 mice occurs within weeks—as opposed to the delay of many months observed in the NOD strain—allowing relatively rapid evaluation of genetic influences on the autoimmune process<sup>139</sup>. BDC2.5 heterozygous for MHC class II molecule H-2<sup>g7/b</sup> are protected from the disease because of the influence of non-diabetogenic clones selected by H-2b molecules<sup>138</sup>.

To evaluate the effect of ADAP on autoimmunity, we introduced ADAP null allele into BDC2.5 (on the C57BL/6 background and congenic for MHC H-2<sup>g7</sup>) TCR transgenic mice<sup>137</sup>. We observed that ADAP deficiency results in a dramatically higher incidence of diabetes in both diabetes prone (BDC2.5 g7/g7) and resistant (BDC2.5 g7/b) mice. Further, we found that the enhanced disease correlates with both decreased numbers of diabetogenic T cells in the periphery and with augmented T cell homeostatic proliferation in ADAP-deficient BDC2.5 mice. Upon intravenous transfer of syngenic leukocytes, ADAP-deficient mice are protected from disease, strongly suggesting a causal influence of lymphopenia on the enhanced rate of diabetes. Pre-diabetic ADAP-deficient BDC2.5 mice display markedly reduced numbers of mature CD4<sup>+</sup> single-positive (SP) thymocytes. Our data reveal that loss of ADAP results in a defect in peripheral T cell homeostasis that is causally linked to enhanced autoimmune diabetes in the BDC2.5 model. Further, our findings strongly suggest that ADAP promotes efficient thymocyte positive selection and thymic output, processes important for support of peripheral T cell numbers in BDC2.5 mice. This study emphasizes that adaptor proteins are essential in thymic output-dependent lymphopenia in autoimmunity.

## **Materials and methods**

### **Mice and diabetes diagnosis**

BDC2.5 TCR Tg (C57BL/6) mice were generous gifts from D. Mathis and C. Benoist, of Harvard University. ADAP-deficient mice ( $\times 7$  cross to C57BL/6)<sup>46</sup> were interbred with BDC2.5 mice and housed in conventional or SPF facilities. The MHC haplotype was determined by blood cell expression of I-A<sup>b</sup> and I-A<sup>g7</sup>. NOD/SCID mice were purchased from the Jackson laboratory. Detection of glycosuria with Diastix strips (Bayer Corporation, Elkhart, Indiana) during weekly screening was confirmed by blood glucose measurement (glucometer from Ascensia Elite XL, Bayer). Diabetes was defined by the occurrence of two consecutive weekly blood glucose measurements greater than 200 mg/100 mL. Animal work was done in compliance with regulations of the IACUC at the University of Minnesota.

### **Histology**

Pancreata isolated from 5.5-week-old, prediabetic mice were fixed in 10% formaldehyde, paraffin-embedded, and sectioned for hematoxylin and eosin staining per established protocol<sup>117</sup>. At least 100  $\beta$ -islets from each pancreas were categorized as “aggressive,” “non-aggressive,” “peri-insulinitis,” or “no infiltration” in a blinded fashion by a single trained observer (N.O.-M.) using light microscopy<sup>140</sup>.

### **Antibodies, flow cytometry**

CD44-FITC (IM7), CD45.2-FITC (104), Foxp3-PE (FJK-16s), V $\alpha$ 2-FITC (B20.1) CD4-APC (GK1.5), CD45.1-APC (A20), TCR $\beta$ -fict, TNF $\alpha$ -Fitc, IL4-PE, IFN $\gamma$ -APC and CD25-biotin (PC61) were purchased from

eBioscience (San Diego, CA). CD8b.2-FITC (Ly-3.2), I-A<sup>k</sup>-FITC (A $\beta$ k 10-3.6), V $\beta$ 4-PE (KT4), and I-A<sup>b</sup>-PE (AF60120.1) were purchased from BD PharMingen (San Diego, CA). CD1d-tetramer (PBS57) was purchased from NIH. Cells were stained in FACS buffer [2% FBS in PBS with FcBlock (2.4G2)] and subjected to flow cytometric analysis using a Becton-Dickinson FACSCalibur.

### **T cell purification**

Approximately  $100 \times 10^6$  unfractionated splenocytes and LN cells were incubated with FITC-conjugated mAb to B220, CD11b, and NK1.1 (1.5  $\mu$ g/ $10 \times 10^6$  cells) for 20 minutes on ice. After being washed twice, cells were resuspended in 500  $\mu$ L FACS buffer containing BioMag anti-FITC conjugated magnetic beads (Polyscience Inc., Warrington, PA, 1.5 ml/ $100 \times 10^6$  cells) and rocked at room temperature for 30 min. Magnetic depletion then yielded >90% pure CD3<sup>+</sup> T cells.

### **Intracellular staining**

For cytokine intracellular staining, cells were first stimulated with different stimuli as indicated in media containing brefeldin A (10 $\mu$ g/ml) at 37° C. At different time points, cells were fixed with 2% Formaldehyde for 20 minutes. After 2x washes, cells were blocked with 2.4G2 for 20 minutes and permeabilized with saponin (0.25% saponin, 12.5% FBS) for 30 minutes at room temperature. Then cells were stained with individual antibodies (dilution: 1.5 $\mu$ g/ml) followed by washes.

### **T cell proliferation and survival assays**

To measure endogenous DNA synthesis, mice were fed BrdU (Sigma, St. Louis, MO) dissolved in drinking water (0.8 mg/mL). Intracellular staining for BrdU in harvested lymphocytes was performed

according to kit manufacturer protocol (BD PharMingen). To measure proliferation of adoptively transferred cells, splenocytes were labeled with CFSE (5  $\mu$ M in PBS) for 10 min at 37°C; after being washed,  $3 \times 10^6$  CFSE-labeled cells were transferred via tail vein. Proliferation was determined by FACS-detected CFSE dilution amongst transferred cells. To examine T cell survival, pooled, RBC-depleted “reporter” splenocytes and LN cells from wild-type CD45.1<sup>+</sup> BDC2.5 g7b mice were labeled with CFSE, and  $10 \times 10^6$  labeled cells were injected into CD45.2<sup>+</sup>, non-Tg recipients. After four days, LN cells were recovered for FACS analysis.

### **Diabetes and lymphopenia suppression by leukocyte transfer**

Pooled, ADAP<sup>-/-</sup> BDC2.5 g7b splenocytes and LN T cells were transferred into 4-5-week-old ADAP<sup>-/-</sup> BDC2.5 g7b recipients and mice were monitored up to age 11 weeks. To detect suppression of lymphopenia, BrdU was fed to recipients for four days starting seven days after injection of  $240 \times 10^6$  cells into ADAP<sup>-/-</sup> BDC2.5 g7b mice; BrdU-positive splenocytes and LN cells were detected by flow cytometric analysis.

### **Treg purification and functional assessment**

BDC2.5 g7b splenocytes were stained with biotin-anti-CD25 mAb (PC61.5 clone, ebioscience; 1ul anti-CD25/mouse in 100 ul resuspension) on ice for 20 minutes. After 2x washes, cells were resuspended with streptavidin-conjugated magnetic beads (315ul cell suspension and 35 ul beads per mouse) on ice for 15 minutes. With one wash, cells were passed through LS midiMACS columns (Miltenyi Biotec, Auburn, CA) for positive selection. CD25<sup>+</sup> fraction were collected as Tregs while CD25<sup>-</sup> were collected as effectors. Diabetogenic effectors (column-non-adherent

CD25<sup>-</sup> cells) were mixed with CD25<sup>+</sup> cells (Treg) at indicated ratios and were injected by tail vein into NOD/SCID recipients. Recipients were followed for diabetes 7-28 days after injection.

## Results

### **ADAP-deficient BDC2.5 mice display increased incidence of autoimmune diabetes.**

To evaluate the function of ADAP in T cell-dependent autoimmune disease, we crossed ADAP-deficient mice with diabetes-prone BDC2.5 (C57BL/6 background) TCR transgenic mice<sup>137</sup>. Animals homozygous for I-A<sup>g7</sup> (g7g7), the MHC class II selecting element for the diabetogenic TCR, were monitored for diabetes from age 4 to 20 weeks. As previously reported<sup>149</sup>, we observed that wild-type BDC2.5 g7g7 mice develop diabetes between 4 and 10 weeks of age with an incidence of 60% in conventional environment (Fig. 2.1A). Greater numbers of ADAP-deficient BDC2.5 g7g7 mice (94%) become diabetic over the same age range in a conventional facility (Fig. 2.1A). We observed an even greater difference between diabetes incidence rates for control (26%) and ADAP-deficient mice (78%) when we examined BDC2.5 mice heterozygous for I-A<sup>b</sup> and I-A<sup>g7</sup> (g7b) in a conventional facility (Fig. 2.1A). Expression of I-A<sup>b</sup> exerts a protective effect against disease in wild-type BDC2.5 mice<sup>150</sup>, but this effect was ablated by ADAP deficiency (Fig. 2.1A). Taken together, the diabetes incidence data indicate that ADAP-deficient BDC2.5 mice exhibit enhanced incidence of autoimmune diabetes in a conventional facility.

Previous studies showed that environmental factors influence the development of autoimmune diseases, including autoimmune diabetes<sup>151,152</sup>, so we extended our observation of diabetes into the SPF condition. Again for the g7/g7 mice, ADAP-deficient BDC2.5 mice showed a higher incidence of diabetes than wild-type controls as in conventional condition (Fig. 2.1A). Overall, for ADAP-deficient and sufficient mice, the magnitude of diabetes is lower in SPF than in conventional conditions, suggesting environment-independent regulation of autoimmune diabetes by ADAP. Strikingly, there is no difference of diabetes incidence observed between ADAP-deficient and sufficient BDC2.5 g7b mice when housed in an SPF condition in contrast to conventional BDC2.5 g7b mice (Fig. 2.1A). Collectively, these data suggest that ADAP regulates autoimmune diabetes in BDC2.5 mice in environmental dependent and independent manners.

To rule out the possibility that a potential genetic difference contributes to diabetes difference observed between SPF and conventional mice, we transferred some breeding cages from our SPF colony into a conventional facility. Offspring from the transferred cages were monitored for diabetes development. Just like the original conventional mice, diabetes incidence (80%) of ADAP-deficient BDC2.5 g7b mice from transferred breeding cages is much higher than that (20%) of ADAP sufficient controls (Fig. 2.2). These data suggests that the difference of diabetes between SPF and conventional mice is not due to a genetic difference.

To search for a mechanism of enhanced diabetes resulting from ADAP deficiency, we elected to focus further investigation upon the

BDC2.5 g7b mice housed in a conventional condition, given the wide disease incidence gap between ADAP-deficient and control animals of this strain. Prior to development of overt hyperglycemia, BDC2.5 mice display progressive pancreatic islet lymphocyte infiltration<sup>139</sup>. To determine whether ADAP deficiency regulates islet immune cell infiltration, we evaluated hematoxylin and eosin-stained pancreata<sup>117,140</sup> from age-matched, 5.5-week-old control and ADAP-deficient, prediabetic BDC2.5 g7b mice (Fig. 2.1B). We found no significant difference in the fraction of ADAP-deficient islets exhibiting either non-aggressive (“level 1”;  $p=0.15$ ) or aggressive (“level 2”;  $p=0.27$ ) insulinitis. These findings suggest that the difference in diabetes incidence between control and ADAP-deficient BDC2.5 g7b mice could not be explained by altered kinetics of islet infiltration by inflammatory cells.

### **ADAP-deficient BDC2.5 g7b mice show reduced diabetogenic T cell numbers.**

Disordered T lymphocyte homeostasis resulting in lymphopenia has been causally linked to autoimmunity<sup>145</sup>. ADAP-deficient lymph node and spleen display modestly decreased T cell numbers in non-Tg mice<sup>46,148</sup>. To test whether ADAP deficiency results in altered T cell homeostasis in BDC2.5 g7b mice, we enumerated peripheral T cells expressing TCRV $\beta$ 4 and CD4, markers expressed by diabetogenic, Tg cells<sup>138</sup>. In both draining (pancreatic) and non-draining (inguinal/axillary) LN, as well as in spleen, we observed a 50-66% reduction in the percentages (Fig. 2.3A) and numbers (Fig. 2.3B) of V $\beta$ 4<sup>+</sup>CD4<sup>+</sup> T cells. Total cellularity of spleen and LN was not significantly affected by ADAP deficiency. However, total

T cell (combined CD4<sup>+</sup> and CD8<sup>+</sup> cells; wild-type  $12 \times 10^6 \pm 2 \times 10^6$  versus ADAP-deficient  $5.8 \times 10^6 \pm 1.2 \times 10^6$ ;  $p < 0.01$ ) and total CD4<sup>+</sup> cell numbers (wild-type  $11 \times 10^6 \pm 1.6 \times 10^6$  versus ADAP-deficient  $4.4 \times 10^6 \pm 0.9 \times 10^6$ ;  $p < 0.01$ ) in the LN are also significantly decreased in the ADAP-deficient animals. We concluded from these data that enhanced diabetes incidence is associated with reduction in the numbers of CD4<sup>+</sup> diabetogenic T cells in ADAP-deficient, BDC2.5 g7b peripheral lymphoid tissues.

### **Lymphopenia associated with loss of ADAP promotes homeostatic proliferation.**

Current models of lymphoid homeostasis hold that T cells in a lymphopenic environment display increased propensity to proliferate due to decreased interclonal competition for finite antigen/MHC and cytokine resources<sup>153</sup>. Given the CD4<sup>+</sup> T cell lymphopenia in ADAP-deficient BDC2.5 g7b mice, we hypothesized that ADAP-deficient animals would support enhanced homeostatic T cell expansion. To test this idea, we injected CFSE-labeled, congenic (CD45.1<sup>+</sup>), non-Tg “reporter” lymphocytes into wild-type or ADAP-null (CD45.2<sup>+</sup>) BDC2.5 g7b mice. Transferred lymphocytes, distinguished by congenic marker expression, were recovered from inguinal LN and spleen 4 days after injection. We observed that significantly higher percentages of reporter CD4<sup>+</sup> and CD8<sup>+</sup> T cells recovered from ADAP-deficient recipients show evidence of CFSE dilution (indicating cell division) compared with cells from control BDC2.5 recipients (Fig. 2.4A and B). These data indicate that diminished T cell numbers correlate with the presence of an ADAP-deficient BDC2.5 g7b

tissue environment that supports enhanced T cell proliferation. To examine basal proliferation of endogenous, autoreactive T cells, we fed BrdU to pre-diabetic BDC2.5 g7b mice. FACS analysis revealed BrdU uptake in a greater percentage of splenic and LN CD4<sup>+</sup>Vβ4<sup>+</sup> cells from ADAP-deficient animals than in cells from wild-type mice (Fig. 2.5A and B).

### **ADAP-deficient T cells show enhanced memory phenotype.**

Previous studies suggested that homeostatic proliferation renders T cell memory phenotype, including CD44<sup>high</sup> expression, but not the intermediate activation makers such as CD69 and CD25<sup>29</sup>. When we examined the expression of CD44 and CD69 on CD4<sup>+</sup>Vβ4<sup>+</sup> LN T cells, we observed a significantly higher proportion of cells expressing CD44<sup>high</sup> from non-draining lymph nodes and draining lymph nodes (Fig. 2.6A and B). In contrast, CD69 expression only showed difference on cells from draining but not from non-draining lymph nodes (Fig. 2.6A and B). Together, these data strongly suggest that ADAP-deficient BDC2.5 g7b mice support enhanced homeostatic T cell proliferation.

### **Syngeneic lymphocytes rescue ADAP-deficient BDC2.5 g7b mice from diabetes and block endogenous T cell proliferation.**

T cell lymphopenia can be relieved, and diabetes prevented, by transfer of syngeneic splenocytes and T cells into NOD mice<sup>29</sup>. We used a similar lymphocyte replacement approach to investigate whether lymphopenia plays a causal role in ADAP-dependent diabetes. We transferred ADAP-deficient BDC2.5 g7b leukocytes into 4-5-week-old,

pre-diabetic syngeneic recipients. We studied the effect of a “replacement” cell dose ( $240 \times 10^6$  splenocytes) calculated to restore total numbers of  $CD4^+V\beta4^+$  T cells in prediabetic, ADAP-deficient mice to levels observed in age-matched controls (not shown). A dosage of  $80 \times 10^6$  cells was chosen for an alternative “low dose” cell infusion.

We observed that transfer of a low dose of syngeneic leukocytes into ADAP-deficient BDC2.5 g7b mice results in delayed onset and partial prevention of disease (Fig. 2.7A). Strikingly, replacement dose leukocyte transfer reduces diabetes incidence in ADAP-deficient mice to the rate observed in wild-type animals. Transfer of purified splenic T cells results in a similar dramatic reduction in diabetes incidence (Fig. 2.7A), strongly suggesting that the protective effects of cell transfer are determined by the T cell compartment. Taken together, these data suggest that reconstitution of T cell lymphopenic BDC2.5 g7b mice with either unfractionated leukocytes or with purified T cells is sufficient to protect animals from ADAP-dependent enhanced diabetes.

To investigate the mechanism of protection from disease conferred by leukocyte transfers, we examined basal T cell proliferation in transfer recipients. BrdU was fed to transfer recipients at seven days after replacement dose transfer. Spleen and lymph node cells were then analyzed for BrdU uptake. We observed that after the transfer of syngeneic cells, the percentage of  $BrdU^+$  cells in ADAP-deficient recipients is reduced to levels observed in unmanipulated BDC2.5 g7b wild-type mice (Fig. 2.7B). We concluded that disease-preventing transfer of syngeneic leukocytes also reduces  $CD4^+$  T cell homeostatic expansion in ADAP-deficient BDC2.5 g7b mice.

**ADAP is required for efficient positive selection of diabetogenic T cells, but is dispensable for their survival.**

The T cell lymphopenia observed in ADAP-deficient BDC2.5 g7b mice might be caused by decreased thymic output or by reduced T cell survival in the periphery. To address the first possibility, we counted thymocytes. While overall thymic cellularity of young, pre-diabetic BDC2.5 g7b mice is unaffected by ADAP deficiency, we found a marked reduction in both the percentage and absolute numbers of ADAP-deficient CD4<sup>+</sup> SP thymocytes (Fig. 2.8A and B). Similar impaired production of CD4 SP thymocytes has also been noted in other ADAP-deficient TCR Tg mice<sup>47</sup>. The deficit in ADAP-deficient CD4<sup>+</sup> SP correlates with increased numbers of CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) thymocytes (not shown). These data suggest a developmental block in ADAP-deficient BDC2.5 g7b mice at the DP to SP transition, a stage that correlates with positive selection of CD4<sup>+</sup> thymocytes prior to migration into peripheral tissues.

We next addressed the possibility that altered survival capacity of ADAP-deficient BDC2.5 g7b T cells contributes to lymphopenia. Mixtures of congenically marked control or ADAP-deficient CD4<sup>+</sup>Vβ4<sup>+</sup> T cells (harvested from pre-diabetic BDC2.5 g7b donors) were transferred into non-Tg hosts (see “Input” percentages, left hand panel, Fig. 2.9A). We then determined the contributions of either control or ADAP-deficient T cells to the donor T cell population in peripheral lymphoid organs after nine days. We found no difference between control and ADAP-deficient T cells in their abilities to survive (Fig. 2.9 A, B) or to proliferate (Fig. 2.9 C, D) in either non-draining or draining lymph nodes. Together, these

data strongly suggest that a thymic production deficit, and not reduced T cell survival, is the underlying cause of T cell lymphopenia in ADAP-deficient BDC2.5 g7b mice.

### **Loss of ADAP alters transgene BDC2.5 TCR expression on T cells.**

Central tolerance has been implicated as a central mechanism of self-tolerance. During thymic selection, T cells bearing TCR showing a strong affinity to self-antigen go to negative selection<sup>62</sup>. Since ADAP has been demonstrated as a regulator in thymic selection<sup>49</sup> and regulates thymic selection in BDC2.5 mice, we sought to determine if the expression of transgene BDC2.5 TCR is altered in ADAP-deficient mice. When we stained thymocytes with anti-CD4 and CD8 antibodies and BDC2.5 clonotype, we observed increased intermediate BDC2.5 staining on ADAP-deficient thymocytes (Fig. 2.10 A). When we stained cells from spleens and lymph nodes, we observed the similar pattern of BDC2.5 staining on peripheral T cells as on thymocytes (Fig. 2.10A). Further, the mean fluorescence intensity shows a significantly lower value on ADAP-deficient lymphocytes, indicating that loss of ADAP downregulates transgene BDC2.5 TCR expression (Fig. 2.10B).

### **Loss of ADAP results in a higher proportion of V $\alpha$ 2 expressing T cells.**

It is suggested that the MHC I-A b allele suppresses autoreactive T cells through the selection of regulatory cell population expressing V $\alpha$ 2 segment<sup>154</sup>. To explore whether ADAP supports the selection of V $\alpha$ 2 segment, we stained cells from thymus, spleen, and lymph nodes with

anti CD4, CD8, and V $\alpha$ 2 antibodies and detected a higher proportion of V $\alpha$ 2 expression within CD4<sup>+</sup>CD8<sup>-</sup> T cells from thymus and peripheral lymph organs (Fig. 2.11). Together these data implicate that enhanced diabetes in ADAP-deficient BDC2.5 mice is probably not due to the preferential selection of V $\alpha$ 2 segment usage.

### **Loss of ADAP does not alter differentiation of T cells.**

Since ADAP regulates cytokine like IL2 secretion by T cells <sup>46</sup>, we asked if ADAP regulates T cell differentiation. For this purpose, we stimulated spleen cells from ADAP sufficient and deficient BDC2.5 g7/b mice with PMA and ionomycin for 6 hours and measured the IL4 and IFN $\gamma$  secretion by intracellular staining. IFN $\gamma$  and IL4 are two signature cytokines for T cell helper 1 and helper 2 lineages respectively. With gating on CD4<sup>+</sup>CD44<sup>hi</sup> (CD44<sup>hi</sup> is a marker for memory T cells), we detected comparable IL4 and IFN $\gamma$  secretion by ADAP-deficient BDC2.5 T cells and controls in terms of intensity and frequency (Fig. 2.12A and B). To extend this observation, we also examined IFN $\gamma$  and IL4 secretion by T cells from nontransgenic Blab/C mice and detected no difference of IFN $\gamma$  and IL4 staining between ADAP-deficient T cells and controls (Fig. 2.12C and D). As a conclusion, ADAP does not regulate T cell differentiation in terms of cytokine secretion.

### **ADAP-deficient T cells show moderate reduced capacity in diabetes induction on a per cell basis.**

Since ADAP is required for the thymic selection and altered thymic selection may preferentially support autoreactive T cell development <sup>49</sup>,

we tried to ask if ADAP-deficient diabetogenic T cells are more potent to induce diabetes with a transfer system. When 0.04 million total spleen cells were transferred into immune deficient NODscid recipients, there was no diabetes detected among recipients of either ADAP wild-type or deficient cells (Fig. 2.13A). When 0.2 million cells were transferred, 20% of mice receiving ADAP-deficient cells showed diabetes in two weeks, while 40% of mice receiving ADAP-sufficient cells manifested diabetes syndrome (Fig. 2.13A). To rule out the contribution to diabetes development by non-T cells, like B cells and NK cells contained in total splenocytes and to compensate lower number of autoreactive T cells in ADAP deficient spleen cells, we purified T cells with negative depletion of B220<sup>+</sup>, cell, NK1.1<sup>+</sup> and transferred into NODscid. Again, purified ADAP-deficient T cells (50%, diabetes incidence) showed reduced capacity for diabetes induction compared with control cells (90%, diabetes incidence) (Fig. 2.13B). Together these data suggest that the enhanced diabetes in ADAP-deficient BDC2.5 mice is not due to hyperactive autoreactive T cells.

**ADAP is dispensable for development and in vivo regulatory function of Tregs.**

Thymus-derived regulatory T cells (CD4<sup>+</sup>foxP3<sup>+</sup>; Treg) play an essential disease-suppressive role in the BDC2.5 model<sup>87</sup>. As ADAP is required for efficient selection of CD4<sup>+</sup> T cells<sup>47</sup>, we asked whether loss of ADAP regulates Treg number or function in BDC2.5 g7b mice. We observed increased (approximately twofold) fractions of the CD4<sup>+</sup> T cell subset expressing CD25 (Fig. 2.14 A, B) and Foxp3 (Fig. 2.14 A, B) and

in spleen and LN from pre-diabetic, 5.5-week-old, ADAP-deficient BDC2.5 g7b mice. However, absolute numbers of Treg are not significantly altered by ADAP deficiency since T cell number is reduced in ADAP-deficient BDC2.5 g7b mice. These observations strongly suggest that the enhanced diabetes incidence in ADAP-deficient mice cannot be explained by altered development of Tregs.

To ask if ADAP is required for Tregs function, we first performed a T cell proliferation suppression assay. When CFSE labeled effectors (CD25<sup>-</sup> splenocytes) were co-cultured with Tregs (CD25<sup>+</sup>) at different ratio in medium containing mimotope, we detected comparable suppression by ADAP-sufficient and deficient Tregs, suggesting that ADAP-deficient T cells function normally in vitro (Fig. 2.15A). Further, to evaluate cell-intrinsic Treg function in vivo, we co-transferred purified CD25<sup>+</sup> BDC2.5 g7b splenocytes (Tregs) in combination with CD25<sup>lo</sup> BDC2.5 g7b (effector) T cells into NOD/SCID mice and monitored the recipients for diabetes. We observed that co-transfer of Tregs derived from control mice results in cell dose-dependent protection of recipients from effector-induced diabetes for up to 30 days (Fig. 2.15B). ADAP-null Tregs show a non-significant trend toward enhancement of disease-suppressive capacity when co-transferred in low numbers. These data strongly suggest that the increased diabetes in ADAP-deficient mice is not caused by defects in BDC2.5 g7b Treg development or function.

**ADAP-deficient bone marrow derived dendritic cells show normal cytokine TNF $\alpha$  secretion after TLR stimulation.**

Antigen-presenting cells are the connection between environmental stimuli and T cells, and Toll-like receptors have been implicated in autoimmunity<sup>108,109 103,104,106,107</sup>. Environmental stimuli enhanced autoimmune diabetes in ADAP BDC2.5 g7/b mice and ADAP is also expressed in dendritic cells and macrophages, two major antigen-presenting populations<sup>38,39</sup>. It is possible that the loss of ADAP alters the function of antigen-presenting cells, which accounts for the increased autoimmune diabetes in ADAP-deficient mice. To test this possibility, first, we investigated cytokine TNF $\alpha$  secretion by bone marrow derived dendritic cells after TLR stimuli. We detected normal TNF $\alpha$  secretion by intracellular staining in ADAP-deficient dendritic cells stimulated with either Poly I:C (TLR3 ligand) or LPS (TLR4 ligand) (Fig. 2.16-17), indicating that ADAP is not required for TLR signaling in dendritic cells.

It was reported recently that Card9/Bcl-10/Malt1 is required for innate immunity against fungi through dectin-1, a non-TLR receptor for fungi-derived zymosan<sup>155</sup>, and that ADAP is required for assembly and membrane translocation of CARMA-1/ BCL-10/MALT-1 complex, which is required for NF $\kappa$ B nuclear translocation in T cells<sup>45</sup>. These findings led us to ask if ADAP is required for dectin-1 signaling in bone marrow derived dendritic cells. When we stimulated dendritic cells with zymosan at different dosages and examined dectin-1 signaling with TNF $\alpha$  intracellular staining as a reading out, we failed to detect any defective TNF $\alpha$  secretion in ADAP-deficient dendritic cells, suggesting a dispensable role of ADAP in dectin-1 signaling (Fig. 2.18).

**ADAP-deficient APCs prime T cells sufficiently in vitro and in vivo.**

To examine the function of ADAP in APCs more carefully, we investigated ADAP-deficient APCs in T cell priming. To evaluate ADAP-deficient APCs in T cell priming in vitro, CFSE-labeled purified BDC2.5 T cells were co-cultured with nontransgenic spleen cells as APCs pulsed with mimotope of different dosages. After four days, flow cytometry revealed comparable T cell proliferation primed by either ADAP-deficient or sufficient spleen cells at different concentrations of mimotope, suggesting that loss of ADAP does not alter APC function in T cell priming in vitro (Fig. 2.19).

To ask if ADAP is required for APCs in T cell priming in vivo, we performed adoptive transfer experiments.  $10 \times 10^6$  CD45.1<sup>+</sup> spleen cells were labeled with CFSE and transferred into ADAP-deficient and wild-type CD45.1<sup>-</sup> nontransgenic mice. On day 3, transferred cells were recovered from draining and nondraining lymph nodes and cell proliferation was evaluated with CFSE dilution. Transferred CD4<sup>+</sup>V $\beta$ 4<sup>+</sup> cells recovered from both ADAP-deficient and wild-type nondraining lymph nodes showed no CFSE dilution, while cells from draining lymph nodes showed significant CFSE<sup>low</sup> population, suggesting that BDC2.5 T cells were primed by endogenous antigen presented by APCs (Fig. 2.20A and B). However, there is no difference detected of CFSE dilution on cells recovered from ADAP-deficient and wild-type recipients (Fig. 2.20A and B), indicating dispensable ADAP for APCs to prime T cells in vivo.

**ADAP-deficient hosts show comparable susceptibility to diabetes induction.**

Antigen-presenting cells, including dendritic cells and macrophages, are essential in regulation of autoimmune diabetes<sup>127,132</sup>. To evaluate ADAP-deficient APCs in diabetes development, we crossed an ADAP-deficient allele into a Rag-deficient genetic background and transferred  $1 \times 10^6$  spleen cells into these Rag-deficient recipients, which were monitored for diabetes development. All of the animals, including both ADAP-deficient recipients and wild-type controls, showed diabetes rapidly (Fig. 2.21A). To exclude the effect from exogenous APCs, we purified and transferred  $0.3 \times 10^6$  T cells into Rag-deficient mice. Both ADAP-sufficient and deficient recipients developed diabetes rapidly and there was no difference of diabetes detected in terms of incidence and kinetics, regardless of ADAP expression (Fig. 2.21B). As a conclusion, ADAP-deficient APCs show comparable ability to promote diabetes induction and suggest that the enhanced diabetes in ADAP-deficient BDC2.5 mice is not due to hyperactive APCs.

### **Transgene BDC2.5 TCR excludes NKT cell development.**

As an essential regulatory cell population, NKT cells have been implicated in the suppression of autoimmune diabetes<sup>116</sup>, and the ADAP-binding protein Fyn is required for NKT cell development<sup>156</sup>. So we sought to ask if loss of ADAP impairs NKT cell development. When cells from thymus, spleen, lymph nodes, and liver were stained with anti-CD24 (NKT cells are enriched in CD24<sup>-</sup> leukocytes), TCR $\beta$  antibodies, and CD1d tetramer (PBS-57 loaded), we did not detect significant NKT cell population (CD1d Tetramer<sup>+</sup>TCR $\beta$ <sup>+</sup>) in either ADAP-deficient or wild-type BDC2.5 mice compared with nontransgenic controls. Lack of NKT cells

may due to allele exclusion during NKT cell development, since NKT cells require a restricted TCR segment<sup>114</sup>. Hence, NKT cells are negligible in BDC2.5 background although this population has been implicated in autoimmune diabetes previously<sup>116</sup>.

## **Discussion**

Results presented here corroborate recent findings that lymphopenia is a causative influence in T cell-dependent diabetes<sup>29</sup>. In ADAP-deficient BDC2.5 g7b mice, reduced numbers of diabetogenic T cells and increased homeostatic proliferation of both transferred and endogenous T cells suggest the presence of functional lymphopenia. That such lymphopenia is causally linked to disease is strongly suggested by the finding that simple enhancement of peripheral T cell numbers normalizes both increased diabetes incidence and T cell turnover in ADAP-deficient BDC2.5 g7b mice. Our data implicate a marked defect in thymic output of CD4<sup>+</sup> T cells as the source of lymphopenia in ADAP-deficient mice.

T lymphocyte homeostatic proliferation is critical to the restoration of host immune competence<sup>153</sup>. However, in the genetically predisposed host, the lymphopenia-driven lymphocyte expansion may translate into destructive autoimmune responses<sup>145</sup>. For example, recent work showed that transferred, islet antigen-specific T cells are normally tolerated in NOD recipients. However, the same cells proliferate and cause severe disease when transferred into NOD/SCID lymphopenic recipients<sup>24</sup>. Furthermore, transfer of diabetogenic leukocytes into modestly

lymphopenic NOD recipients protects mice from disease, suggesting that relief of lymphopenia can restrain the diabetogenic potential of T cells <sup>29</sup>.

A key novel finding arising from our studies is that defects in thymic autoreactive cell productive capacity can result in autoimmune diabetes-inducing lymphopenia. Others have shown that total ablation of thymic output through thymectomy accelerates murine diabetes <sup>28,46</sup>. However, for these studies, numerous thymus-dependent populations known to regulate the course of diabetes were removed by experimental manipulation. For example, numbers of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, recently shown capable of suppressing diabetes in the BDC2.5 model <sup>87</sup>, are reduced in thymectomized animals <sup>116</sup>. Others have shown disease regulatory properties for thymus-derived NKT cells and CD8 $\alpha\alpha$  intraepithelial lymphocytes in autoimmune diabetes <sup>28,157</sup>. However, we found no differences between control and ADAP-deficient BDC2.5 g7b lymphoid tissues in Treg, in numbers of NKT cells (as detected by CD1d tetramers) or in CD8 $\alpha\alpha$  expressing intraepithelial lymphocytes (data not shown). Finally, enhanced usage of alternative (non-Tg) TCR $\alpha$  chains by Tg V $\beta$ 4<sup>+</sup>-expressing cells has been associated with reduced diabetes in BDC2.5 g7b mice <sup>138</sup>. Surprisingly, we observed a modest increase in the percentage of peripheral CD4<sup>+</sup> T cells expressing alternative TCR $\alpha$  in ADAP-deficient BDC2.5 g7b mice (not shown). There is no literature precedent to support the notion that such alternative TCR $\alpha$ -bearing cells could account for enhanced diabetes, however. Thus, the T cell lymphopenia driving enhanced diabetes in ADAP-deficient BDC2.5 g7b mice is likely not attributable to loss of important regulatory populations.

Severity of the T cell lymphopenia in ADAP-deficient BDC2.5 g7b mice is dependent upon expression of the BDC2.5 TCR transgene. Indeed, thymi from ADAP-deficient H-Y, DO11.10, and AND TCR Tg mice all display reduced numbers of SP thymocytes, similar to observations in the BDC2.5 mice <sup>47</sup>. However, nontransgenic, ADAP-deficient mice show only mild deficits in peripheral CD3<sup>+</sup> and CD4<sup>+</sup> lymphocyte numbers <sup>46,148</sup>, and do not support increased spontaneous proliferation of transferred reporter T cells (not shown). The possibility that ADAP regulates development of T cell-dependent, but TCR transgene-independent, autoimmune responses such as diabetes (in the NOD model) or systemic lupus (exemplified by MRL/*lpr* mice) remains under study.

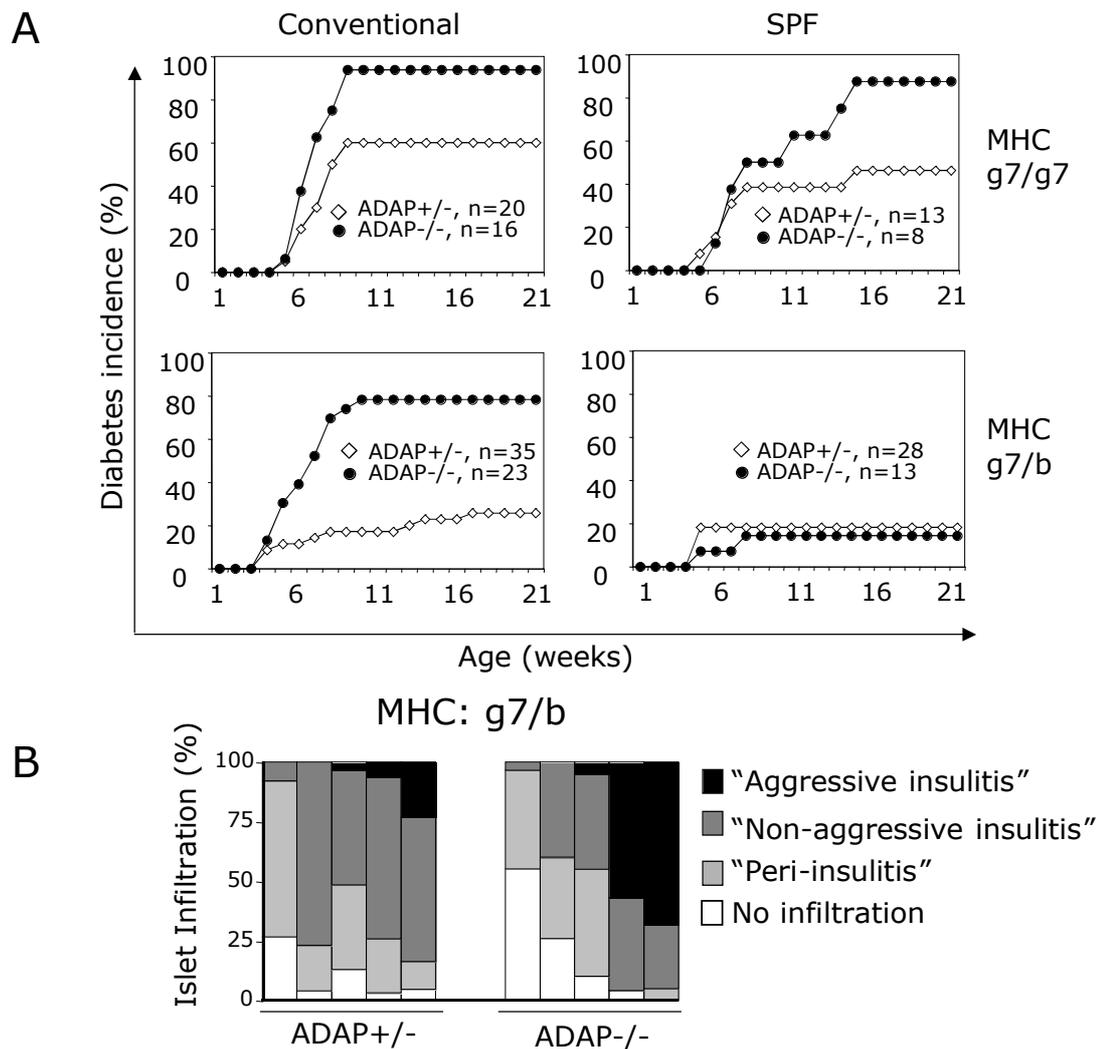
The molecular mechanism whereby ADAP regulates positive selection of CD4 SP thymocytes remains incompletely understood. ADAP is a known modulator of TCR signaling, which is critical for efficient thymocyte selection <sup>158</sup>. ADAP is dispensable for TCR-mediated ERK activation and Ca<sup>2+</sup> flux in thymocytes <sup>47</sup>. However, through association with the adapter SKAP55, ADAP regulates Rap1-dependent TCR-stimulated integrin activation and thymocyte conjugate formation <sup>45,159</sup>. Recent work has also established a requirement for ADAP in TCR-dependent formation of a CARMA-1-containing complex and subsequent activation of the NF- $\kappa$ B pathway. Experiments to determine whether immature thymocytes also require ADAP to activate NF- $\kappa$ B upon receipt of a selecting TCR stimulus are underway.

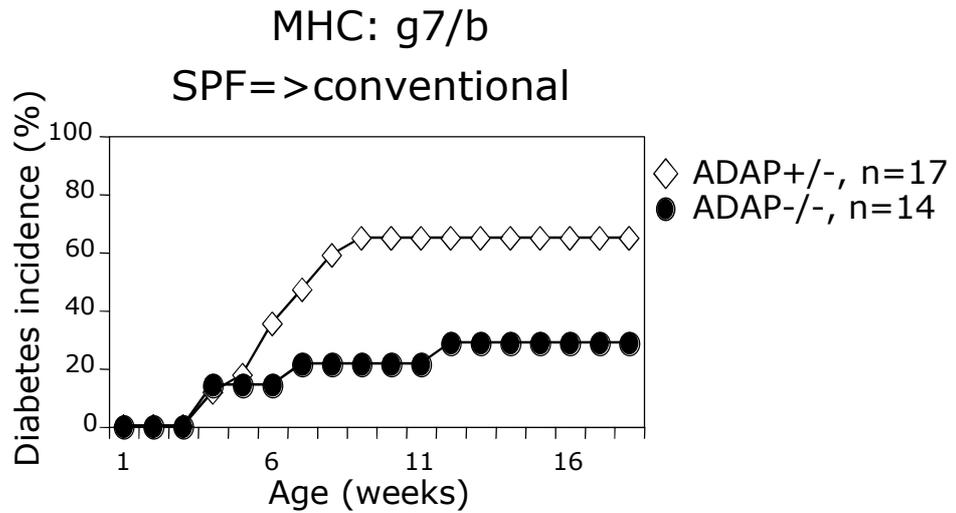
Although Treg defects do not account for enhanced diabetes in ADAP-deficient BDC2.5 mice (Fig. 2.14 and 2.15), they may be a contributing factor in the observed amelioration of ADAP-dependent

diabetes by syngeneic leukocyte transfer (Fig.2.7). Recent studies have suggested that Tregs can control both CD4<sup>+</sup> homeostatic proliferation and disease expression in transfer models of autoimmunity<sup>160,161</sup>. Further, Herman et al.<sup>87</sup> showed that co-transfer of CD25<sup>+</sup> Tregs could block BDC2.5 T cell-transfer induced diabetes in NOD-Scid recipients in a dose-dependent manner. However, unlike Herman et al., we have transferred Tregs (in the context of leukocytes or T cells) into ADAP-deficient mice harboring an already-intact, homeostatically proliferating, autoreactive repertoire and displaying pancreatic infiltration. Because we transferred ADAP-deficient BDC2.5 g7b leukocytes cells, the ratio of Tregs to effector T cells in the recipients was not changed. Thus, if Tregs are playing a role in disease suppression by transferred ADAP-deficient cells, an enhanced Treg absolute number in recipient mice is likely the major determinant of their effect. Quantification of the role of Tregs in the suppression of disease and/or suppression of homeostatic expansion in our model must await completion of experiments achieving Treg depletion by physical or genetic means.

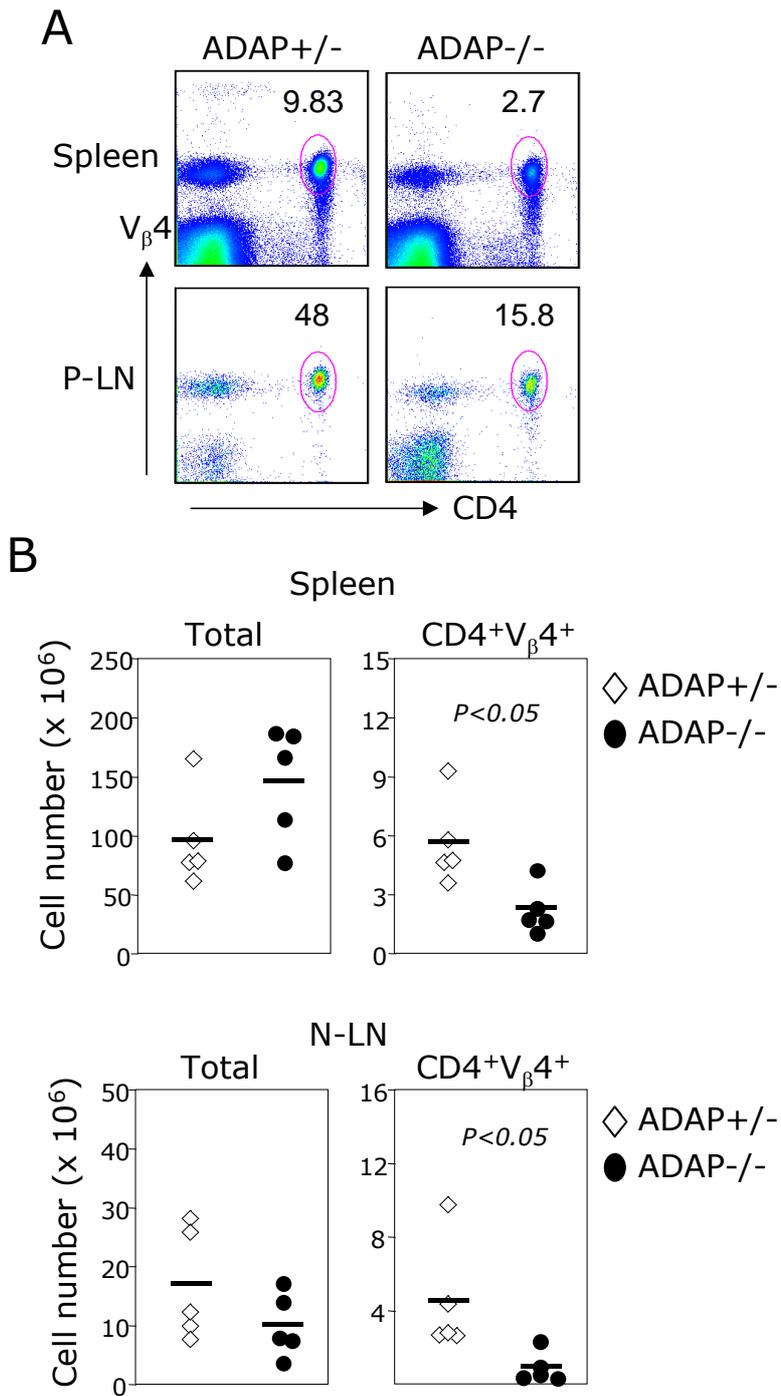
Although all of our data including in vitro and in vivo on antigen presenting cells suggests that ADAP is dispensable for APC function, we still cannot rule out the possibility that ADAP-deficient APCs may contribute to autoimmunity. Rag-deficient mice may be not an appropriate system to evaluate ADAP-deficient APCs in diabetes regulation since lack of lymphocytes generates too strong of a lymphopenic stimulus. Conditional depletion of ADAP gene in myeloid cells will be valuable on this point.

Previous studies demonstrate the essential influence of environmental factors, including bacteria and viral infections on autoimmunity <sup>162</sup>. But how environmental factors regulate autoimmunity is inconclusive. Different environmental factors may regulate autoimmunity by different mechanisms, such as molecular mimicry and bystander effect <sup>151,152</sup>. Particularly, King and his co-workers reported that treatment with complete (heat-killed mycobacterium-containing) Freund's adjuvant (CFA) relieved lymphopenia and autoimmune diabetes in NOD mice. They argued that a "dirty" environment protects humans and animals from autoimmune diseases through suppression of lymphopenia <sup>29</sup>. Interestingly, in our BDC2.5 g7/b background, the dramatically higher diabetes incidence in ADAP-deficient mice observed in a conventional facility disappeared in an SPF environment, suggesting that ADAP regulation of autoimmunity is environment-dependent. To answer this question, lymphopenia should be evaluated in SPF mice in terms of cell number and homeostatic proliferation. In contrast, in a BDC2.5 g7g7 background, ADAP-deficient BDC2.5 mice showed enhanced autoimmune disease in both conventional and SPF facilities, suggesting that ADAP also regulates autoimmunity in an environment-independent manner. Thus, ADAP regulates autoimmune diabetes in both environment-dependent and independent manners. The mechanism of regulation of autoimmune diabetes by environmental factors is under investigation.

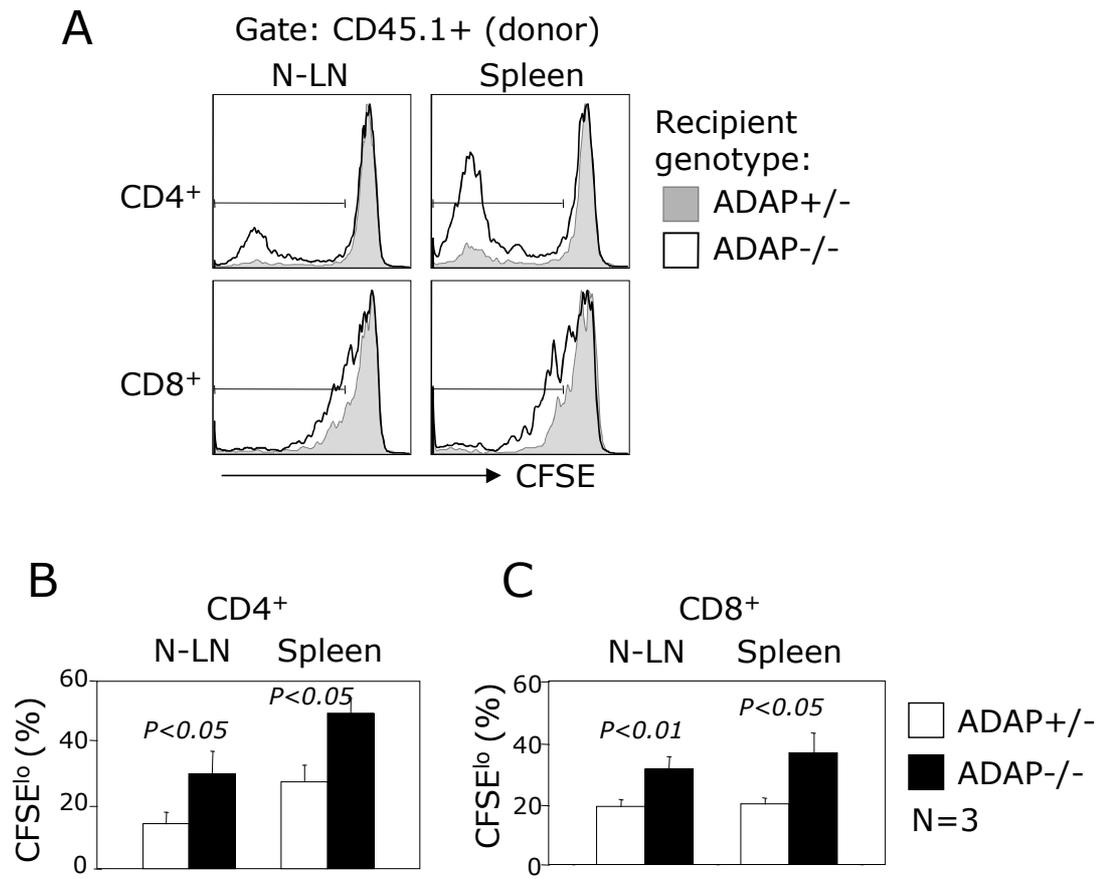




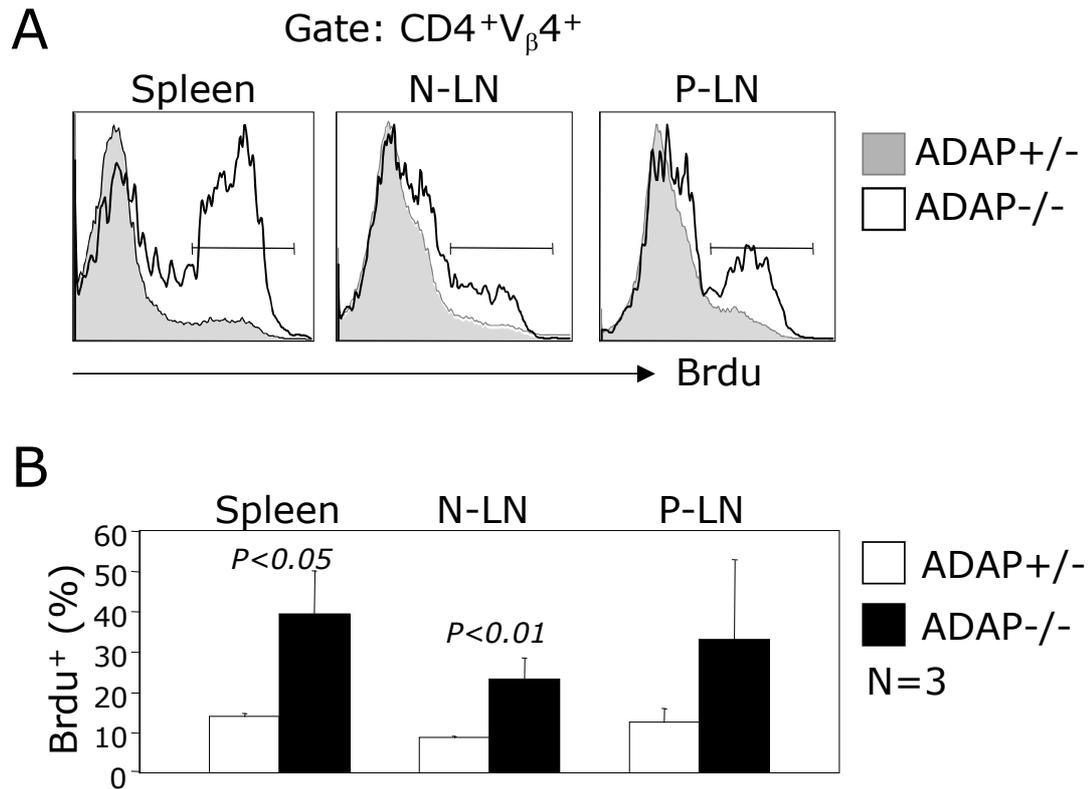
**Figure 2.2. Diabetes difference between SPF and conventional environments is not due to genetic difference.** Breeding cages from an SPF BDC2.5 g7/b colony were transferred into a conventional facility. The offspring were monitored for diabetes development, and diabetes incidences of 17 controls and 14 knockout mice are shown.



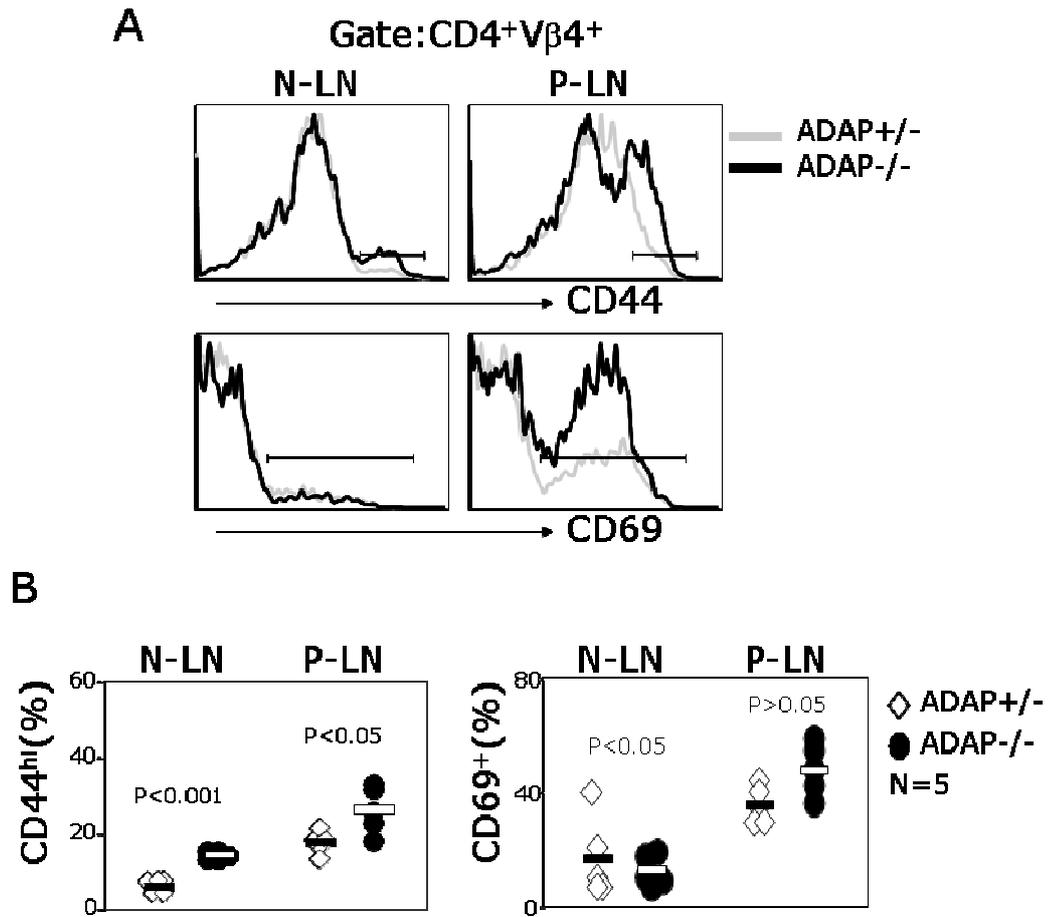
**Figure 2.3. ADAP<sup>-/-</sup> BDC2.5 g7b peripheral lymphoid tissues contain reduced numbers of CD4<sup>+</sup> T cells.** A) Percentages of live cells in indicated tissues (P-LN = pancreatic lymph nodes) from control or ADAP<sup>-/-</sup>, 5-week-old BDC2.5 g7b mice staining positive for CD4 and V<sub>β</sub>4 are shown. B) Comparison of control and ADAP<sup>-/-</sup> BDC2.5 g7b absolute CD4<sup>+</sup>V<sub>β</sub>4<sup>+</sup> T cell counts in spleen and inguinal, non-draining (N-LN) lymph nodes. Graph shows individual and Mean (indicated by bars) cellularity of five mice per group.



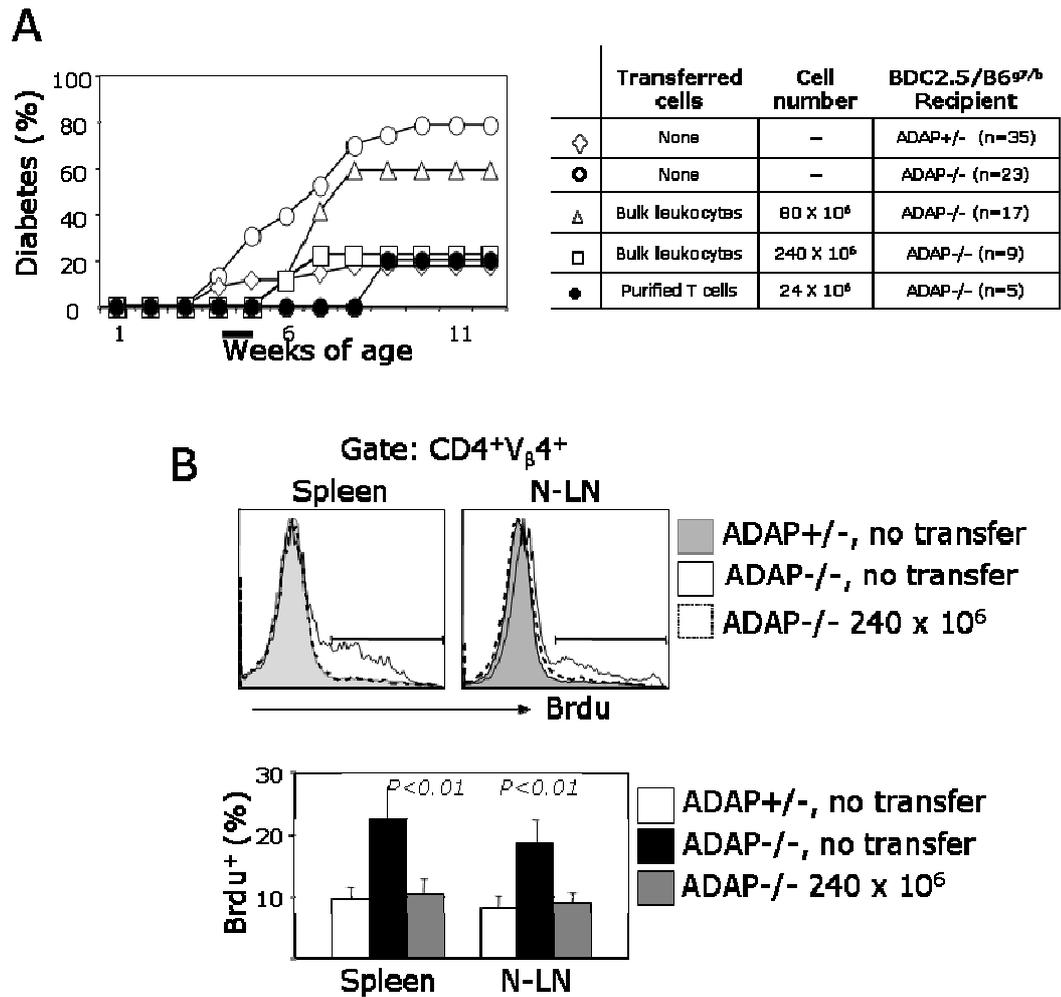
**Figure 2.4. Enhanced homeostatic proliferation of exogenous T cells transferred into ADAP<sup>-/-</sup> BDC2.5 g7b mice.**  $3 \times 10^6$  purified, CFSE-labeled, wild-type CD45.1<sup>+</sup> nontransgenic B6 g7b T cells were transferred into either ADAP<sup>+/-</sup> (Ctl) or ADAP-deficient (<sup>-/-</sup>), CD45.2<sup>+</sup> transgenic BDC2.5 g7b 4-week-old, pre-diabetic recipients. Representative histograms (A) show CFSE dilution by donor (CD45.1<sup>+</sup>) CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes harvested 4 days after transfer. Graphs (B and C) display mean (+ S.D.) percentage of CFSE-diluting donor cells from 3 individual recipients<sup>163</sup>.



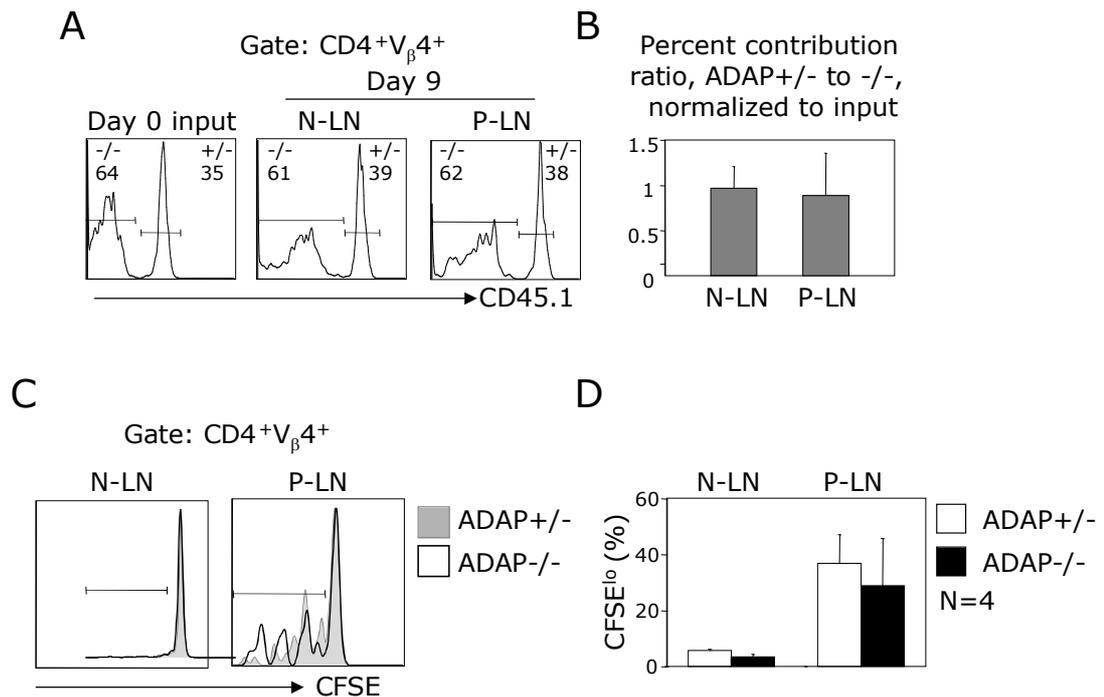
**Figure 2.5. Endogenous diabetogenic T cells show enhanced homeostatic and antigen specific proliferation in ADAP<sup>-/-</sup> BDC2.5 g7b mice.** ADAP<sup>-/-</sup> BDC2.5 g7b 5-week-old, pre-diabetic BDC2.5 g7b mice were fed Brdu for 5 days. CD4<sup>+</sup>V<sub>β</sub>4<sup>+</sup> lymphocytes harvested from indicated tissues were stained for Brdu uptake. Representative histograms (top) and graphs (bottom) showing mean (+ S.D.) % Brdu<sup>+</sup> were derived from analysis of 3-5 individual control or ADAP-deficient mice<sup>163</sup>.



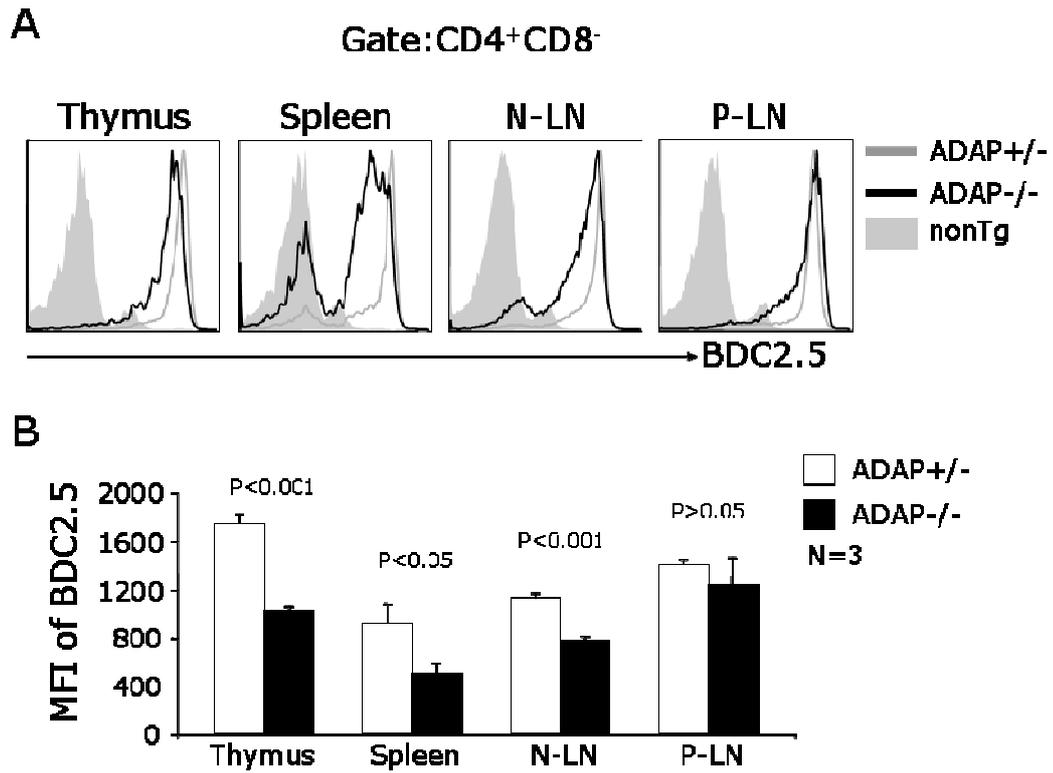
**Figure 2.6. ADAP-deficient T cells showed increased memory phenotype.** Lymphocyte isolated from five-week-old prediabetic BDC2.5 g7/b mice were stained with anti CD4, Vβ4, CD44, CD69 antibodies. Representative histograms (A) and graphs (B) showing percentages (+ mean indicated by bar) of % CD44<sup>hi</sup>, CD69 were derived from analysis of 5 individual control or ADAP-deficient mice.



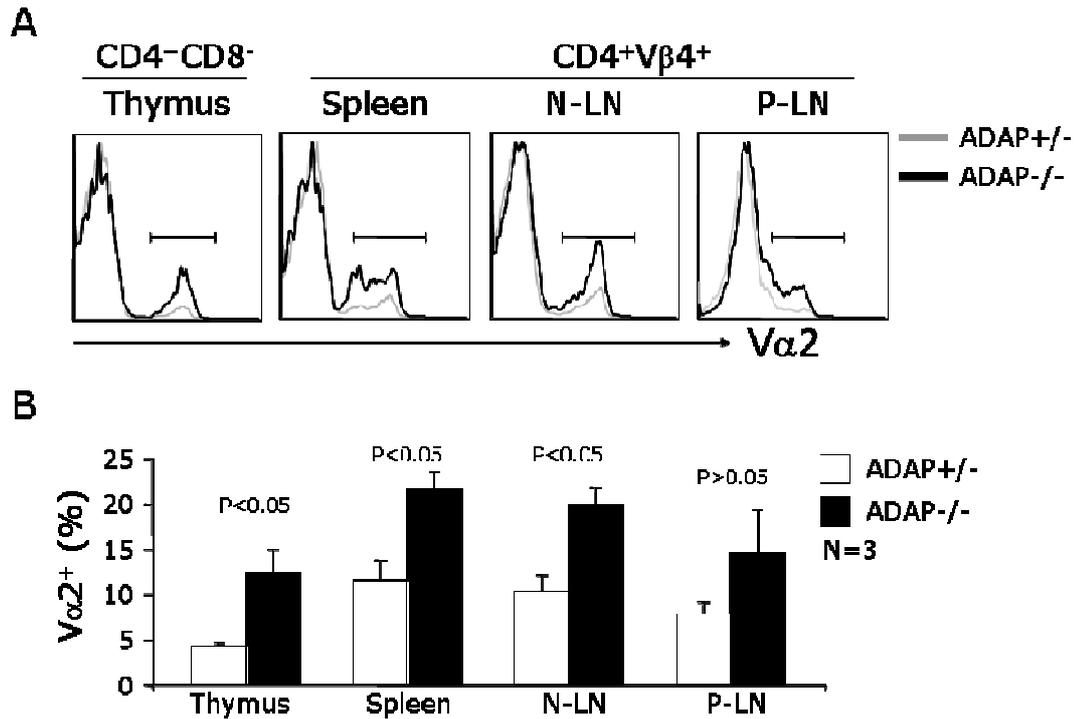




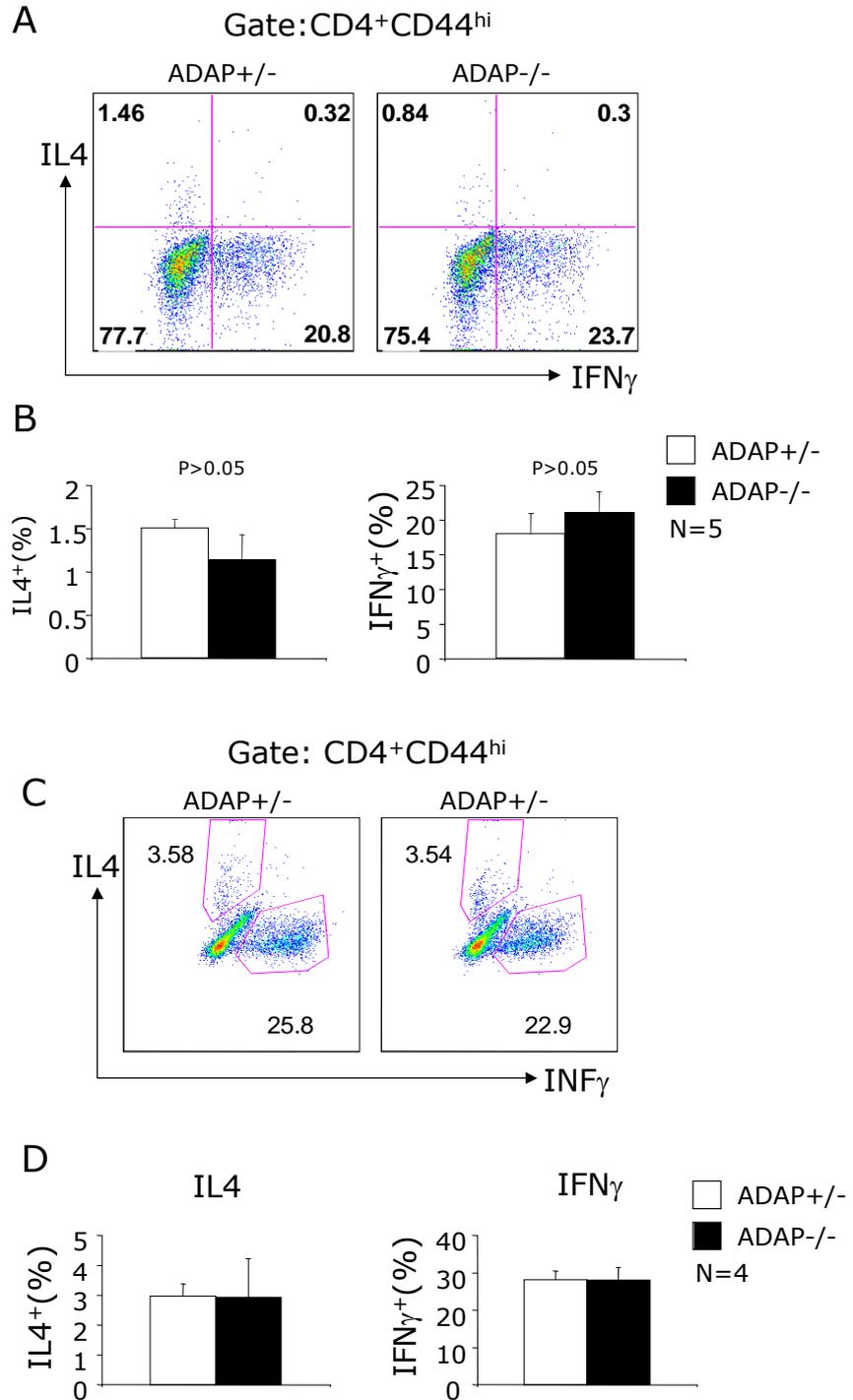
**Figure 2.9. Loss of ADAP has no effect on cell proliferation and short-term survival in vivo.** CFSE-labeled, ADAP-deficient (CD45.1<sup>-</sup>CD45.2<sup>+</sup>) and control (CD45.1<sup>+</sup>CD45.2<sup>-</sup>) CD4<sup>+</sup> T cells, in the context of unfractionated splenocytes, were mixed at a 2 to 1 ratio ("Day 0" histogram at left) and injected into non-transgenic (CD45.1<sup>+</sup>CD45.2<sup>-</sup>) mice. 9 days after injection, inguinal (N-LN) and pancreatic (P-LN) lymph node cells were analyzed. Representative histograms show percent CD4<sup>+</sup>V<sub>β</sub>4<sup>+</sup> T cell contribution by each genotype (A). Graph shows mean (+ S.D.) ratios derived by dividing Ctl contribution (%) by ADAP-deficient contribution (%), normalized to input ratio (B). Results represent analysis of 4 individual recipients. BDC2.5 g7b CD4<sup>+</sup>V<sub>β</sub>4<sup>+</sup> T cells described in were analyzed for CFSE dilution (C and D). Representative histograms (C), and graph (D) show mean (+ S.D.) fraction of CFSE<sup>10</sup> (divided) donor cells in indicated tissues<sup>163</sup>.



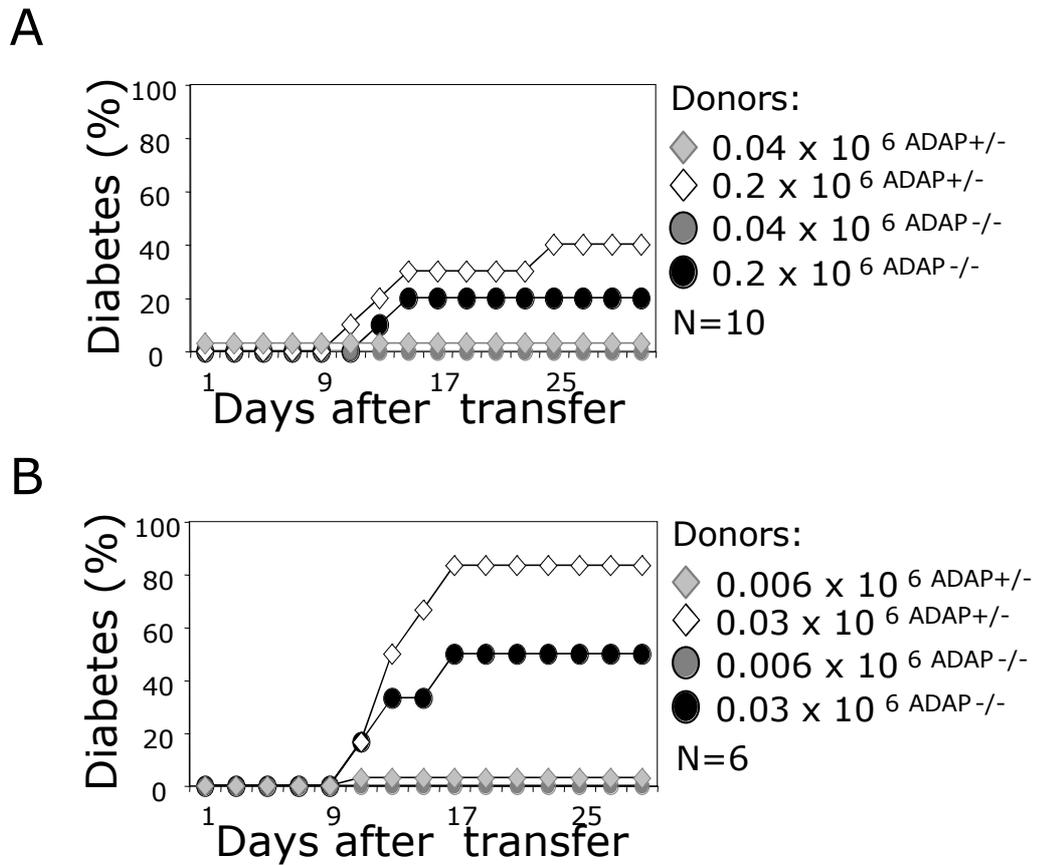
**Figure 2.10. Loss of ADAP decreases BDC2.5 TCR expression.** Cells from thymus, spleen and lymph nodes from BDC.25 g7/b mice were stained with anti-CD4, CD8 and BDC2.5 TCR. Representative histograms (A) of BDC2.5 TCR staining and mean fluorescence intensity (B) of CD4<sup>+</sup>CD8<sup>-</sup> cells are shown from triplicates.



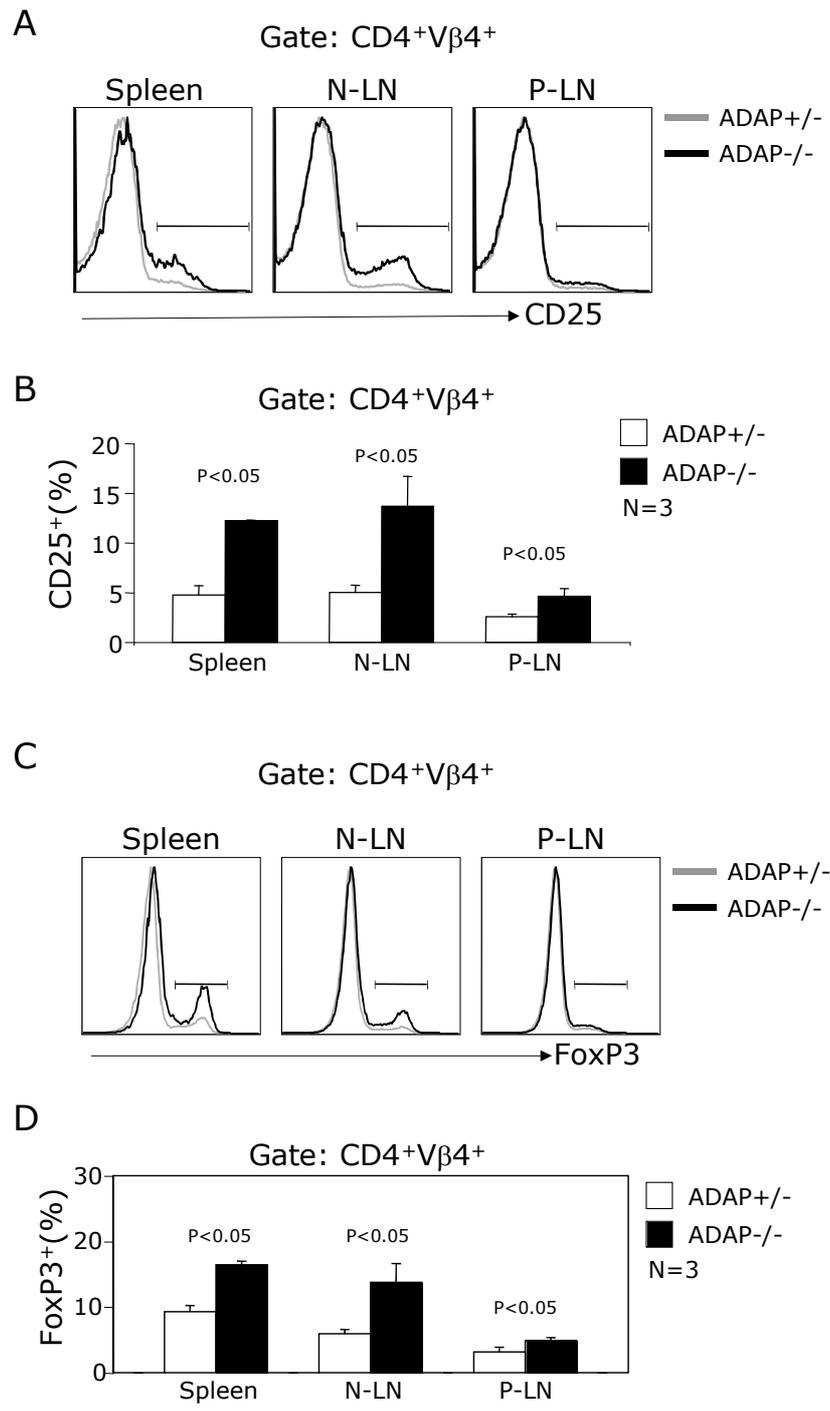
**Figure 2.11. ADAP-deficient T cells showed increased V $\alpha$ 2 expression.** Cells from thymus, spleen, and lymph nodes from BDC.25 g7/b mice were stained with anti-CD4, V $\beta$ 4 and V $\alpha$ 2. Representative histograms (A) of V $\alpha$ 2 staining and mean (+ S.D.) % of V $\alpha$ 2<sup>+</sup> (B) within CD4<sup>+</sup>CD8<sup>-</sup> cells are shown from triplicates.



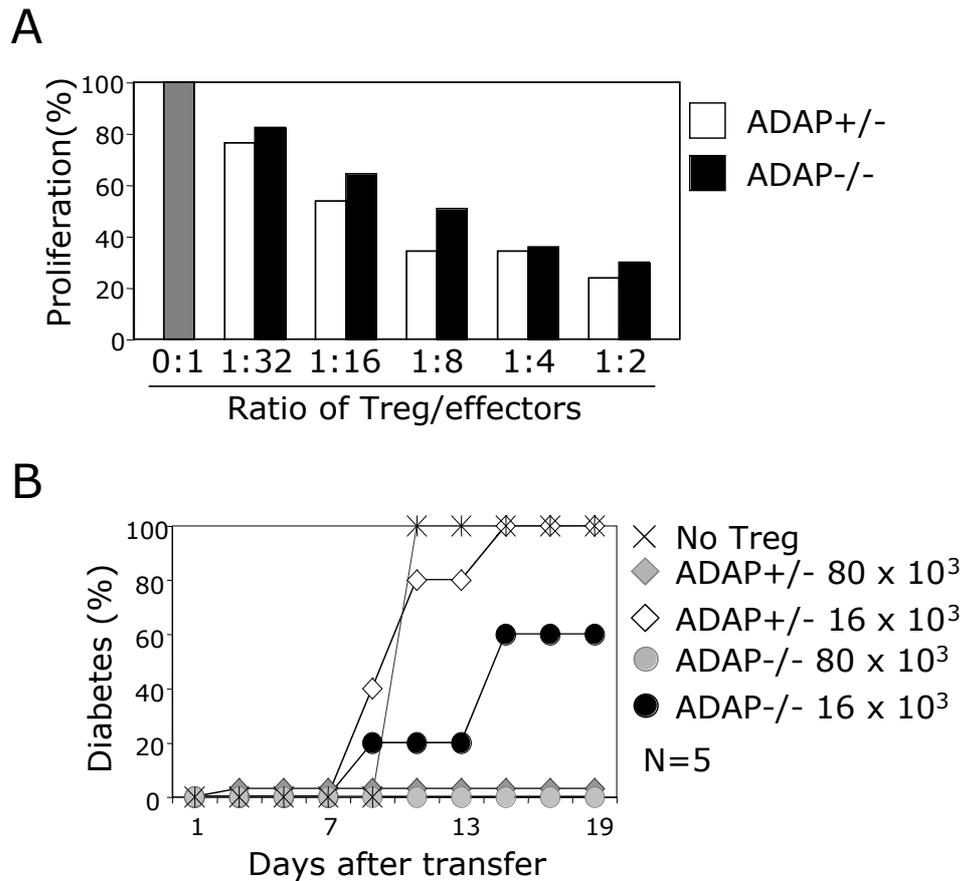
**Figure 2.12. ADAP-deficient memory cells show normal IL4 and IFN $\gamma$  secretion.** Spleen cells were stimulated with PMA and ionomycin for 6 hours and stained for CD4, CD44, IL4 and IFN $\gamma$ . Representative staining of IL4 and IFN $\gamma$  in BDC2.5 memory T cells (CD4<sup>+</sup>CD44<sup>hi</sup>) and mean (+S.D) % of IL4<sup>+</sup> and IFN $\gamma$ <sup>+</sup> derived from five mice per group are shown in figure A and B. Representative staining of IL4 and IFN $\gamma$  in memory T cells (CD4<sup>+</sup>CD44<sup>hi</sup>) from nontransgenic Balb/c mice and mean (+ S.D.) % of IL4<sup>+</sup> and IFN $\gamma$ <sup>+</sup> derived from four mice per group are shown in figure C and D.



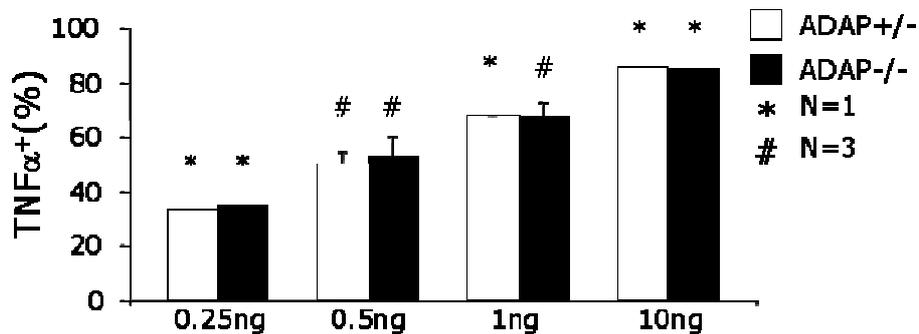
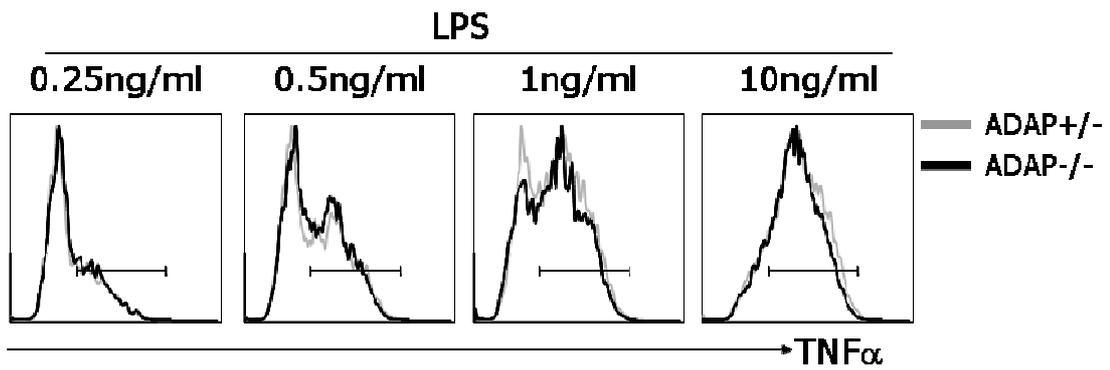
**Figure 2.13. ADAP-deficient spleen cells and T cells show moderate decreased capacity in diabetes induction in NODscid recipients.** Total spleen cells (A) or purified T cells (depleted of B220<sup>+</sup>, NK1.1<sup>+</sup> and CD11b<sup>+</sup>) (B) of different dosages as indicated were I.V. injected into NODscid mice. The development of diabetes derived from 10 recipients (figure A) and 6 recipients (figure B) is shown.



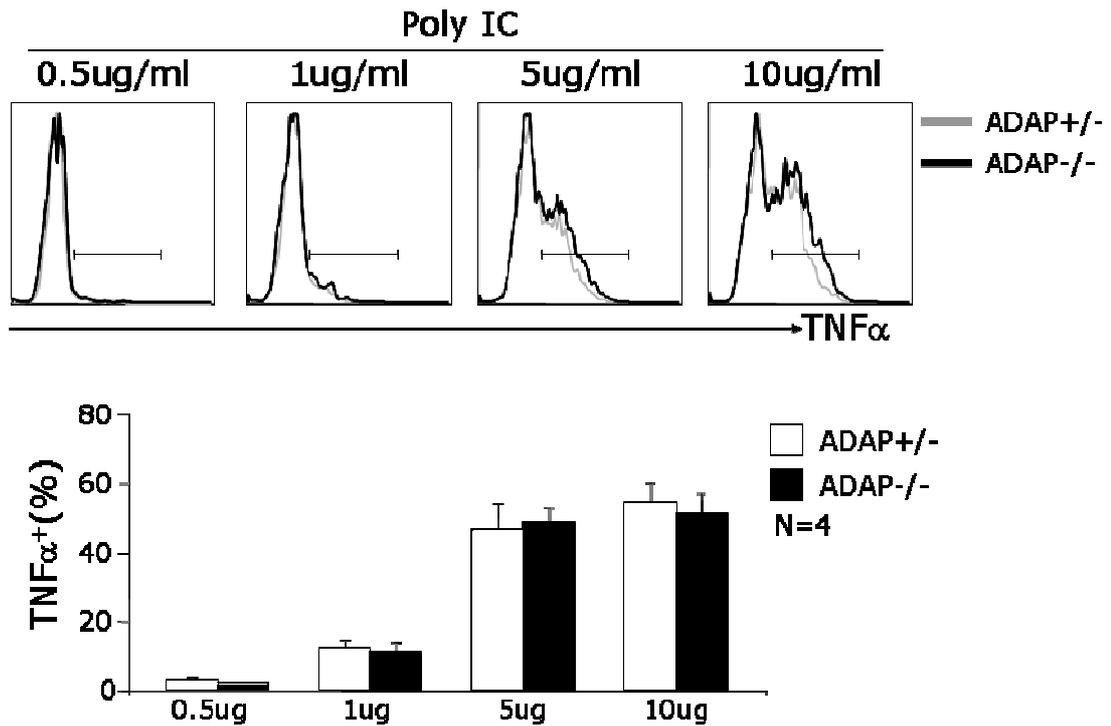
**Figure 2.14. ADAP-deficient BDC2.5 g7b mice show normal Treg number.** Lymphocytes isolated from spleen, N-LN, or P-LN were stained with anti-CD4, CD25, Vβ4 and FoxP3. Representative staining (A) of CD25 and mean % CD25<sup>+</sup>(B) within the CD4<sup>+</sup>Vβ4<sup>+</sup> population derived from three mice per group are shown. Representative staining (A) of FoxP3 derived from three mice per group is shown in figure C, and mean (+ S.D.) % FoxP3<sup>+</sup>(B) within the CD4<sup>+</sup>Vβ4<sup>+</sup> population is shown in figure D.



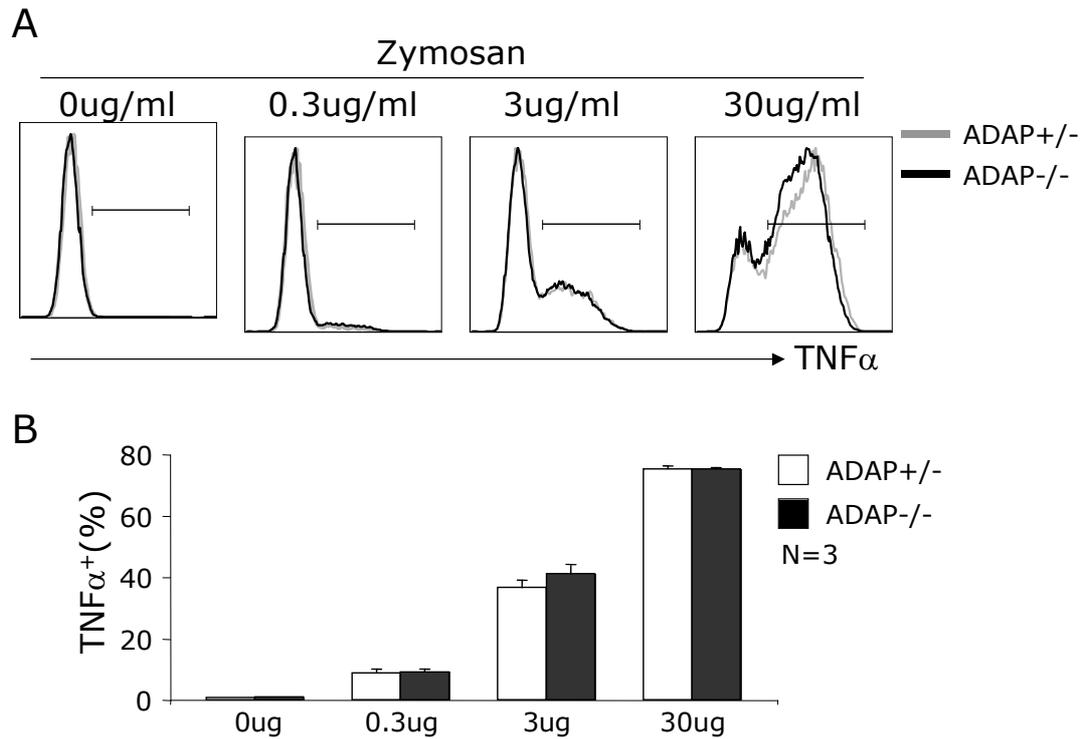
**Figure 2.15. ADAP-deficient Tregs show normal capacity in suppression of T cell proliferation in vitro and diabetes in NODscid recipients.** A) Tregs were isolated by CD25<sup>+</sup> positive selection from mixture of spleen and lymph nodes from either ADAP (+/-) CTL or ADAP-deficient (-/-) mice and ADAP CTL CD25<sup>-</sup> fraction was used as effectors. CFSE labeled effectors (0.6 million) were co-cultured with Tregs at different ratio as indicated in media containing 100ng/ml mimotope for 3 days. CFSE dilution of effectors was evaluated with flow cytometry and normalized to a Treg-free condition. B) Indicated numbers of CD25<sup>+</sup> BDC2.5 g7b splenocytes (Tregs) from ADAP+/- (Ctl) or ADAP-deficient (-/-) were co-transferred with 0.6 X 10<sup>6</sup> wild-type, CD25<sup>lo</sup> BDC2.5 g7b "effector" spleen cells into NOD/SCID mice (n=5 mice per group). Recipients were monitored for 4 weeks.



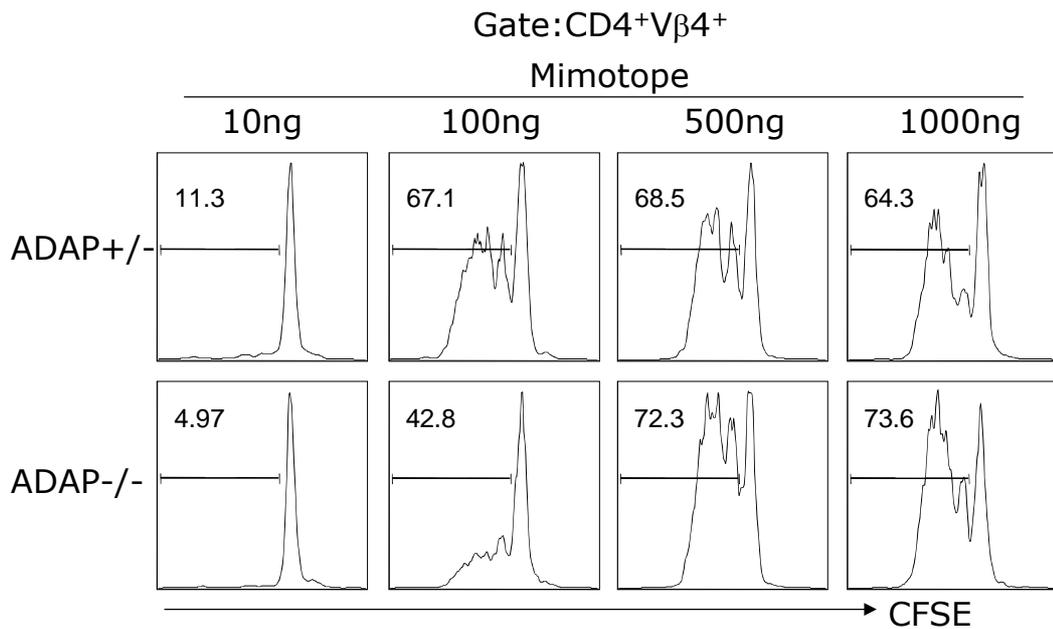
**Figure 2.16. ADAP-deficient bone marrow derived dendritic cells show normal TNF $\alpha$  secretion after LPS stimulation.** Bone marrow derived dendritic cells were stimulated with LPS of different dosages as indicated plus brefeldin A for 14 hours and cells were stained with antiCD11C and TNF $\alpha$ . Representative histograms (A) and mean (+ S.D.) % TNF $\alpha$ + with CD11C+ population from three experiments are shown. Data is derived from one or three mice as indicated .



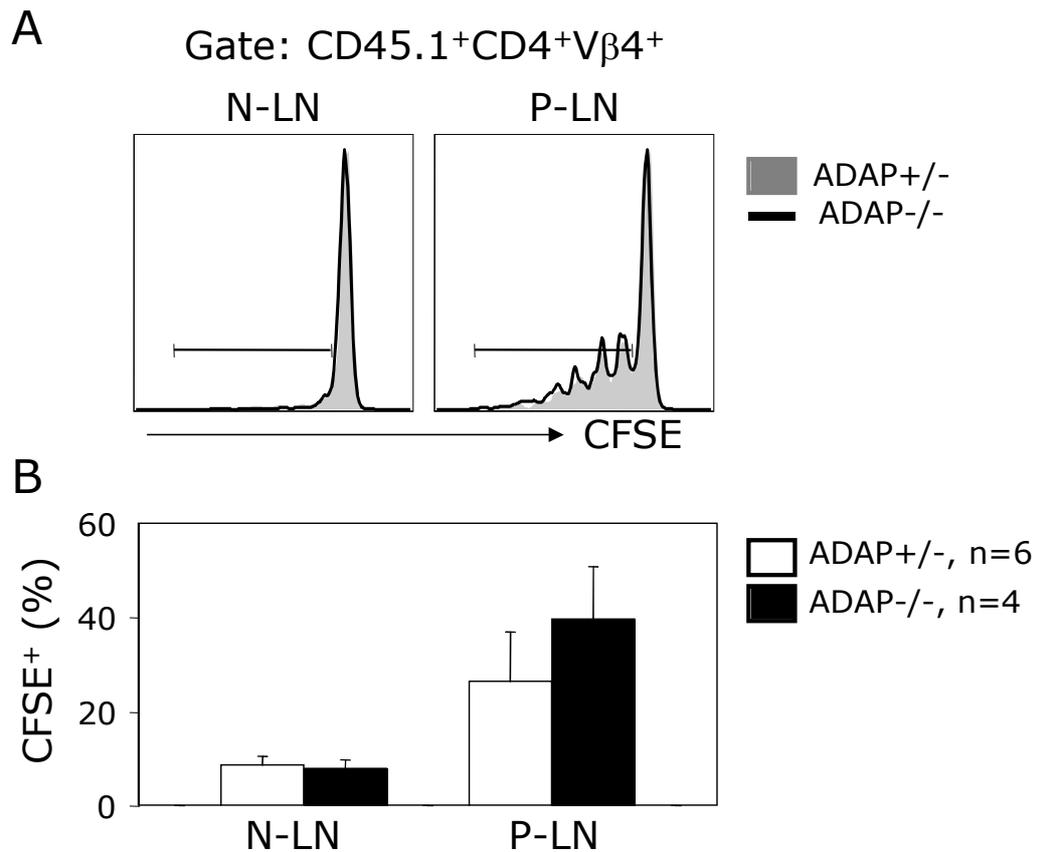
**Figure 2.17. ADAP-deficient bone marrow derived dendritic cells show normal TNF $\alpha$  secretion after Poly IC stimulation.** Bone marrow derived dendritic cells were stimulated with Poly IC of different dosages as indicated plus brafeldin A for 14 hours and cells were stained with antiCD11C and TNF $\alpha$ . Representative histograms (A) and mean (+ S.D.) % TNF $\alpha$ <sup>+</sup> with CD11C<sup>+</sup> population derived from four mice are shown.



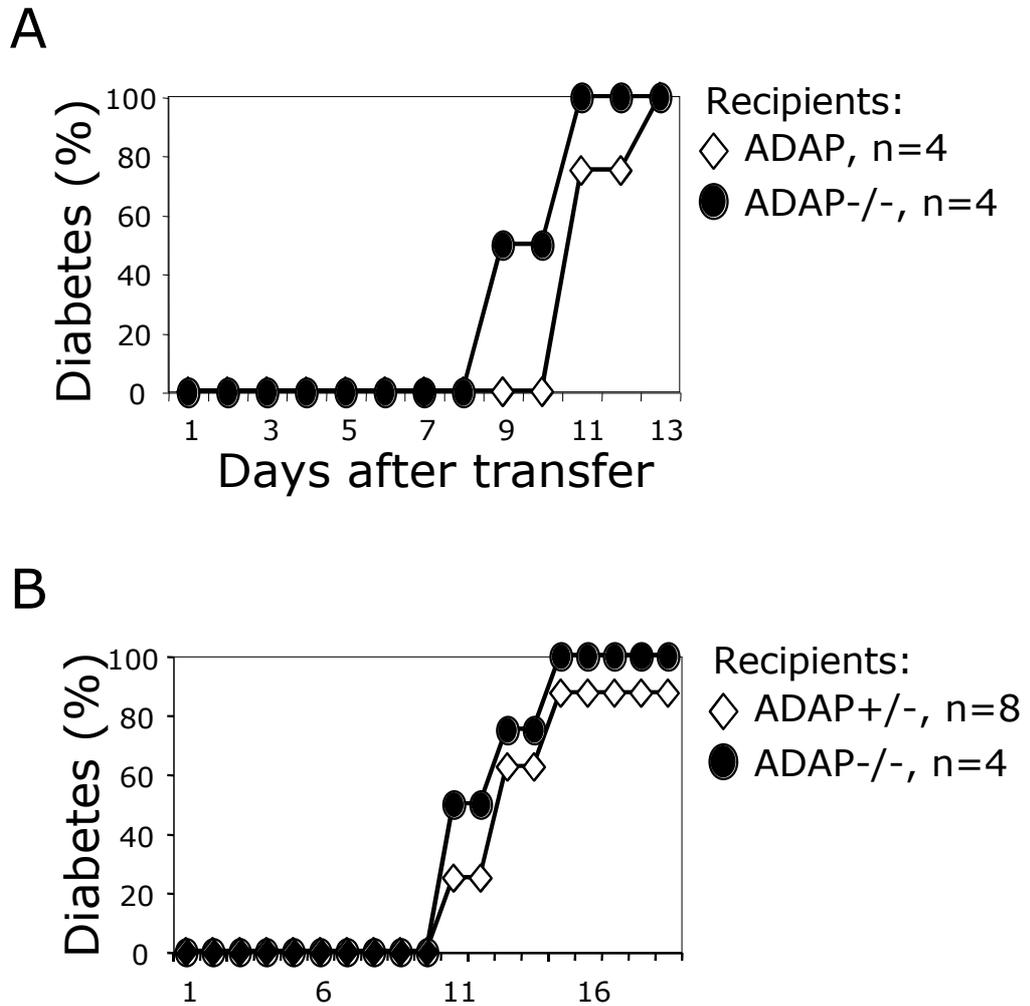
**Figure 2.18. ADAP-deficient bone marrow derived dendritic cells show normal TNF $\alpha$  secretion after zymosan stimulation.** Bone marrow derived dendritic cells were stimulated with zymosan of different dosages as indicated plus brafeldin A for 14 hours and cells were stained with antiCD11C and TNF $\alpha$ . Representative histograms (A) and mean (+ S.D.) % TNF $\alpha$ <sup>+</sup> with CD11C<sup>+</sup> population from two experiments are shown.



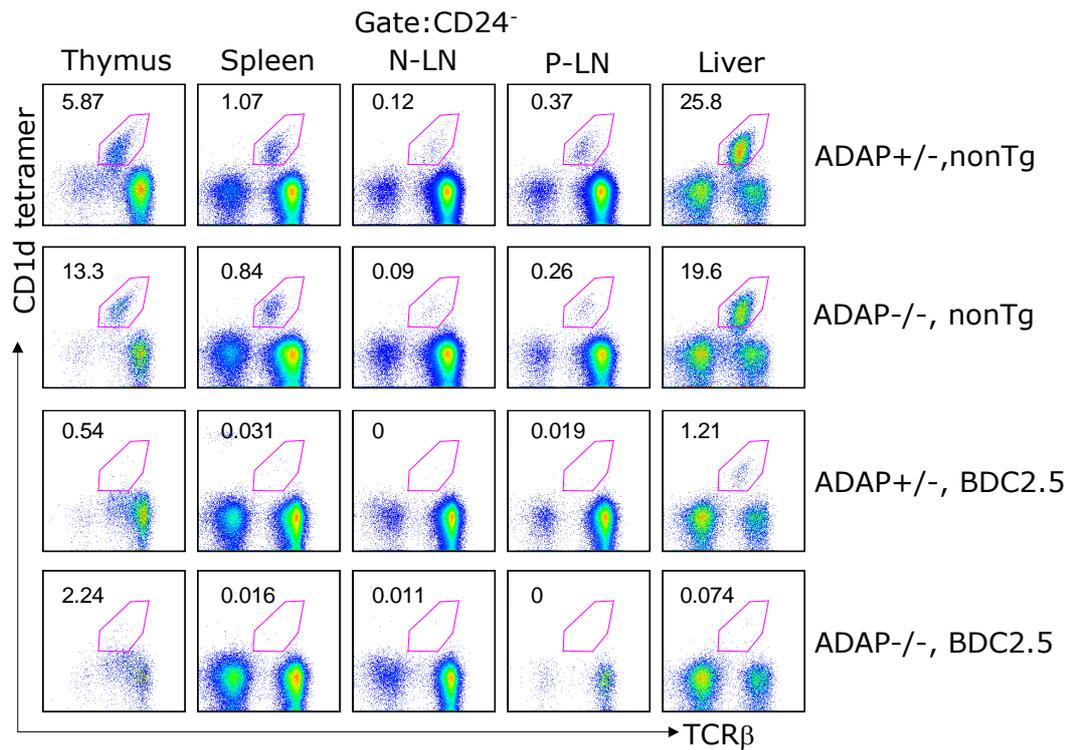
**Figure 2.19. ADAP-deficient APCs function normally in T cell priming in vitro.** T cells were purified with depletion of B220<sup>+</sup>, CD11b<sup>+</sup>, Dx5<sup>+</sup> and CD8<sup>+</sup> from BDC2.5 g7/b mice and APCs were prepared with spleen cells depleted of CD3<sup>+</sup> population from nontransgenic mice. 0.5 million CFSE labeled T cells and 0.5 million ADAP-sufficient (CTL) or deficient (-/-) APCs were co-cultured in medium containing mimotope of 0ng/ml, 10ng/ml or 100ng/ml for three days. Cell proliferation was evaluated by CFSE dilution within CD4<sup>+</sup>Vβ4<sup>+</sup> population. Fractions of CFSE<sup>lo</sup> are shown and one representative from three repeated experiments is shown.



**Figure 2.20. ADAP-deficient APCs function normally in T cell priming in vivo.** Lymphocytes were isolated from spleen and lymph nodes of congenic CD45.1<sup>+</sup> Wild-type BDC2.5 g7/b mice and were labeled with CFSE. 10 million CFSE labeled cells were I.V. injected into ADAP sufficient (CTL) or deficient (-/-) CD45.1- nontransgenic g7/b recipients. Cells were recovered from inguinal (N-LN) and pancreatic lymph nodes on days 4 for CFSE dilution within CD45.1<sup>+</sup>CD4<sup>+</sup>Vβ4<sup>+</sup> population. Representative histograms (A) and mean % CFSE<sup>lo</sup> (+ S.D.) (B) are shown from 6 wild-type controls and 4 ADAP-deficient recipients.



**Figure 2.21. ADAP-deficient recipients in Rag-deficient background show comparable susceptibility to diabetes induced by transferred BDC2.5 spleen cells and T cells.** 1 million spleen cells or 0.3 million purified BDC2.5 T cells (depleted of B220<sup>+</sup>, NK1.1<sup>+</sup> and CD11b<sup>+</sup>) were I.V. injected into ADAP-deficient or wild-type Rag<sup>-/-</sup> g7/b mice, which were housed in SPF condition and monitored for diabetes. Diabetes incidences are shown.



**Figure 2.22. NKT cell development is impaired in BDC2.5 transgenic mice.** Lymphocytes from BDC2.5 transgenic and non-transgenic thymus, spleen, lymph nodes, and liver were stained with anti CD24, CD1d tetramer (PBS-57 loaded) and TCR $\beta$  chain. CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup> Frequencies within CD24<sup>-</sup> population are shown. One representative from two experiments is shown.

### **CHAPTER 3**

## **REGULATION OF INTEGRIN SIGNALING IN PHAGOCYTES: AN OVERVIEW**

### **The role of neutrophils in adaptive and innate immune responses**

Neutrophils, as a myeloid cell population, develop and mature in bone marrow and circulate in blood. When pathogenic microbes such as bacteria and fungi invade mammalian bodies and cause tissue damage, danger signals from injured tissue or/and pathogens will be alarmed and immune responses will be orchestrated to clear the pathogens.

Neutrophils are the first cell population arriving at the inflammation site, where they kill pathogens. Neutrophils can also regulate other cell populations, such as dendritic cells, T cells, and NK cells, in immune responses through cytokines like  $TNF\alpha$  and  $IFN\gamma$ . Once neutrophils take up and kill pathogenic particles they undergo apoptosis. Apoptotic neutrophils can be engulfed by macrophages, which have an essential influence in adaptive responses by secreting cytokines like IL12,  $IFN$  <sup>164</sup>.

Neutrophils can either secrete toxic reagents such as proteases and chromatin to trap and kill pathogenic particles outside, or engulf them through a process called phagocytosis. Neutrophils are able to kill pathogens with Reactive Oxygen Intermediates (ROI) and other cytotoxic materials like proteases <sup>165</sup>. Clinical evidence from study of patients with the Chronic Granulomatous Disease (CGD) emphasizes that capacity to generate ROI is critical for neutrophils to kill microbes. CGD is characterized by severe and recurrent bacterial and fungal infections,

sometimes leading to death. The clinical phenotype of CGD is dependent upon mutations in genes encoding oxidase (ROI machinery).

### **Regulation of cell adhesion, migration, phagocytosis and oxygen burst by integrins**

Integrins have the capacity to integrate extracellular environment and cytoskeleton for cell adhesion, migration, degranulation, and phagocytosis in response to stimuli. Each integrin subunit has a large extracellular domain, a single pass transmembrane domain, and a small cytoplasmic tail. The cytoplasmic domain of integrins is connected to bundles of F-actin through bridging proteins such as vinculin, talin, actinin, tensin, and paxillin<sup>166,167</sup>. Upon attachment to integrin substrates, the cytoskeleton actin will be reorganized and cause clustering of integrins at the focal contact sites.

The recruitment of neutrophils from blood vessel into inflammation sites can be divided into multiple steps: capture (first capture of circulating neutrophils on endothelium), rolling, slow rolling/arrest (decreased rolling due to activation of integrin by chemokines presented on endothelium), spreading/postadhesion strengthening (increased contact area and integrin/receptor clustering at anchoring sites), crawling (movement towards the epithelial tight junction), and transmigration (cells squeeze through the tight junction)<sup>168</sup>. In vitro and in vivo studies demonstrated involvement of integrin in all of these recruitment steps<sup>169,170,171</sup>.

Phagocytosis is initiated by the engagement of phagocytic receptors such as complement receptors, scavenger receptors, Fc

receptors, and toll-like receptors. Opsonization (coating microbes with host-derived proteins) is a prerequisite for phagocytosis of microbes that have no immediate recognizable ligands. In mammals, several members of integrins can serve as receptors for opsonized microbes, of which  $\alpha$ M $\beta$ 2 is the principal receptor for C3bi coated particles<sup>172</sup>. The significance of  $\alpha$ M $\beta$ 2 in pathogen clearance is emphasized by studies of leukocyte adhesion deficiency (LAD). LAD patients lack functional  $\beta$ 2 integrin on leukocytes and suffer bacterial infections due to defective phagocytosis<sup>173</sup>.

The NADPH complex (ROI machinery) is composed of multiple components including cytosolic (p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, Rac2, p29<sup>phox</sup>) and membrane bound (gp91<sup>phox</sup>, p22<sup>phox</sup>, Rap1A) subunits. Once neutrophils are activated, cytosolic subunits can be phosphorylated and translocate to and complex with membrane components to generate ROI such as O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. Although there are multiple ways to trigger oxygen burst in neutrophils, integrins have been intensively studied because they are critical connectors between phagocytosis and oxygen burst, two key steps in bacterial killing. It was shown that integrin engagement activates and mediates membrane translocation of NADPH cytosolic subunits<sup>174</sup> and that integrin engagement promotes cytokine TNF $\alpha$ -mediated oxygen burst<sup>175</sup>. Many signaling modules, including Fgr/Hck, DAP12/FcR $\gamma$ , Syk, and SLP-76, downstream of integrins, have been also implicated in oxygen burst and/or bacterial killing by neutrophils<sup>174,176-179</sup>.

### **Members of integrin family**

Each member of the integrin family has  $\alpha$  and  $\beta$  subunits forming a heterodimer. So far, there are 18 $\alpha$  and 8 $\beta$  subunits identified, of which 24 known pairs can be formed on mammalian cells.  $\beta$ 1 associates with  $\alpha$ 1 to  $\alpha$ 11 plus  $\alpha$ V, forming twelve members of integrins, while  $\beta$ 2 binds to  $\alpha$ L,  $\alpha$ M,  $\alpha$ X, and  $\alpha$ D for four members. Beside  $\beta$ 1,  $\alpha$ V also associates with  $\beta$ 3,  $\beta$ 5,  $\beta$ 6, and  $\beta$ 8 to form four members. Moreover,  $\alpha$ 4 $\beta$ 7,  $\alpha$ E $\beta$ 7,  $\alpha$ 6 $\beta$ 4, and  $\alpha$ I**I** $\beta$ 3 are also identified as integrin family members. Out of these 24 combinations, immune cells express at least 10 members of the integrin family, consisting of  $\beta$ 1,  $\beta$ 2 or  $\beta$ 7. The expression of  $\beta$ 2 and  $\beta$ 7 integrins is limited to leukocytes, while  $\beta$ 1 integrins are expressed on a wide range of tissue <sup>167</sup>. Integrins expressed on phagocytes include  $\alpha$ 4 $\beta$ 1 (CD49d/CD29, VLA4),  $\alpha$ 5 $\beta$ 1 (CD49e/CD29, VLA5)  $\alpha$ 6 $\beta$ 1 (CD49f/CD29, VLA6),  $\alpha$ L $\beta$ 2 (CD11a/CD18, FLA-1),  $\alpha$ M $\beta$ 2 (CD11b/CD18, CR3, Mac-1),  $\alpha$ X $\beta$ 2 (CD11c/CD18, gp150/95), and  $\alpha$ D $\beta$ 2 (CD11d/CD18) <sup>167,180</sup>.

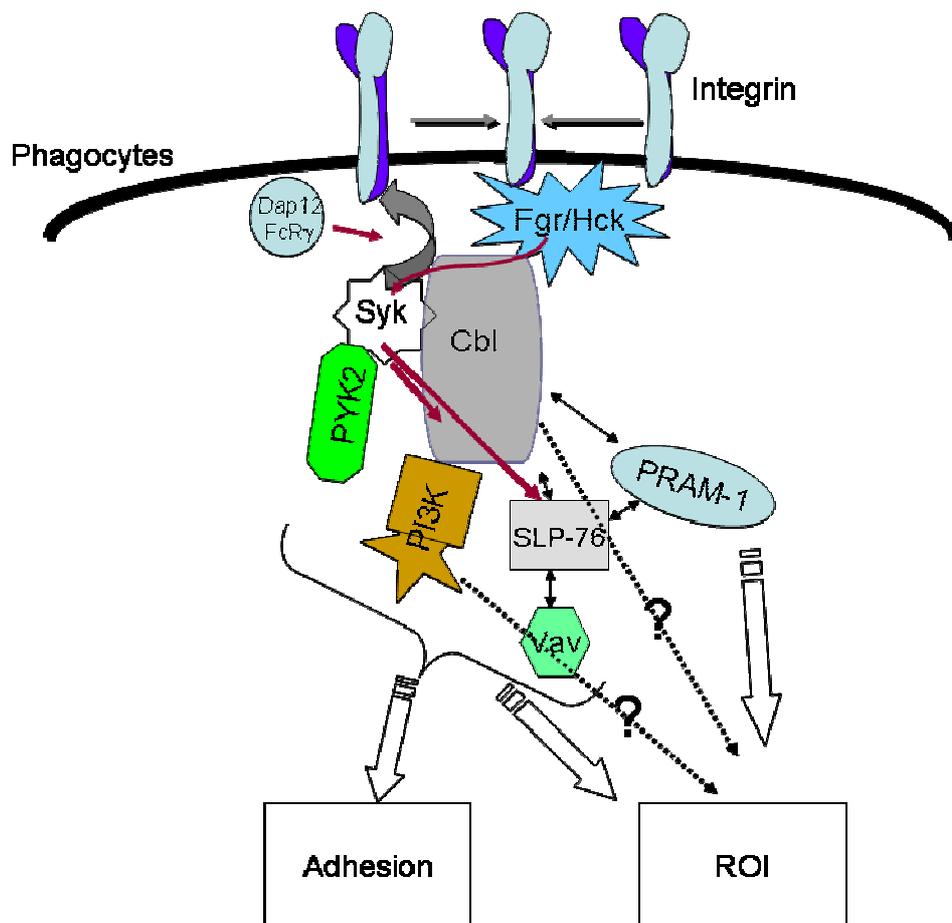
### **Activation of integrins**

The most fantastic feature for integrins is to transduce signals in a bi-directional manner. On the one hand, the extracellular domain can be activated by cytoplasmic tail once integrins are stimulated by a signal from a second receptor like TNF $\alpha$  and Fc receptor ("inside-out" signal). On the other hand, the engaged extracellular domain can regulate cytoplasmic domain and initiate downstream signal events ("outside-in" signal) <sup>167</sup>. Integrin activation can be regulated at two levels: affinity (conformation change) and valency (clustering). On resting cells, two head-pieces of integrins with low affinity are bent (closed). Upon activation, the two bent head pieces will extend up and open the

conformation with high affinity. Beside affinity regulation, inside-out signals can cause integrin clustering at leukocyte contact site to extracellular matrix, resulting in multivalent interactions with ligands. As a result, overall binding strength (avidity) to ligands is increased <sup>167</sup>.

### **Inside-out signal to integrins in phagocytes**

On neutrophils, multiple receptors including fMLP receptors, TNF $\alpha$  receptors, Toll-like receptors, and Fc receptors can give inside-out signals to activate integrins. Signal pathways through chemokine receptors (fMLP receptors) to integrin include PI3 kinase/RhoGEF<sup>181</sup> or DOCK2 <sup>182</sup> or RHOA/PKC $\zeta$  pathways <sup>167,183</sup>. There are several lines of evidence showing crosstalk between Fc receptor and integrin: Physical association between Fc receptor and integrins <sup>184-188</sup> and cooperation between integrins and Fc receptors in many activities including phagocytosis <sup>189</sup> and oxygen burst <sup>190,191</sup>. PI3 kinase has been shown critical for crosstalk between integrin and Fc receptors <sup>192-194</sup>. Similar to Fc receptors, the TNF $\alpha$  receptor can also activate integrins, probably through the Syk kinase and p38 MAP kinase pathway <sup>178,195</sup>. TLR2 and TLR4 have been implicated to activate integrins through PI3K and RAP1 <sup>172,196-198,198</sup>.



**Figure 3.1. Signaling pathways downstream of integrin in phagocytes.** Integrin activation results in activation of Src kinase family members like Fgr/Hck, which activate Syk, of which recruitment to integrins requires ITAM-bearing proteins DAP12/FcR $\gamma$ . Syk may further phosphorylate downstream targets, including PYK2, c-Cbl, SLP-76 for integrin dependent activities including cell adhesion and ROI generation. C-Cbl, as an adaptor protein, may complex with other proteins Syk, SLP-76, PRAM-1, and Vav in phagocytes. PYK2, SLP-76, and Vav have been documented in integrin-dependent cell adhesion and ROI generation while PRAM-1 is only required for integrin-dependent ROI generation, but not for cell adhesion. C-Cbl and PI3K have been implicated in cell adhesion but their roles in integrin-dependent ROI generation have not been characterized yet. This modified diagram is derived from the proposal in Berton, G. et al. *TRENDS in Immunology* 2005 <sup>199</sup>.

## **Outside-in signal downstream of integrins in phagocytes**

The general scheme for integrin mediated outside-in signaling in phagocytes has been established with evidence primarily from macrophages and neutrophils<sup>199</sup>. Tyrosine phosphorylation has been implicated as a critical event for the outside-in signal downstream of integrins<sup>199</sup>. It is established that Src kinase activity is autoinhibited by intramolecular interactions of SH2 and SH3 domains<sup>200-202</sup>. Interaction of SH2 and SH3 domains of Src kinases keeps kinase domain in a inhibited conformation<sup>200-202</sup>. After activation, the phosphorylated tail of integrin  $\beta$  chain binds to SH3 domain and the conformation of kinase domain of Src kinases is open. As a result, Src kinases may be auto-activated<sup>203</sup> and further phosphorylate ITAM bearing proteins such as DAP12 or/and FcR $\gamma$  chain, which in turn mediate the recruitment of Syk (Fig. 3.1)<sup>199</sup>. Syk may be activated through autophosphorylation or by Src kinases<sup>200,201,204-208</sup>. Further Syk and Src kinases will activate downstream substrates including c-Cbl, Pyk2, PI3 kinase, SLP-76 in cell adhesion, migration, degranulation, phagocytosis, and ROI generation (Fig. 3.1). As an adaptor protein, c-Cbl may interact with different proteins including Syk, PI3 kinase, SLP-76 to form a complex (Fig. 3.1). Moreover, Vav/PLC $\gamma$ 2 pathway is proposed downstream of SLP-76 in integrin dependent adhesion and ROI generation by neutrophils<sup>174,209</sup>.

Activation of Src kinases is a required early step in integrin signaling in phagocytes<sup>176</sup>. Src family members expressed in myeloid cells include Fgr, Hck, and Lyn. Evidence demonstrated a critical role of Src family kinase in integrin signaling in phagocytes. Engagement of integrin  $\beta$ 2 results in phosphorylation of Fgr, which is not detectable in

neutrophils isolated from leukocyte adhesion deficient (LAD) patients<sup>210,211</sup>. Fgr/Hck/Lyn triple deficient, but not single deficient, neutrophils were defective in adhesion, ROI generation, degranulation, and migration, suggesting a critical, yet redundant role of the Src family in integrin signaling<sup>176,178,212-214</sup>. Close study further revealed that Fgr/Hck specifically regulated outside-in but not inside-out signaling<sup>212-214</sup>. Src kinases may regulate the adhesion through Pyk2, Syk, and c-Cbl/PI3K, which failed to be activated in the absence of Src kinases<sup>214,215</sup>.

Syk is a critical module downstream of integrin. Integrin ligation resulted in activation of Syk in neutrophils, monocyte cell lines, and platelets<sup>216-218</sup>. Defective adhesion, spreading, degranulation, and respiratory burst were observed in Syk deficient neutrophils stimulated by integrin ligands, suggesting an indispensable role of Syk in integrin signaling<sup>219</sup>. Syk plays an essential role in outside-in, but not inside-out, signaling downstream of integrin, since TNF $\alpha$ -mediated CD18 upregulation and L-selectin shedding were normal in Syk deficient cells<sup>219</sup>. Interestingly, Syk deficient neutrophils showed normal migration in vitro and in vivo. Intracellular staining and immunoprecipitation assays showed association and colocalization of Syk and integrin, suggesting a direct interaction between Syk and integrin<sup>219</sup>. Lack of activation of Syk in Fgr/Hck/Lyn triple mutant neutrophils after integrin ligation suggested that Syk functions downstream of Src family kinases<sup>219</sup>. The activation of c-Cbl, Vav and Pyk2 was dependent on Syk, indicating that they are regulated by Syk downstream of integrins<sup>219</sup>.

How Syk is recruited to integrins was appreciated with the study of ITAM bearing adaptor protein DAP12 and Fc $\gamma$  receptor in phagocytes.

ITAM represents immunoreceptor tyrosine-based activation motif of consensus sequence YxxI/Lx<sub>(6-8)</sub>YxxI/L. ITAM-bearing proteins have been identified in many cell types (e.g. CD3 $\zeta$  in T cells, DAP12 and Fc receptor  $\gamma$  chain in osteoclasts, DAP12, CD3 $\zeta$  and Fc receptor  $\gamma$  chain in NK cells). Fc $\gamma$ -, Fc $\alpha$ - receptors, DAP12 bearing ITAM motif are expressed in neutrophils and macrophages<sup>220,221</sup>. Neutrophils and macrophages depleted of DAP12 and FcR $\gamma$  showed fundamental defects in oxygen burst, degranulation, and adhesion upon integrin ligation, but not in nonintegrin-mediated responses such as migration to fLMP, suggesting specific regulation of outside-in signal by DAP12 and FcR $\gamma$  downstream of integrins<sup>177</sup>. Multiple signaling pathways, including activation of Syk, were impaired in response to integrin stimulation on DAP12/FcR $\gamma$  double deficient cells, suggesting that DAP12 and FcR $\gamma$  serve upstream of Syk<sup>177</sup>.

Integrin-dependent activation of and colocalization of Pyk2 with talin, paxillin, and  $\beta$ 2 integrin in podosome of migrating macrophages suggests a potential role of Pyk2 in integrin signaling<sup>222,223</sup>. Pyk2 is required in macrophage migration since knockdown by antisense RNA or genetic depletion of Pyk2 hampered macrophage migration in vivo<sup>224,225</sup>. When C-terminal fragment of Pyk2 fusion protein was incubated with and taken up by human neutrophils, the fragment dominantly inhibited integrin dependent oxygen burst but not the degranulation and bacterial killing functions of neutrophils, suggesting a selective regulation of integrin signaling by Pyk2<sup>225</sup>. Moreover, the dominant interfering Pyk2 suppressed activation of both endogenous Pyk2 and Syk, indicating cross talk between Pyk2 and Syk<sup>225</sup>. Regulation of integrin signaling by Pyk2

may include Rho GTPase and PI3 kinase and  $\text{Ca}^{2+}$  flux (see below for details)<sup>224</sup>.

Vav family members Vav1, Vav2, and Vav3 are expressed in myeloid cells. Evidence from Vav triple and double (Vav1/3) deficient mice showed that the Vav family was required for integrin dependent oxygen burst and adhesion<sup>174,209</sup>. One way for the Vav family to regulate integrin dependent ROI generation is to regulate activation of PLC- $\gamma$ , which is also required for integrin mediated oxygen burst<sup>174</sup>. PLC- $\gamma$  can metabolize PIP2 into diacylglycerol (DAG) and IP3. The latter triggers  $\text{Ca}^{2+}$  flux from ER stock.  $\text{Ca}^{2+}$  and DAG cooperatively activate PKCs, which have been implicated in phosphorylation of NADPH subunits<sup>174</sup>. The Vav family may also regulate cell adhesion through Pyk2 and paxillin, since phosphorylation of these two proteins is impaired in the absence of Vav1 and Vav3<sup>209</sup>. As guanine nucleotide exchange factors, Vavs can also activate Rho GTPases (Rho, RhoG, Rac and Cdc42)<sup>226</sup>, which may regulate different aspects of cytoskeleton rearrangement, including lamellipodia formation, focal adhesion, and phagocytosis<sup>227</sup>. One can speculate that the Vav family mediates integrin-dependent ROI generation through Rac1 and Rac2, since Rac2 is one component of the NADPH complex<sup>228</sup> and both Rac1 and Rac2 have been implicated in activation of NADPH<sup>229-231</sup>.

### **Adaptor protein regulation of integrin signaling in phagocytes**

Beside antigen receptor signaling in T cells, adaptor proteins also regulate integrin signaling in phagocytes. Adaptor proteins characterized in integrin signaling in phagocytes (neutrophils and macrophages) include

SLP-76, WASP, c-Cbl and PRAM-1. The indispensable role of SLP-76 in integrin signaling in neutrophils was clearly demonstrated in experiments where SLP-76 deficient neutrophils showed decreased adhesion to integrin ligand and defective ROI generation<sup>232</sup>. Interestingly, SLP-76 was not required for migration of and bacterial killing by neutrophils<sup>233</sup>. Moreover, SLP-76 was also essential for Fc receptor dependent Ca flux and oxygen burst<sup>232</sup>. Western blot data suggested that SLP-76 regulates integrin signaling through activations of Vav, P38 and PLC- $\gamma$ 2<sup>232</sup>. However normal degranulation upon TNF or fMLP stimulations of SLP-76 null neutrophils suggested specific regulation of outside-in signal downstream of integrins by SLP-76. Recently the adaptor protein WASP has been implicated in regulation of outside-in integrin signals in neutrophils. WASP deficient neutrophils showed defective integrin clustering, integrin-dependent adhesion, Ca<sup>2+</sup> flux, and Syk activation in response to TNF $\alpha$  stimulation<sup>234</sup>.

### **Regulation of integrin signaling in phagocytes by adaptor protein c-Cbl**

C-Cbl is a 120 KD cytoplasmic protein which was first identified as a proto-oncogene in hematopoietic tumors<sup>235</sup>. To date, there are three members of the Cbl family identified in mammals, including c-Cbl, Cbl-b, and Cbl-3<sup>235</sup>. All of these members share a highly conservative sequence in the N-terminal. C-Cbl is ubiquitously expressed in different tissues with the highest level in hematopoietic cells and testis. C-Cbl contains a tyrosine kinase binding (TKB) domain, a ring finger domain, a proline rich domain, and an ubiquitin-associated (UBA)/LZ domain from

N- to C- terminals, plus several tyrosine residues<sup>235</sup>. Of the tyrosine residues, Tyr700, Tyr731 and Tyr774 can be phosphorylated by Syk and Src family kinases<sup>236</sup>.

C-Cbl regulates signal transduction downstream of a wide range of cell surface receptors, including immunoglobulin receptors, hormone receptors, cytokine receptors, growth factor receptors, and integrins<sup>235</sup>. Downstream of many receptors, c-Cbl functions as an ubiquitin ligase and negatively regulates signal transduction. For example, overexpression of c-Cbl protein enhanced the ubiquitination and degradation of Lyn tyrosine kinase and inhibited FcεRI receptor dependent mast cell activation, suggesting a negative role of c-Cbl downstream of FcεRI receptor<sup>237</sup>. In contrast, some evidence also suggested a positive function for c-Cbl downstream of integrins. As a substrate of Src family kinases<sup>236,238</sup>, c-Cbl can be phosphorylated Syk after integrin engagement on phagocytes<sup>215,239-242</sup>. It was shown that c-Cbl was associated with Src/Pyk2 complex and that the phosphorylation of c-Cbl by Src kinase was dependent on Pyk2 in osteoclasts<sup>242</sup>. C-Cbl deficient macrophages were defective in adhesion to fibrinogen-coated surface, emphasizing the importance of c-Cbl in integrin-mediated adhesion<sup>215</sup>. Also, c-Cbl plays a role in macrophage chemotaxis<sup>243</sup>. C-Cbl, as an adaptor protein, associates with a large pool of other proteins, including Src kinases, Syk, Pyk2, SLP-76, Vav, and PI3 kinase<sup>235</sup>. It was shown that cell membrane translocation of PI3 kinase was compromised in the absence of c-Cbl in macrophages, suggesting that c-Cbl may regulate integrin-mediated adhesion through recruitment of PI3 kinase to cell membrane<sup>215</sup>.

Whether c-Cbl is required for activation of Vav and SLP-76 in neutrophils and macrophages has not been documented yet.

## **Regulation of integrin signaling in phagocytes by adaptor protein**

### **PRAM-1**

PML-RARa target gene encoding an Adaptor Molecule-1 (PRAM-1) was identified by subtraction cloning from acute promyelocytic leukemia cells in 2001<sup>244</sup>. As an intracellular protein, PRAM-1 is highly expressed in the NB4 cell line after All-Trans-Retinoic Acid (ATRA) treatment, and it is predominantly expressed in neutrophils. PRAM-1 encodes a protein of 683 amino acids with 97 kDa molecular weight. As an adaptor protein, PRAM-1 shares high homology (57% similarity) and similar protein structures with another adaptor protein ADAP<sup>244</sup>. PRAM-1 has an N-terminal proline-rich domain containing multiple type I (RXPXXP) and type II (PXXPXR) SH3 recognition motifs, a proline rich region in the center, and a C-terminal SH3-like domain, plus several tyrosine residues<sup>244</sup>. Similar to ADAP, PRAM-1 associates with SKAP55-hom through the proline-rich domain constitutively and binds to SLP76 in an inducible manner. PRAM-1 does not bind to Src kinase member Lyn but not Fyn through SH3-like domain; the latter associates with ADAP. A recent study demonstrated that PRAM-1 interacted with the SH3 domain of HIP55, which has been implicated in regulation of c-JNK activity<sup>245</sup> in arsenic trioxide treated NB4 APL cell line. Whether PRAM-1 regulates the activity of HIP55 and c-JNK in primary neutrophils, and whether this regulation has any effect on integrin mediated oxygen burst, are still unknown.

PRAM-1-deficient mice have been generated, and they are viable and fertile. The development of neutrophils is normal in terms of cell number, morphology with giemsa staining, and the expression of integrin and Fc receptors in PRAM-1-deficient mice<sup>246</sup>. Normal Ca<sup>2+</sup> flux and oxygen burst were detected after Fc receptor ligation in those cells<sup>246</sup>. Although the adhesion to integrin ligand fibrinogen was normal, PRAM-1-deficient neutrophils were defective in integrin-mediated oxygen burst and degranulation<sup>246</sup>. The activation of signaling components, including Erk, P38, Vav, and Pyk2 were normal in the absence of PRAM-1<sup>246</sup>. Hence the molecular mechanism regulated by PRAM-1 remains mysterious.

## **CHAPTER 4**

### **REGULATION OF INTEGRIN SIGNALING IN NEUTROPHILS BY ADAPTOR PROTEINS PRAM-1 AND C-CBL**

#### **Introduction**

As the first line of defense, neutrophils are critical in clearance of bacteria and fungi, and in the initiation of adaptive immune responses. Integrins play essential roles in neutrophil migration from blood vessels into inflammation sites, phagocytosis of pathogenic particles, and subsequent ROI generation<sup>199</sup>. ROI is one of critical weapons for neutrophils to kill pathogenic microbes; loss of capacity to produce ROI results from mutations in NADPH complex in GCD patients who suffer from severe, recurrent bacterial and fungal infections<sup>247</sup>. Integrin activation is regulated in a two-signal model. First, ligation of a cell surface receptor such as Fc receptor or TNF $\alpha$  receptor can give an intracellular signal leading to integrin conformation change and clustering (inside-out signal). Second, activated integrins bind to their ligands and initiate downstream signal cascades (outside-in signal)<sup>167</sup>.

Several key molecules have been implicated in integrin-mediated adhesion and ROI generation in neutrophils. For instance, Src kinases (Fgr/HCK/Fyn), Syk, Vavs, SLP76, c-Cbl, and PRAM-1 are all required for cell adhesion to integrin ligands and ROI generation<sup>176,199,209,219,232,248</sup>. Of these modules downstream of integrins, SLP76, c-Cbl, and PRAM-1 are three adaptor proteins, although c-Cbl can also function as an ubiquitin ligase. Evidence from macrophages also suggested a positive function of c-Cbl, as a substrate of Src kinases, in integrin mediated adhesion

<sup>215,242,248,248</sup>. But the role of c-Cbl in neutrophil adhesion has not been documented yet although several studies showed phosphorylation of c-Cbl upon ligation of integrin on neutrophils <sup>215,239-242</sup>. Moreover, whether c-Cbl is required for integrin-mediated ROI generation by neutrophils is still unknown. It was recently demonstrated that PRAM-1-deficient neutrophils were normal in adhesion to integrin ligands but defective in integrin-mediated ROI generation <sup>246</sup>, suggesting selective regulation of integrin signaling pathways by PRAM-1. However, how PRAM-1 specifically regulates integrin-mediated oxygen burst remains unexplored.

In order to understand the role of adaptor proteins in signal transduction, we sought to investigate the molecular mechanisms of integrin signaling in neutrophils regulated by PRAM-1. Activity of Src and Syk family kinases is required for integrin signaling, and PRAM-1 is tyrosine phosphorelated after stimulation with pervanadate. Thus, we initially hypothesized that PRAM-1 as, an adaptor protein, forms a complex with tyrosine phosphorylated proteins after integrin ligation. To test this, we performed immunoprecipitation assay and demonstrated that a protein of 120 kDa weight co-immunoprecipitated by PRAM-1 is tyrosine phosphorylated after integrin ligation. Further we found that another adaptor protein, c-Cbl, accounts for the majority of this pp120. With a genetic approach, we demonstrated that c-Cbl is required for integrin-mediated ROI generation by neutrophils. Additionally, we observed that c-Cbl is required for Fc receptor dependent Ca<sup>2+</sup> flux. The findings of c-Cbl involvement in integrin-dependent ROI generation and Fc receptor mediated Ca<sup>2+</sup> flux by neutrophils indicate that c-Cbl plays

totally opposite roles downstream of different receptors on the same cell type.

## **Materials and methods**

### **Mice**

C-Cbl deficient mice were purchased from Taconic farm and housed in a specific pathogen free environment under regulation of the Institutional Animal Care and Use Committee at the University of Minnesota.

### **Antibodies and flow cytometry**

Gr-1 APC (clone RB6-8c5, ebioscience), CD11b-PE (M7/4, ebioscience), CD11c-fitc (clone HL3, BD pharmingen,) CD18-fitc (clone C71/16, ebioscience), CD16/CD32 (2.4G2, lab made). Surface expression of antigens was determined by flow cytometry.

### **Wright-Giemsa staining**

100 ul purified neutrophils of 1 million/ml was plated on a glass slide by side-spin. Staining was performed with dipping into solution I, II, III ten times for each, followed by gentle washing. After air drying, samples were sealed with permanent mounting and photos were taken under 10 x oil power.

### **Arthritis evaluation**

150ul of K/NxB serum was I.P. injected on day 0, followed with a second injection on day 3. Clinic score was evaluated daily, based on evidence of redness and swelling, using a scale ranging from 0-4<sup>249</sup>. Thickness of ankles of hind legs was measured by caliper, and data are

presented as the mean of individual ankle thicknesses within a group of mice (3–6 mice per group).

### **In vivo migration assay**

5-6-week-old mice are injected with 1 ml of 3% (m/v) thioglycollate per mouse, and leukocytes were collected at different time points as indicated. To collect cells, 30 ml PBS per mouse was used to lavage the peritoneum. Neutrophil numbers were determined with Gr-1<sup>hi</sup> population by flow cytometry.

### **Neutrophil isolation**

Bone marrow cells from femur and tibia were depleted of red cells with one-minute incubation in ACK lysis buffer and neutralized with HBSS prep (5.4 mM KCl, 0.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.8 mM KH<sub>2</sub>PO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 137 mM NaCl, 5.6 mM dextrose, 20 mM HEPES). Neutrophils were separated in percoll gradient at 2000 RPM for 30 minutes and harvested from the interface of two layers. (Percoll was first balanced with 10X PBS in a ratio of 9:1. 62.5% Percoll top layer: 6.25 ml balanced Percoll plus 3.75 HBSS prep, 81% Percoll bottom layer: 8.1 ml balanced Percoll plus 1.9 HBSS prep, Cells were resuspended in 62.5% percoll.) Purity was checked with Gr1 expression by flow cytometry.

### **Measurement of Fc receptor mediated Ca<sup>2+</sup> flux**

Neutrophils of 10<sup>7</sup>/ml were resuspended in HBSS prep containing 3µg/ml of indo-1 (molecular probes, Eugene, Oreg) and 4 mM probenecid and incubated at 37° C for 20 minutes. After two washes with HBSS prep, cells were stained with 2µg/ml 2.4G2 (anti-Fc<sub>γ</sub>RII/III antibody) in PBSg buffer (125 mM sodium chloride, 8 mM sodium phosphate, 2 mM sodium phosphate monobasic, 5 mM potassium chloride, 5 mM glucose) for 15

minutes at 4° C, followed with three washes. Neutrophils were resuspended in ice-cold PBSg at 10<sup>7</sup>/ml. Right before stimulation, neutrophils were diluted into 0.5 million/ml KRP buffer (PBSg, 1 mM **Ca**<sup>2+</sup> chloride, 1.5 mM magnesium chloride) and warmed up in 37° C water bath. Fc receptor was cross-linked by rabbit anti-rat immunoglobulin G at different dosages as indicated right before cells were run through flow cytometry. Fluorescences by free indo-1 and **Ca**<sup>2+</sup> chelated indo-1 were recorded by LSR flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, California). **Ca**<sup>2+</sup> flux was indicated with the ratio of free and chelated indo-1.

### **Adhesion oxygen burst**

96-well immulon 4 HBX plates (Thermo Lab systems) were coated with 150ug/ml of fibrinogen (Sigma F-9754), 15ug/ml of poly-RGD (Sigma f-8141) or 20ug/ml of ICAM-Fc recombinant in carbonate buffer at 4° C overnight. Before assay, wells were blocked with FCS 10% for 30-120 minutes and washed with PBS three times. Neutrophils of 2-4 x 10<sup>6</sup>/ml were resuspended in HBSS containing 10mM HEPES, 100mM ferricytochrome c (Sigma), 0.5 mM Mg<sup>2+</sup>, 0.5 mM Ca<sup>2+</sup>. After 5 minutes of warming in a 37° C water bath, 100ul cell resuspension was added to each well. For some conditions, 50ng/ml of murine TNF $\alpha$ , 10uM fMLP, 100 nM PMA were added. ROI was measured by through oxidation-induced changes in ferricytochrome c absorbance.

### **Immunoprecipitation and western blot**

For co-immunoprecipitation assay, NB4 cells were treated with all trans-retinoic acid for 3 days. The NB4 cells were stimulated stained with serum (1:10) containing antibodies anti integrin  $\beta$ 1 (Ts2/16) or  $\beta$ 2

(Ts2/22) on ice for 30 minutes. After being washed with cold PBS, the NB4 cells were resuspended in cold PBS containing rabbit anti-mouse secondary antibody. Cells were warmed at 37° C and collected at different time points. Then NB4 cells were lysed in 1% NP40 lysis buffer containing protease inhibitors (Roche, Indianapolis) and 1 mM PMSF on ice for 10 minutes, followed by centrifugation at 14000 RPM for 10 minutes to remove insoluble debris. Supernatant then was tumbled with GammaBind Plus Sepharose beads (Life Science, New Jersey) conjugated with anti-human PRAM-1 antibody (10ul sheep anti-hPRAM-1 serum and 5ul beads resuspended in 1% NP40 lysis buffer are used for conjugation for 2 hours at 4° C) at 4° C overnight. After three washes, beads were resuspended in reducing buffer which was subject to SDS-PAGE resolution.

### **Western blot**

For coating, a 6 well plate was coated with 15ug/ml of poly RGD or 5% BSA in carbonate buffer at 4° C overnight and washed with PBS 3 times. Neutrophils at 10 million/ml were resuspended in HBSS prep with 0.5 mM Ca<sup>2+</sup> and 0.5 mM Mg<sup>2+</sup> and then 0.6 ml cell suspension was added into each well. The plate was floated on a 37° C water bath; at different time points, neutrophils were recovered and lysed in 50 ul radioimmunoprecipitation assay-based buffer. For PMA stimulation, resuspended cells were stimulated with PMA 100 nM for 10 minutes and lysed after being collected. Insoluble debris was removed with high speed spinning (14000 rpm) for 10 minutes. Cell lysate were mixed with 5x reducing buffer (0.5 M Tris-HCl [pH 6.8], 277 mM sodium dodecyl sulfate, 40% glycerol, 20% 2-mercaptoethanol, 1% bromophenol blue) and

boiled for 7 minutes. After being separated by SDS PAGE gel, the samples were transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% bovine serum albumin in PBS for one hour, then probed overnight with the appropriate antibody diluted in PBS containing 0.2% tween and 5% bovine serum albumin. After three 10-minute washes with PBS, antibody binding was detected by IRdye conjugated secondary antibody (Bio-Rad) and Licor Odyssey system.

## **Results**

### **A p120 coimmunoprecipitated with PRAM-1 is phosphorylated after integrin activation.**

To test if PRAM-1 regulates integrin signaling through tyrosine phosphorylation cascade, we performed an immunoprecipitation assay with NB4 cells. NB4 cells are human promyelocytic leukemia tumor cells that can differentiate towards a neutrophil like phenotype after All-Trans-Retinoic Acid (ATRA) treatment<sup>244,250</sup>. NB4 cells treated with ATRA for three days were stimulated with integrin ligands, lysed and subjected to immunoprecipitation with anti-PRAM-1. After SDS PAGE separation and transfer onto a PVDF membrane phosphorylation was detected with 4G10 antibody. After  $\beta$ 1 integrin stimulation, a strong tyrosine phosphorylated band at 120 kDa position was detected at 2 and 5

minutes and declined at 20 minutes with crosslinking antibody (Fig. 4.1). Similarly,  $\beta 2$  integrin stimulation also generated a phosphorylated 120 kDa band at 2 minutes and slightly decreased at 5 minutes (Fig. 4.1). This findings suggest that a protein coimmunoprecipitated with PRAM-1 and this protein was phosphorylated after integrin ligation in ATRA treated NB4 cells.

### **C-Cbl accounts for the majority of pp120 co-immunoprecipitated by PRAM-1.**

To identify the pp120 coimmunoprecipitated by PRAM-1, we screened several potential candidates of 120 kDa molecular weight (data not shown). Finally we identified this pp120 as c-Cbl. ATRA-treated NB4 cells were subjected to anti-PRAM-1 IP with or without integrin stimulation, then immunoblotted with anti-c-Cbl antibody, a strong band at 120 kDa position was detected, suggesting an association of c-Cbl and PRAM-1 (Fig. 4.2A). There was no increase in the amount of , c-Cbl coimmunoprecipitating with PRAM-1 after integrin stimulation, suggesting a constitutive association between these two proteins (Fig. 4.2A). To determine whether c-Cbl accounts for the majority of pp120 immunoprecipitated by PRAM-1, NB4 lysates were first depleted of c-Cbl anti-c-Cbl antibody coated beads. The lysate was then used for immunoprecipitation with anti PRAM-1, followed by immunobolt for tyrosine phosphorylation with 4G10 antibody. Very little pp120 was detected in PRAM-1 co-IPs from c-Cbl depleted lysates (Fig. 4.2B). Instead, a strong phosphorylated protein of 120 kDa weight was detected in condition containing elute from c-Cbl depletion beads (Fig. 4.2B).

together, these data show that c-Cbl constitutively associates with PRAM-1, and accounts for the majority of pp120 co-immunoprecipitated by PRAM-1 in ATRA-treated NB4 cells.

### **C-Cbl is required for integrin mediated ROI generation in neutrophils.**

Previous studies demonstrated that c-Cbl is required for macrophage spreading and adhesion to integrin ligands, and that PRAM-1 is required for integrin mediated ROI generation in neutrophils<sup>246</sup>. Given the physical association of PRAM-1 and c-Cbl, we asked if c-Cbl is also involved in ROI generation in neutrophils. To do this, we performed ROI assays with purified neutrophils stimulated with integrin ligands. When the neutrophils were stimulated with  $\beta 2$  specific ligand, fibrinogen plus a second stimulation such as  $\text{TNF}\alpha$  (Fig. 4.3A), or fMLP, (Fig. 4.3B), again c-Cbl deficient cells were significantly defective in ROI generation compared with wild-type controls. To more specifically ask if c-Cbl regulates outside-in signals downstream of integrin, neutrophils were plated on the integrin-specific ligand poly-RGD<sup>219</sup>. Stimulated by poly-RGD, wild-type neutrophils generated significant ROI (Fig. 4.3C). In contrast, c-Cbl-deficient cells showed dramatically reduced ROI production (Fig. 4.3C). The same observation applied to stimulation ICAM-1, the ligand for FLA-1 (Fig. 4.3D). In contrast, c-Cbl deficient cells showed normal ROI generation with PMA stimulation (Fig. 4.3E). Together, these data strongly suggest that c-Cbl is selectively required for integrin-mediated ROI generation.

### **C-Cbl deficient neutrophils display normal development.**

A recent study showed that C-Cbl is required for optimal development of myeloid cell precursors<sup>251</sup>. To rule out the possibility that the defective ROI generation is due to abnormal development of c-Cbl deficient neutrophils, we stained neutrophils with Wright-Giemsa staining to examine the morphology of nucleus. Mature neutrophils are characterized by the presence of large and multi-lobed nuclei. Wright-Giemsa staining of blood samples showed normal morphology of c-Cbl deficient neutrophils compared with wild-type cells (Fig.4.4A). Another possibility for the decreased ROI generation by c-Cbl deficient neutrophils is altered surface expression of integrins. To test this, we stained Gr1<sup>hi</sup> cells for CD18 ( $\beta$ 2), CD11a (LFA-1 subunit), and CD11b (mac-1 subunit). C-Cbl deficient neutrophils showed comparable expression of CD11a, CD11b, and CD18 compared to controls (Fig. 4.4C). Considering the crosstalk between Fc receptors and integrins<sup>189-191</sup>, we also evaluated the expression of Fc $\gamma$ II, III receptors with 2.4G2 staining. C-Cbl deficient neutrophils also showed normal expression of Fc $\gamma$ II and III receptors (Fig. 4.4C). From these data, we conclude that impaired ROI generation by c-Cbl deficient neutrophils is not due to gross alterations in phenotype or integrin expression.

### **C-Cbl deficient neutrophils show normal integrin-dependent activation of P38 and Erk.**

To understand the mechanisms of integrin-mediated ROI generation by neutrophils regulated by c-Cbl, we evaluated the activation of signaling components downstream of integrins. Previous studies

showed that integrin ligation on neutrophils can activate Erk through the Rac/Raf pathway<sup>215,252</sup>. First we examined the activation of Erk in neutrophils. Integrin stimulation by poly RGD for 15 or 30 minutes showed a strong band of phosphorylated Erk in wild-type neutrophils compared to the BSA stimulation control (Fig. 4.5A). However, activation of Erk even was slightly increased in c-Cbl deficient cells (Fig. 4.5A). Since stress activated mitogen activated kinase p38 is required for integrin dependent adhesion and oxygen burst<sup>159</sup>, we tested whether the activation of p38 is altered in the absence of c-Cbl. With immunoblot assay, comparable P38 phosphorylation was detected with lysate from c-Cbl deficient neutrophils compared with wild-type cells (Fig. 4.5B). These data suggest that impaired ROI generation is not due to impaired Erk and p38 activation.

### **C-Cbl is required for Fc receptor dependent Ca<sup>2+</sup> flux in neutrophils.**

Since Fc receptors and integrins share similar signaling components like Syk and DAP12/Fc<sup>253</sup>, and cooperate in multiple neutrophil activities<sup>189</sup>, we wanted to ask if c-Cbl is involved in FcR signaling. Ca<sup>2+</sup> modulation is a well established result of FcR crosslinking, we evaluated c-Cbl function downstream of FcR with Ca<sup>2+</sup> flux as readout. After neutrophils were labeled with endo-1 and stained with 2.4G2 antibodies against Fc receptors, followed by crosslinking, flow cytometry revealed a dosage-dependent response of Ca<sup>2+</sup> flux in wild-type neutrophils. Surprisingly, c-Cbl deficiency resulted in enhanced Fc receptor dependent Ca<sup>2+</sup> flux in neutrophils. With low dosages of 4 and 6

ug/ml 2.4G2 antibodies, no significant  $\text{Ca}^{2+}$  flux was detectable in wild-type cells. In contrast, the lowest dosage of 4 ug/ml 2.4G2 antibodies induced detectable  $\text{Ca}^{2+}$  flux and 6 ug/ml 2.4G2 triggered a strong  $\text{Ca}^{2+}$  spike in c-Cbl deficient neutrophils (Fig. 4.6). As a conclusion, c-Cbl negatively regulates Fc receptor-dependent  $\text{Ca}^{2+}$  flux in neutrophils.

### **C-Cbl is not required for neutrophil migration in vivo.**

Integrins have a critical function in cell migration. Especially in leukocyte translocation from blood flow into the inflammation tissue, integrins are required for leukocytes to migrate through the endothelial barrier<sup>171</sup>. To ask if c-Cbl is required for neutrophil migration, we evaluated thioglycollate-mediated recruitment of neutrophils to the peritoneum. Thioglycollate can cause tissue damage and initiate an inflammation response, which recruits leukocytes to the injection sites<sup>254</sup>. Based on the cell number collected at injection sites, cell migration in vivo can be evaluated. After injection of 1 ml of 3% thioglycollate into peritoneum, leukocytes were harvested from peritoneum at different time points of 2, 6, or 20 hours. At the 2-hour point, peritoneal lavage resulted in an average of 0.5 million of neutrophils both in c-Cbl wild-type and deficient mice (Fig. 4.7). At the 6-hour time point, the numbers of neutrophils migrating to the peritoneum dramatically increased to 7 million in wild-type mice and 5 million in c-Cbl-deficient animals (Fig. 4.7). Neutrophil numbers (4 million for wild-type, 3 million for c-Cbl-deficient mice) collected from inflammation sites at the 20-hour time point were slightly decreased compared with the 6-hour time point (Fig. 4.7). There was no statistically significant difference between c-Cbl

deficient and control animals in numbers of migrating neutrophils at the time points examined, although c-Cbl deficient neutrophils showed a trend toward reduced migration. These observations suggest that c-Cbl is not required for neutrophil migration in vivo.

### **C-Cbl deficient mice showed normal K/NxB serum induced arthritis.**

K/NxB mice are a animal model for study of neutrophil dependent inflammatory arthritis<sup>255</sup>. K/NxB mice are NOD/B6 F1 offspring with transgene TCR KRN specific for bovine RNase (42-56) expressed on T cells. These mice develop spontaneous arthritis between 3 and 5 weeks of age. An attractive feature of these mice is that their serum can induce arthritis once injected into recipient mice. Since we showed that c-Cbl has an essential role in neutrophil function, we asked whether c-Cbl is required for K/NxB serum induced arthritis. For this purpose, we injected c-Cbl wild-type and deficient mice with 150ul K/NxB serum on day 0 and again on day 3. Arthritis was measured by joint thickness and clinical scores. Overall, c-Cbl deficient mice showed slightly but not statistically significant reduced serum-induced arthritis compared to controls(Fig. 4.8). These data suggest that c-Cbl is not required for K/NxB serum-induced arthritis (Fig. 4.8).

### **Discussion**

This study shows that c-Cbl associates with PRAM-1 in NB4 cells and is tyrosine phosphorylated upon integrin ligation. Further, similar to

neutrophils lacking PRAM-1, c-Cbl-deficient neutrophils are defective in integrin-mediated reactive oxygen species generation. In contrast, c-Cbl-deficient neutrophils showed dramatically enhanced  $\text{Ca}^{2+}$  flux after ligation of Fc receptors, indicating a negative regulatory role for c-Cbl in FcR mediated  $\text{Ca}^{2+}$  flux. These data suggest that c-Cbl plays a positive role downstream of integrin but a negative role downstream of Fc receptors. Impaired ROI generation by c-Cbl deficient neutrophils is not due to cell surface expression of integrins (and Fc receptors as well). Moreover, c-Cbl is dispensable for thioglycollate-mediated neutrophil migration in vivo. The molecular mechanism and biological significance remains to explore.

Previous studies demonstrated that c-Cbl functions as a positive regulator downstream of integrin<sup>215,239,240,243</sup>. In line with this, our ROI data clearly show that c-Cbl plays a positive role downstream of integrins, which is consistent with previous findings<sup>215,242,248,248</sup>. C-Cbl deficient macrophages were demonstrated defective in adhesion to integrin<sup>215</sup>. It is possible that impaired ROI generation by c-Cbl deficient neutrophils may be due to defective adhesion. However, normal activation of Erk and P38 in c-Cbl deficient neutrophils plated on poly RGD coated surface suggests sufficient engagement of integrins on c-Cbl deficient cells. So it is less likely that defective ROI generation by c-Cbl deficient neutrophils is due to defective adhesion. Although c-Cbl is required for macrophage adhesion<sup>215</sup>, it is still not clear if c-Cbl is required for adhesion by neutrophils. Hence, adhesion assay should be performed to test if c-Cbl is required for neutrophil adhesion.

It was demonstrated that c-Cbl mediated cell adhesion through regulation of cell membrane trans-location of PI3 kinase<sup>215</sup>, whether this is the case for integrin mediated ROI generation is not characterized yet. To answer this question, we examined ROI generation by P110 $\gamma$  (a subunit of PI3 kinase) deficient neutrophils with integrin specific ligand poly RGD stimulation and failed to detect any defective ROI generation by P110 $\gamma$  deficient cells compared to wild type control cells (data not shown). Therefore, c-Cbl does not regulate PI3 kinase activity for integrin dependent ROI generation. Previously, multiple c-Cbl associated proteins including Syk, SLP-76, Vav and PLC $\gamma$ -2 were also implicated on neutrophil adhesion and integrin dependent oxygen burst<sup>174,219,232</sup>. To identify the signaling pathway regulated by c-Cbl downstream of integrins, the activity of SLP-76, Vav, PLC $\gamma$ -2 in c-Cbl deficient cells is under investigation.

Since we identified the association of c-Cbl and PRAM-1 in NB4 cells, which is a human leukemia cell line, whether c-Cbl binds to PRAM-1 in mouse primary neutrophils is still unknown. To this end, immunoprecipitation assay should be performed with mouse neutrophils. With human version of PRAM-1, our preliminary data owed that c-terminal (313-583) of PRAM-1 is critical for the association with c-Cbl (data not shown). Further mapping is required to define the binding site(s) of PRAM-1 to c-Cbl. Although both PRAM-1 and c-Cbl are required for integrin mediated ROI generation by neutrophils, whether this association is critical for the integrin signaling in ROI generation is not determined yet. To evaluate the biological significance of c-Cbl/PRAM-1 association, ROI rescue assay serves for this purpose. If this association

of c-Cbl/PRAM-1 is critical for integrin-mediated ROI generation, we expect that neutrophils isolated from PRAM-1 mutant (lacking binding activity to c-Cbl) reconstituted chimeras will not generate sufficient ROI upon integrin stimulation compared to cells isolated from wild-type reconstituted controls.

Although c-Cbl and its homolog protein Cbl-b play a redundant role in several aspects of immune responses, such as the development of B and T cells <sup>256,257</sup>, evidence also indicate that these two proteins may function differently. For instance, c-Cbl-deficient mice showed more a profound problem in the development of thymocytes than Cbl-b-deficient mice, while Cbl-b is more important for peripheral T cell function <sup>235</sup>. To test if Cbl-b is also involved in integrin-mediated ROI generation by neutrophils, we performed an oxygen burst assay and failed to detect any defect of ROI generation in Cbl-b deficient cells, suggesting that, unlike c-Cbl, Cbl-b is not required for ROI generation (data not shown).

With consideration of impaired ROI production in c-Cbl deficient neutrophils, the increased  $Ca^{2+}$  flux in c-Cbl deficient neutrophils stimulated by 2.4G2 antibody provokes great interest to ask if c-Cbl play opposite functions of different receptors in neutrophils: positive downstream of integrin (impaired ROI generation) and negative downstream of Fc receptors. It is shown that c-Cbl defective mast cells showed comparable, or slightly decreased  $Ca^{2+}$  flux triggered by ligation of high-affinity IgE receptors <sup>258</sup>, further experiment should be taken to differentiate which Fc receptor is regulated by c-Cbl. Further investigation should be taken to elucidate the molecular mechanism regulated by c-Cbl downstream of Fc receptors. It is especially interesting to compare the

regulation by c-Cbl of signaling downstream of integrin and Fc receptor. Since Fc receptor engagement can also trigger oxygen burst and degranulation, more assays should be used to address the significance of enhanced Fc receptor mediated  $Ca^{2+}$  flux in c-Cbl deficient neutrophils. It is interesting to know how c-Cbl regulates integrin-triggered  $Ca^{2+}$  flux in neutrophils. Moreover 2.4G2 antibody recognizes  $Fc\gamma RII$ ,  $Fc\gamma RIII$  receptors and  $Fc\gamma RII$  is subdivided into  $Fc\gamma RIIA$ , B and C, of which  $Fc\gamma RIIB$  bears an inhibitory signaling motif ITIM, while  $Fc\gamma RII$  A and C harbor an ITAM motif in their cytoplasmic domains <sup>259</sup>. So one of interesting question is to ask which isoform of  $Fc\gamma Rs$  is regulated by c-Cbl.

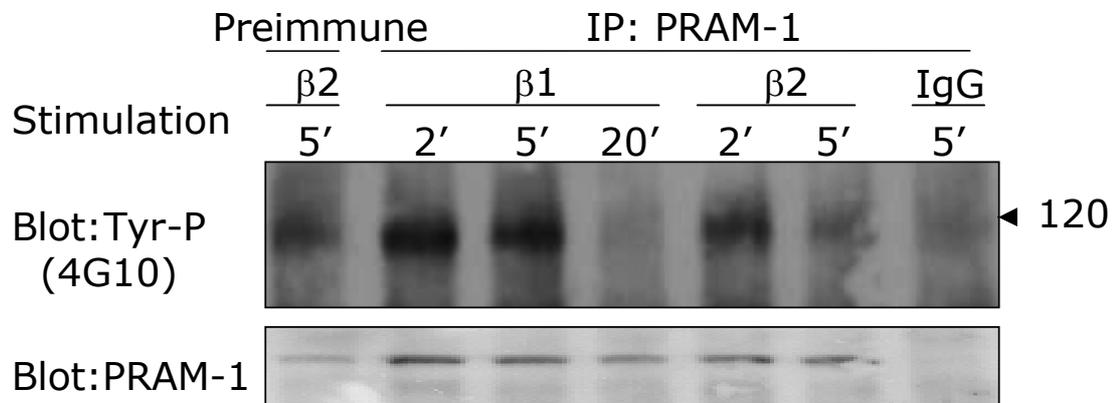
It was shown that loss of Cbl-b, but not c-Cbl, resulted in dramatic enhanced  $Ca^{2+}$  flux upon ligation of  $Fc\epsilon RI$  in mast cells, suggesting a differential requirement of c-Cbl and Cbl-b in  $Ca^{2+}$  flux downstream of Fc receptor <sup>258</sup>. In contrast, c-Cbl deficient neutrophils showed a dramatic defect in  $Ca^{2+}$  flux upon  $Fc\gamma RII$ ,  $Fc\gamma RIII$  receptor ligation compared with wild-type control cells. This difference of  $Ca^{2+}$  flux observations raises an intriguing question: Do c-Cbl and Cbl-b function differentially downstream of different Fc receptors? To answer this question, further characterization of  $Ca^{2+}$  flux in Cbl-b deficient neutrophils is required. Additionally, compared to the positive role of c-Cbl downstream of integrin in term of ROI generation, c-Cbl negatively regulates Fc receptor signaling.

Our in vivo migration data suggest that c-Cbl is dispensable for neutrophils to migrate in vivo, although a slightly reduced number of neutrophils were recovered from c-Cbl deficient peritoneum. However, a previous study indicated that c-Cbl is required for chemotaxis of

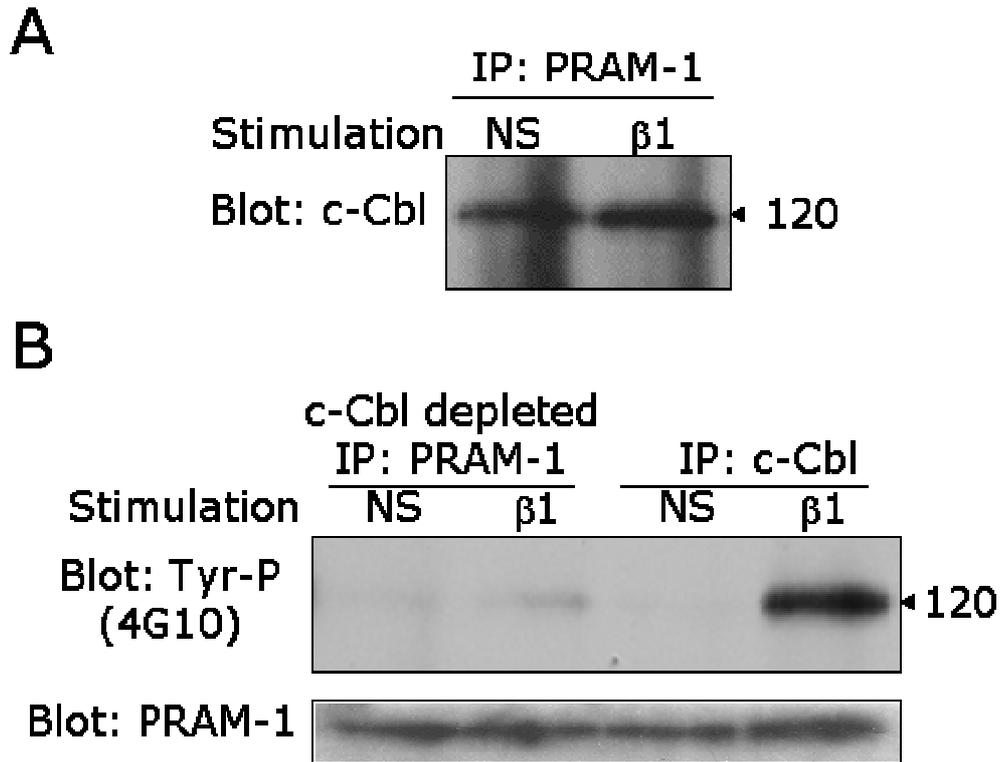
macrophages<sup>243</sup>. This discrepancy can be explained either with the difference of in vivo and in vitro systems or with difference of cell types. There are two caveats in our studies: 1) only two mice were tested for each condition, 2) migration was evaluated in the whole straight mice in which other factors may compensate and mask the migration defect in c-Cbl deficient mice (e.g. cytokine profile in tissue and accessible neutrophils to peritoneum). In fact, the higher blood neutrophil count in c-Cbl deficient mice supports the possibility of the second cavity (data not shown). For the first caveat, more mice need to be examined. The second caveat can be resolved by co-transfer of c-Cbl-deficient and wild-type neutrophils into the same host for thioglycollate-mediated migration assay.

Clearly ADAP is required for thymocyte development. The molecular mechanisms are still mysterious, although it has been implicated that ADAP was required for positive and negative thymic selection. In peripheral T cells, ADAP is required for assembly and membrane translocation of CARMA-1/ BCL-10/MALT-1 complex<sup>44,45</sup>. One of the interesting questions is whether ADAP regulates the assembly and translocation of CARMA-1/ BCL-10/MALT-1 complex in thymocytes. Moreover, we are dissecting the signaling pathways regulated by ADAP with different mutants of ADAP. In this way, we can understand which domain of ADAP mediates thymic selection.

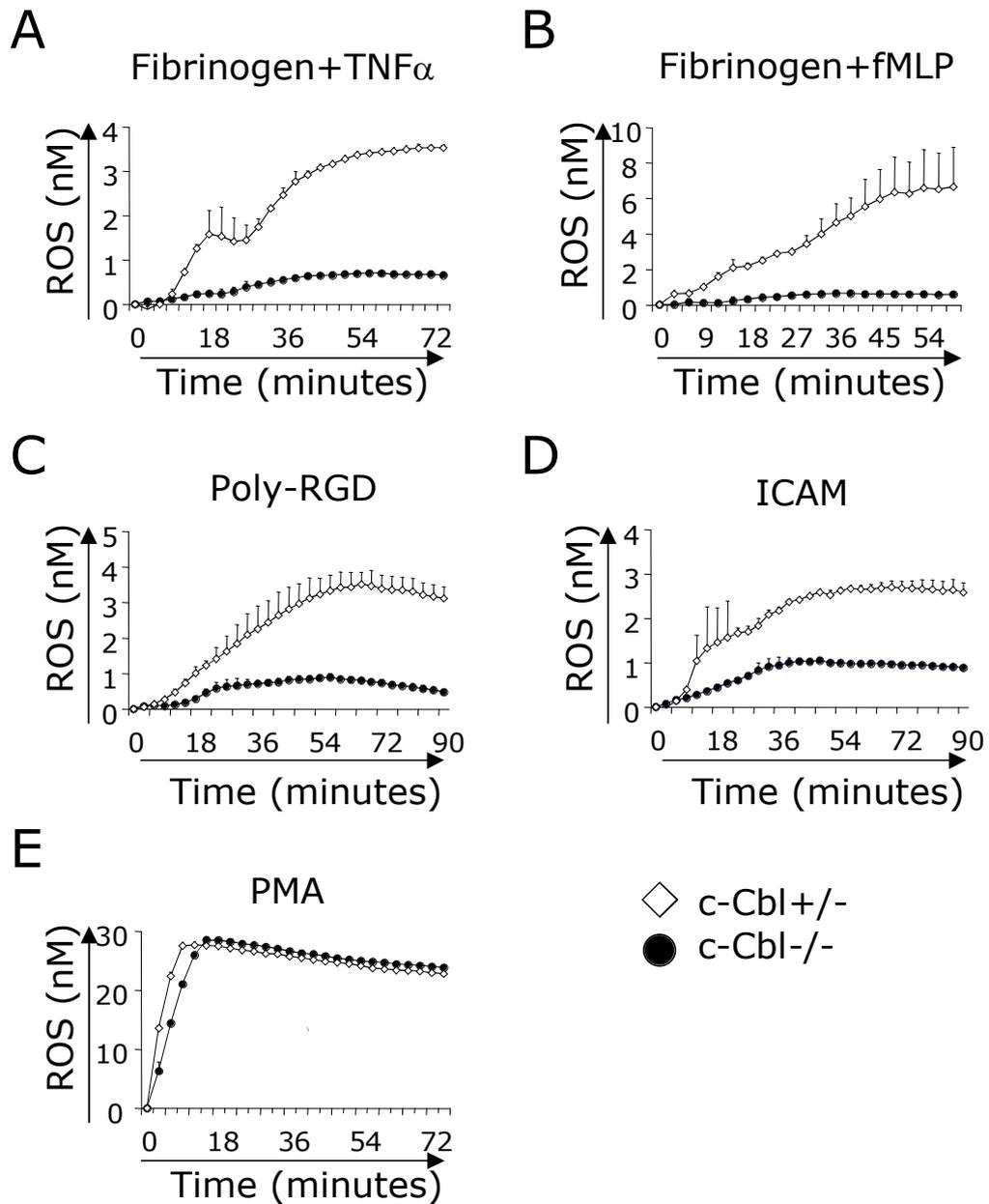
**FIGURE**



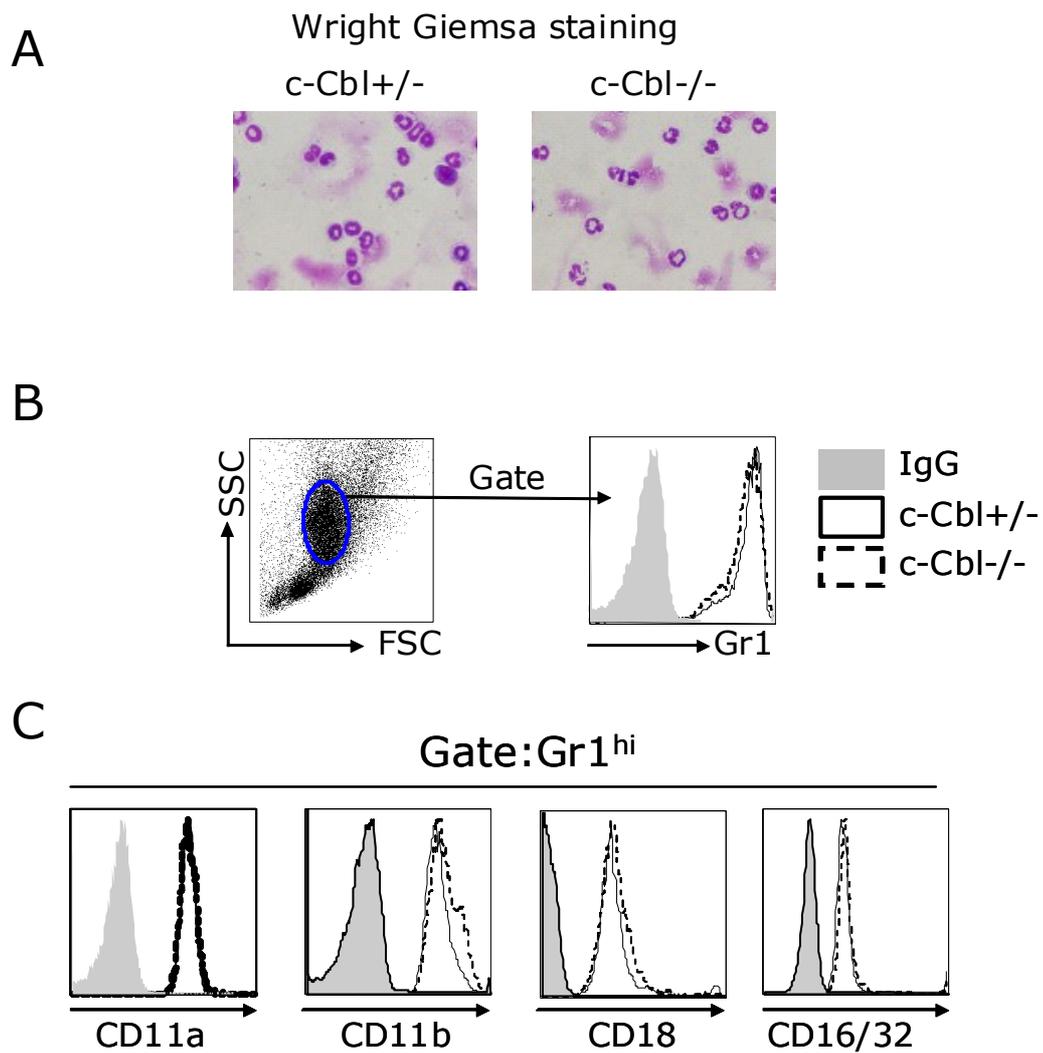
**Figure 4.1. A 120 kda phosphoprotein associates with PRAM-1 after integrin stimulation.** NB4 cells treated with all-trans retinoic acid (ATRA) for 3 days were stimulated with antibodies against integrin subunits ( $\beta 1$ ,  $\beta 2$ ) or IgG control antibodies. At different time points, cell lysates were subjected to immunoprecipitation with anti-PRAM-1 and western blot anti-phosphorylated tyrosine residue. One representative out of three experiments is shown.



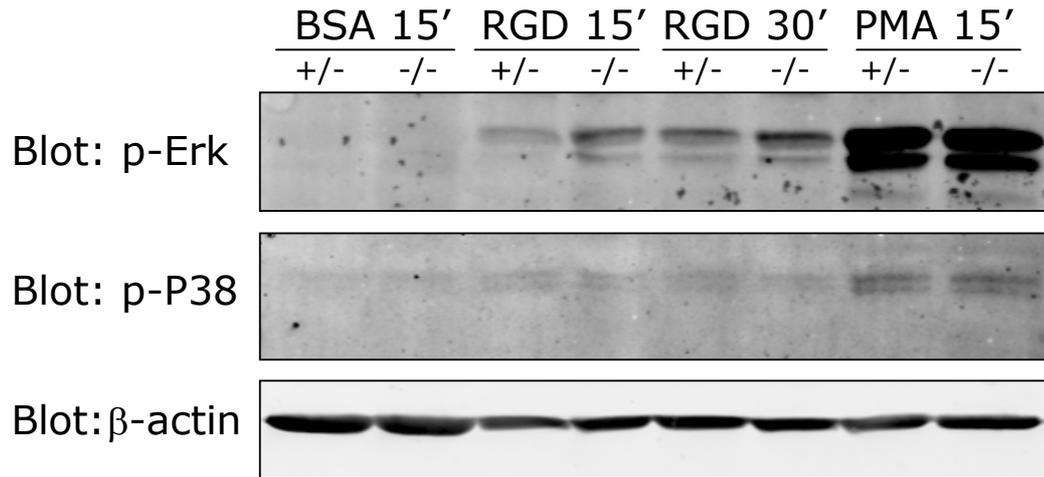
**Figure 4.2. C-Cbl constitutively associates with PRAM-1 and accounts for the majority of pp120.** A) ATRA-treated NB4 cells were subject to IP anti-PRAM-1, with or without integrin stimulation, then to western blot anti c-Cbl. B) ATRA-treated NB4 cells, with or without integrin stimulation, were depleted of c-Cbl, subject to IP anti-PRAM-1 and western blot anti phosphorylated tyrosine residue. The beads for Cbl depletion were used to blot anti phosphorylated tyrosine residue (4G10). After stripping, the membrane was blotted for PRAM-1. One representative out of three experiments is shown.



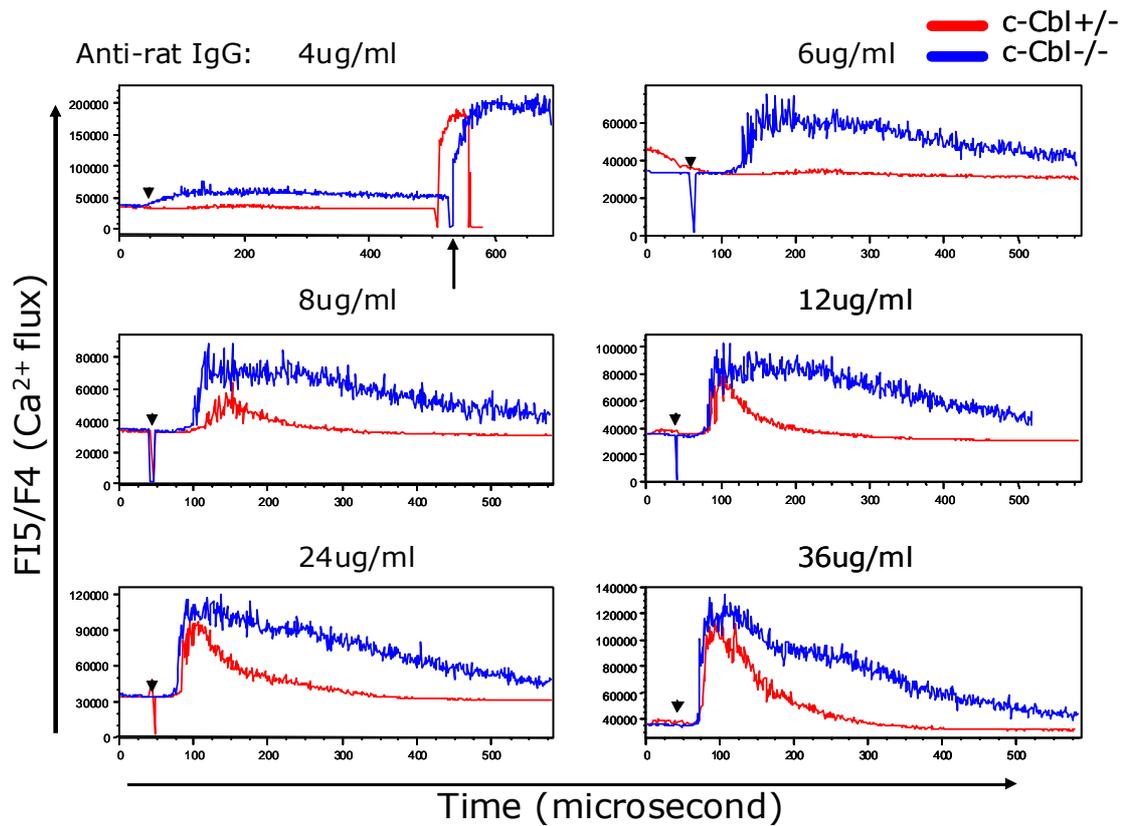
**Figure 4.3. C-Cbl is required for optimal integrin-mediated ROS production in neutrophils.** Purified bone marrow derived neutrophils resuspended in HBSS buffer containing 10mM Heps, 0.5mM Ca<sup>2+</sup>, 0,5mM Mg<sup>2+</sup> were stimulated as indicated in Immulon 4 HBX 96 well Plate and ROS was detected in oxidation induced change of ferri-cytochrome C absorbance. Stimulations: plates were coated with either 15ug/ml poly-RGD, 150ug/ml Fibrinogen or 15ug/ml ICAM recombinant overnight. For some experiments, soluble 50ng/ml TNF $\alpha$ , 10uM fMLP, 100nM PMA were applied. One representative out of at least three experiments is shown. Error bar represents deviation from triplicated wells of one condition.



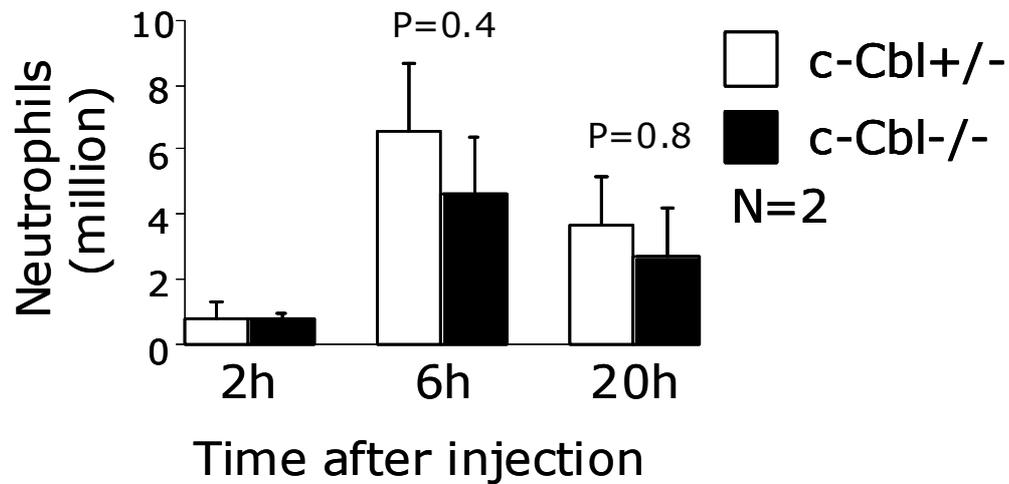
**Figure 4.4. C-Cbl deficient neutrophils show normal surface protein expression.** A) Purified neutrophils from bone marrow were stained with Wright-Giemsas stain. B) Bone marrow cells were gated for neutrophil population based on FSC/SSC as indicated and Gr1 expression is shown. C) The expression of CD11a, CD11b, CD18, CD16/32 on Gr1<sup>hi</sup> population is shown. One representative out of three experiments is shown.



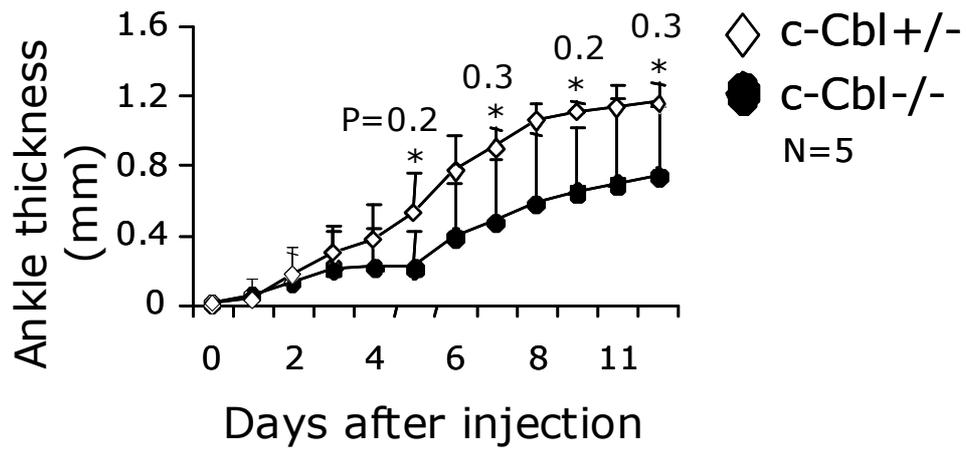
**Figure 4.5. C-Cbl is not required for integrin-mediated phosphorylation of Erk and p38 in neutrophils.** In 6 well tissue culture plates, bone marrow derived neutrophils were stimulated with plate bound polyRGD (15ug/ml for coating), BSA or soluble PMA (100nM) in HBSS buffer containing 10mM Hepes, 0.5mM Ca<sup>2+</sup>, 0,5mM Mg<sup>2+</sup>. At different time points as indicated, cells were harvested for western blot anti phospho-Erk, phospho-P38 and β-actin. One representative out of two experiments is shown.



**Figure 4.6. C-Cbl deficient neutrophils are defective in Fc receptor dependent Ca<sup>2+</sup> flux.** C-Cbl-deficient and wild-type neutrophils were labeled with Indo-1 and incubated with an antibody against Fc $\gamma$ R. Ca<sup>2+</sup> flux was measured by flow cytometry following cross-linking of the receptors with anti-rat immunoglobulin G (head arrow) and ionomycin (long arrow). One representative out of four experiments is shown



**Figure 4.7. C-Cbl is not required for thioglycollate-mediated neutrophil migration in vivo.** Age-matched mice were I.P. injected thioglycollate (1ml of 3% per mouse) and sacrificed for leukocytes collection from peritoneum at different time points as indicated. Neutrophils (Gr1<sup>hi</sup>) numbers were counted with reference beads by flow cytometry and shown. Mean (+ S.D.) neutrophil numbers are shown from two mice per condition.



**Figure 4.8. C-Cbl is not required for K/BxN serum induced arthritis.** Age-matched mice were I.P. injected with 150  $\mu$ l K/BxN serum on day 0 and again on day 3. Arthritis was monitored with ankle thickness and clinic score every day. Mean (+ S.D.) ankle thickness are derived from five mice per group.

## **FUTURE DIRECTIONS**

### **1) To investigate how environmental factor(s) regulates diabetes development in BDC2.5 g7/b mice**

Methods: With different treatments including toll like receptor ligands, bacteria, and virus, we aim to identify the treatment which can enhance diabetes in ADAP deficient BDC2.5 mice but not in controls.

### **2) To investigate molecular mechanism in integrin signaling in regulated by c-Cbl neutrophils**

Methods: Evaluate the phosphorylation of potential candidates including Vav, PLC $\gamma$ 2, SLP-76 and Pyk2 in c-Cbl deficient neutrophils with integrin stimulation.

### **3) To evaluate the significance of association of c-Cbl and PRAM-1**

Methods: First, bone marrow chimera need to be generated with PRAM-1 deficient bone marrow cells infected with a retrovirus encoding PRAM-1 mutant, which lacks the capacity of binding to c-Cbl or PRAM-1 wild-type control; Second, evaluate ROI generation of neutrophils rescued with PRAM-1 wild type and mutant.

### **4) To evaluate the role of c-Cbl in Fc receptor signaling in neutrophils**

Methods: Evaluate the ROI generation by c-Cbl deficient neutrophils with Fc receptor stimulation.

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