MERTK MEDIATES IMMUNE HOMEOSTASIS: EFFECTS UPON DENDRITIC CELL FUNCTION AND TYPE 1 DIABETES

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ABSTRACT

MARK A. WALLET: MerTK Mediates Immune Homeostasis: Effects upon Dendritic Cell Function and Type 1 Diabetes
(Under the direction of Roland Tisch, PhD)

The receptor tyrosine kinase Mer (MerTK) is expressed by dendritic cells (DC) in mice. The heretofore undefined role of MerTK in DC was investigated in two major studies. First, the role of MerTK in the inhibition of DC activation by apoptotic cells was investigated. It was found that apoptotic cells inhibited DC activation by bacterial lipopolysaccharide or CD40 crosslinking via MerTK. Inhibition of DC was characterized by blockade of IL-12p70 secretion, co-stimulatory molecule expression and stimulation of $CD4^+$ and $CD8^+$ T cells. The MerTK ligand growth arrest specific factor-6 (Gas6) was expressed on the surface of apoptotic cells, and Gas6 was essential for the inhibitory effects of apoptotic cells upon DC. Non-obese diabetic (NOD) mice, lacking MerTK expression (NOD.$MerTK^{KD/KD}$), demonstrated aberrant expansion of type 1 $CD4^+$ and $CD8^+$ T cell effectors in response to apoptotic cell death *in vivo*. Furthermore, NOD.$MerTK^{KD/KD}$ mice expressing a diabetogenic T cell receptor transgene, developed diabetes at an accelerated rate and a greater frequency than mice expressing MerTK. These findings support a role for MerTK in peripheral immune homeostasis.

Secondly, the impact of MerTK-deficiency in NOD mice was investigated. NOD.$MerTK^{KD/KD}$ mice were protected from diabetes onset and protection was due to lack of self-specific type 1 $CD4^+$ and $CD8^+$ T cell effectors. Thymic selection was enhanced in

NOD.MerTK$^{KD/KD}$ mice resulting in deletion of pathogenic, autoreactive T cells. The change in thymic selection efficiency was mediated by bone marrow-derived cells and was likely due to dysregulation of thymic DC. These data indicate that MerTK mediates immunoregulation of DC in both the periphery and the thymus.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Ab	antibody
AC	apoptotic cell
APC	antigen presenting cell
APC	allophycocyanin (used in the context of fluorescent labeled antibodies, e.g. APC-anti-Cd11c)
BM	bone marrow
BMDC	bone marrow dendritic cell
CCL	chemokine ligand
CCR	chemokine receptor
cDC	conventional dendritic cell
CFSE	carboxyfluoroscein succinimidyl ester
ConA	concavilin A
CTE	cortical thymic epithelial
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen
DC	dendritic cell
DN	double-negative
DP	double-positive
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate

FTOC	fetal thymic organ culture
GAD65	glutamic acid decarboxylase (65 kilodalton isoform)
Gas6	growth arrest specific factor-6
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony stimulating factor
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IA-2	insulinoma-associated protein 2
ICA	islet cell antibodies
Idd	insulin-dependent diabetes susceptibility locus (murine)
IFN	interferon
IGRP	islet-specific glucose-6-phosphatase catalytic subunit related protein
IL	interleukin
InsB	insulin B chain
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MTE	medullary thymic epithelial
NF-κB	nuclear factor-kappa B
NK	natural killer
NOD	non-obese diabetic
OS	outer segment
Ova	ovalbumin

pDC	plasmacytoid dendritic cell
PE	phyco-erythrin
PerCP	peridinin-chlorophyll protein
PI3K	phosphatidylinositol 3-kinase
PLN	pancreatic lymph node
PS	phosphatidylserine
PSR	phosphatidylserine receptor
RA	rheumatoid arthritis
RPE	retinal pigment epithelial
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SAV	streptavidin
sDC	splenic dendritic cell
SLE	systemic lupus erythemetosus
SP	single positive
SR-A	scavenger receptor-A
STZ	streptozotocin
T1D	type 1 diabetes
TCR	T cell receptor
TGF	transforming growth factor
TH2	type 2 T helper
TLR	toll like receptor
TNF	tumor necrosis factor

Treg	regulatory T cell
VNTR	variable number of tandem repeats
α	alpha
β	beta
β2m	β_2-microglobulin (gene written as *b2m*)
γ	gamma
μ	micro

CHAPTER I

INTRODUCTION

Type 1 Diabetes

Type 1 diabetes (T1D) is a chronic disease which manifests as dysregulation of glucose metabolism due to the loss of insulin-producing pancreatic β cells within the Islets of Langerhans. T1D is classified as either type 1A or type 1B. Type 1B diabetes is a rare disease characterized by the idiopathic loss of β cell function (Abiru et al., 2002). The more common form of T1D is Type 1A which is caused by autoimmune destruction of the β cells (Tisch and McDevitt, 1996). In both types of T1D, insulin deficiency and resultant hyperglycemia induce many chronic pathologies including diabetic nephropathy, diabetic retinopathy (Fong et al., 2004) and cardiovascular disease (Daneman, 2006). Treatment of T1D is currently achieved through subcutaneous administration of various insulin preparations. The use of fast-acting and long-acting insulins are implemented to better control blood glucose levels (Daneman, 2006) as it has been found that poor glycemic control is associated with increased frequency of complications (1993; 1994).

To date, prevention of T1D in at-risk individuals or reversal of T1D in patients has been largely ineffective. Replacement of β cell mass has been attempted through transplantation of the whole pancreas or purified-islets. Typically, whole pancreas and kidney transplants are combined and this approach has been somewhat effective in reversal of T1D. The risks,

however, of such major surgery and lifelong immune suppression are high (Sutherland, 2003; Sutherland et al., 2001). In contrast, islet transplantation carries a much lower risk, although short- and long-term insulin independence is rare due to allogeneic and auto-immune rejection of donor β cells (Balamurugan et al., 2006). Advancements in transplant efficacy will require a greater understanding of the underlying defects which contribute to autoimmunity in T1D patients.

T1D Inheritance

T1D is a complex, multifactoral disease with genetic and environmental factors contributing to susceptibility and induction (Tisch and McDevitt, 1996). Extensive study of human T1D patients and animal models, such as the non-obese diabetic (NOD) mouse, has identified several genetic loci which confer susceptibility or resistance to T1D (Anjos and Polychronakos, 2004). Additionally, evidence suggests that environmental factors play a key role in T1D development as indicated by the discordant rate of T1D onset among identical twins. Although monozygotic twins of type 1 diabetics develop diabetes at a significantly greater frequency than dizygotic twin or non-twin siblings of type 1 diabetics, the frequency of onset for monozygotic twins is less than 25%. (Redondo et al., 1999). This suggests that environmental factors contribute to β cell autoimmunity. Indeed, evidence indicates a potential role for viral infections, including Coxsackievirus B virus and rubella virus (Coulson et al., 2002; Honeyman et al., 2000), in triggering and/or exacerbating T1D (Filippi and von Herrath, 2005). Multiple factors have made it difficult to conclusively implicate viral infections or other environmental factors, such as diet and various toxins, in T1D

development. T1D patients are, for the most part, a heterogeneous population with significant genetic variability. Furthermore, subtle environmental influences, possibly occurring years before T1D diagnosis, are difficult to track.

Inheritance of T1D in humans and rodents is highly complex and, to date, not well understood. The most important locus for T1D inheritance in humans is the human leukocyte antigen (HLA) gene cluster. Specific alleles of HLA class II molecules confer 40-50% of T1D susceptibility (Anjos and Polychronakos, 2004). T1D is most often associated with specific HLA-DQ and HLA-DR β chain alleles which lack an aspartic acid residue at amino acid position 57. Substitution of alanine, valine or serine at position 57 results in altered peptide binding by those HLA class II molecules, which in turn is proposed to promote the development of pathogenic, β cell-specific T cells (Tisch and McDevitt, 1996). Inheritance of T1D in the NOD mouse is similarly conferred mostly by a major histocompatibility complex (MHC) class II I-A allele (Wicker et al., 1995). The I-A^{g7} MHC β chain molecule carries an amino acid substitution at position 57 similar to diabetogenic human HLA-DQ and HLA-DR alleles (Acha-Orbea and McDevitt, 1987; Todd et al., 1987).

In addition to HLA and MHC class II, many genetic loci in humans and mice have been associated with T1D inheritance. To date, only 2 additional human genes have been identified among the many candidate genes within the susceptibility loci. Allelic variants of cytotoxic T lymphocyte antigen 4 (CTLA-4) and alterations in the variable number of tandem repeats (VNTR) upstream of the insulin gene have been directly linked to T1D susceptibility (Bain et al., 1992; Julier et al., 1991; Marron et al., 2000). The vast majority of causal genes within T1D susceptibility loci in humans and NOD mice remain to be identified.

T1D Pathology

Autoantibody production in T1D

The autoimmune process, within the islets of pre-diabetic and diabetic humans, remains ill-defined. Tissue samples from T1D patients are rare, and as such, much of our understanding of T1D in humans comes from analysis of autoantibodies and autoreactive T cells detected in peripheral blood. The presence of circulating β cell-reactive antibodies is not considered to be a factor in islet destruction, however, these antibodies serve as a predictor for T1D onset in at-risk individuals (Lieberman and DiLorenzo, 2003). Islet cell antibodies (ICA) were first observed within islets of diabetic human pancreatic tissue sections (Bottazzo et al., 1974). Since then, many studies have identified predictive autoantibodies in human diabetics including antibodies specific for insulin (Lieberman and DiLorenzo, 2003; Palmer et al., 1983; Yu et al., 2000), glutamic acid decarboxylase 65 (GAD65) (Baekkeskov et al., 1990; Hagopian et al., 1993; Lieberman and DiLorenzo, 2003) and insulinoma-associated protein 2 (IA-2) (Lampasona et al., 1996; Lan et al., 1996; Lieberman and DiLorenzo, 2003) The presence of insulin, GAD65 and IA-2 antibodies provide good predictive value for onset of T1D in at-risk individuals. Among siblings of type 1 diabetics, 39% of those with 2 of the 3 antibodies develop T1D within 3 years and greater than 90% of those with all 3 antibodies develop T1D within 3 years (Verge et al., 1996). NOD mice also produce anti-insulin antibodies (Yu et al., 2000), but not significant anti-GAD65 or IA-2 antibodies (Bonifacio et al., 2001; Lieberman and DiLorenzo, 2003).

Autoimmune destruction of pancreatic islets

While autoantibodies provide measurable diagnostic markers for predicting T1D onset, actual β cell destruction is carried out by $CD4^+$ and $CD8^+$ T cells. Little is known about the local cellular events which contribute to human pre-diabetes and eventual development of overt diabetes. Pancreatic tissue is usually only available from post-T1D autopsy samples. Thus, animal models such as the NOD mouse have been used to gain an understanding of early disease pathology. Questions of relevance have been raised for the NOD mouse as it relates to the human disease. Careful and extensive analyses, however, have shown that murine and human T1D bear many similarities. Even if therapeutic approaches may not directly translate from mouse to human, many discoveries of T1D pathology were first described in NOD mice (Shoda et al., 2005).

T1D pathology begins at approximately 3 weeks of age in NOD mice. Islets become surrounded by a population of macrophages and dendritic cells (DC), followed rapidly by $CD4^+$ and $CD8^+$ T cells (Jansen et al., 1994). This early infiltration of islets is known as peri-insulitis (Figure 1.1C). Although β cell destruction is dependent on macrophages, DC and B cells, $CD4^+$ and $CD8^+$ T cells are the key mediators of disease progression (Kay et al., 1997; Wong and Janeway, 1999). Type 1 $CD4^+$ T cells, which produce IFNγ, promote β cell destruction, while TH2 $CD4^+$ T cells, which produce IL-4, IL-5 and IL-10, suppress T1D development (Anderson and Bluestone, 2005; Delovitch and Singh, 1997; Rabinovitch, 1994). Expansion of type 1 $CD4^+$ T cells is induced by the pro-inflammatory cytokine IL-12 (Trembleau et al., 1995), which is produced by macrophages and DC within the islets and/or pancreatic lymph node (PLN). Additionally, pathogenic type 1 CD8 T cells are required for the development of diabetes (Lieberman and DiLorenzo, 2003; Serreze and Leiter, 2001).

This is supported by evidence that Class I MHC genes are associated with T1D susceptibility (Pociot and McDermott, 2002; Serreze and Leiter, 2001).

β cell autoimmunity in NOD mice progresses in relatively well-defined stages or "checkpoints" (Andre et al., 1996). Prior to 3 weeks of age, no islet pathology can be detected. At three weeks of age the first checkpoint of T1D is reached. Checkpoint 1 is characterized by infiltration of the islets by macrophages, DC, B cells and T cells (Andre et al., 1996; Bergman and Haskins, 1994; Kaufman et al., 1993; Tisch et al., 1993) (Figure 1.1A-E). Insulitis continues from approximately 3 to 12 weeks of age, however the majority of β cell mass remains intact and mice remain diabetes-free. Even severe insulitis may persist without sufficient β cell destruction to induce overt diabetes (Andre et al., 1996). Checkpoint 2 is characterized by a qualitative change whereby "benign" insulitis transitions to an "aggressive" or pathogenic form of insulitis resulting in massive β cell destruction and eventual development of overt diabetes (Andre et al., 1996). Complete β cell destruction following Checkpoint 2 is dependent upon expansion of type 1 $CD4^+$ and $CD8^+$ T cells within the islets. (Andre et al., 1996; Liblau et al., 1995; Rabinovitch, 1994) At this point, from approximately 12-30 weeks of age, NOD mice develop overt diabetes, marked by hyperglycemia and glucosuria (Figure 1.1A). The exact mechanisms responsible for progression from Checkpoint 1 to Checkpoint 2 are not well understood, however, it is likely that DC play a role in this process due to three key characteristics: 1. DC are present within the islets throughout both checkpoints (Charre et al., 2002; Jansen et al., 1994; Nikolic et al., 2005), 2. DC efficiently process self-antigens for presentation via MHC and prime T cell expansion (Kubach et al., 2005; Steinman et al., 2005), and 3. DC produce IL-12p70 which is essential for differentiation of type 1 $CD4^+$ T cells and $CD8^+$ CTL effectors (Trembleau et

al., 1995; Trinchieri, 2003; Trinchieri et al., 1993; Trinchieri et al., 1992). The effects of DC dysregulation upon progression from Checkpoint 1 to Checkpoint 2 will be addressed in Chapter 2.

Autoreactive $CD4^+$ and $CD8^+$ T cells that recognize β cell antigens can be found in peripheral blood samples of human T1D patients and NOD mice. Several $CD4^+$ T cell specificities for GAD65 and insulin epitopes have been identified (Lieberman and DiLorenzo, 2003). The pathogenicity of these T cells was proven when adoptive transfer of insulin-specific $CD4^+$ T cell clones was found to accelerate T1D in NOD mice (Daniel et al., 1995). Additionally, autoreactive $CD8^+$ T cells, recognizing epitopes from GAD65 and insulin, can be found in diabetic humans and NOD mice (Lieberman and DiLorenzo, 2003). NOD mice also present with $CD8^+$ T cells which recognize islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) (Lieberman et al., 2003). IGRP-specific $CD8^+$ T cells can be found at high frequencies, up to 40% of $CD8^+$ T cells, in the islets of NOD mice (Anderson et al., 1999).

Type 1 $CD4^+$ and $CD8^+$ T cells are clearly the most important mediators of β cell destruction in T1D. These effectors are, however, dependent upon antigen presenting cells (APC) to locally present antigen and provide inflammatory cytokines. Both B cells (Serreze and Leiter, 2001) and DC (Nikolic et al., 2005) are essential for T1D progression. Indeed DC play key roles in maintenance of self-tolerance in healthy individuals, and defects in DC function may contribute to autoimmune diseases, including T1D (Yoon and Jun, 2005).

The Role of DC in T1D

DC background

In humans and mice DC are identified by expression of the cell-surface marker CD11c. DC are further differentiated into conventional DC (cDC) and plasmacytoid DC (pDC). Murine cDC consist of $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ and $CD11c^+$ $CD11b^-$ $CD8\alpha^+$ subsets (Vremec et al., 1992), which may be derived from either myeloid or lymphoid progenitor cells (Manz et al., 2001). Murine pDC are identified by expression of CD11c and B220. Similar to cDC, pDC can arise from both myeloid and lymphoid lineages (Karsunky et al., 2005; Wang and Liu, 2004). DC are versatile APC, capable of performing many immune functions. Most notably, DC can prime naïve T cells and induce T cell differentiation and clonal expansion (Kubach et al., 2005; Steinman et al., 2005). DC also phagocytose different types of antigen including particulate antigens, pathogenic agents (i.e. bacteria) necrotic tissue and apoptotic cells (Rossi and Young, 2005). The diverse nature of antigenic targets captured by DC requires flexibility in DC responses. It would be undesirable, for example, to have equivalent immune responses to bacteria and self apoptotic cells. It is due to this duality in function that DC are capable of inducing immunity to pathogens, as well as maintaining self-tolerance (Kubach et al., 2005). Indeed, aberrant DC development and function is characteristic of multiple autoimmune diseases including T1D (Yoon and Jun, 2005), systemic lupus erythemetosus (SLE) and rheumatoid arthritis (RA) (Banchereau et al., 2004; Hardin, 2005).

The normal role of DC is to serve as sentinels, scanning for signs of infection. Upon encounter and phagocytosis of infectious agents such as bacteria or viruses, DC undergo functional maturation characterized by up-regulation of co-stimulatory molecules (CD40, CD80 and CD86), production of pro-inflammatory cytokines (TNFα, IL-12p70, IL-1α and

IL-1β) and up-regulation of the chemokine receptor CCR7 (Hardin, 2005; Kubach et al., 2005; Rossi and Young, 2005). Expression of CCR7 mediates DC homing to lymphatic endothelial cells in response to CCL21 and eventually to T cell rich areas of secondary lymphoid tissues in response to CCL19 (Cravens and Lipsky, 2002). Mature DC are well-equipped to prime naïve $CD4^+$ T cells, inducing differentiation to a type 1 phenotype. It is type 1 $CD4^+$ T cells which are integral to β cell destruction during the development of T1D (Cantor and Haskins, 2005; Tisch and McDevitt, 1996). The breakdown of self-tolerance in diabetic humans and animal models is poorly understood, however, many studies provide evidence that atypical DC function is, in part, responsible.

DC mediate early and late events of T1D pathogenesis

As indicated above, the earliest histological pathology of T1D is the development of peri-insulitis. Due to the lack of human tissue samples from recent onset diabetics or pre-diabetic patients, little is know about the initiating events of insulitis in human diabetes. There is, however, much evidence from NOD mice to implicate DC in the initiation of β cell destruction. Infiltration of islets by DC and macrophages begins as early as birth in NOD mice. One day old NOD mice demonstrate increased frequency of $CD11c^+$ DC within the pancreas relative to non-autoimmune mice (Charre et al., 2002). DC become localized to pancreatic islets by 3 weeks of age, before the arrival of lymphocytes. DC expressing high levels of MHC class II, CD80 and CD86 continue to accumulate at parainsular vessels, found adjacent to the islets, and within the islets from 7 to 13 weeks of age. During this period,

accumulation of T cells accompanies DC infiltration and destructive insulitis ensues (Shinomiya et al., 2000) (Figure 1.1E).

The events triggering DC accumulation within the islets remain largely undefined, but some evidence has suggested that a wave of physiological cell death in murine islets may promote DC trafficking. This so-called "ripple" of cell death, at 12 days of age, is believed to be a normal component of tissue remodeling, however, it may lead to DC activation and β cell antigen capture in diabetes-prone animals (Trudeau et al., 2000; Turley et al., 2003). In this model, $CD11c^+CD11b^+$, and to a lesser extent $CD11c^+CD8\alpha^+$ DC acquire antigens from dead cells in the pancreas and traffic to the PLN to stimulate $CD4^+$ and $CD8^+$ T cells (Turley et al., 2003). Notably, humans undergo a similar wave of β cell death at birth, which may also provide access of β cell antigens to the immune system (Kassem et al., 2000).

The specific mechanisms that induce DC maturation within the islets are not completely understood, however, evidence exists which implicates CD40 signaling in this process. Mice expressing CD40 ligand (CD40L, CD154) on β cells demonstrate DC activation in islets and PLN. This DC activation, along with T cell priming eventually leads to the development of destructive insulitis and diabetes in otherwise non-autoimmune mice (Haase et al., 2004). The presence of DC within islets between 4 and 8 weeks of age also correlates with a transient phase of hyperinsulinemia in NOD mice. Interestingly, DC associate preferentially with abnormally large, hyperinsulinemic islets during this period (Rosmalen et al., 2000). In fact, when NOD DC and purified islets are co-cultured *in vitro,* insulin production is significantly increased, whereas non-autoimmune C57BL/6 DC do not induce hyperinsulinemia (Durant et al., 2002). The mechanism responsible for DC-induced insulin production is not understood, however, the phenomenon may contribute to the disease

process by placing β cells under increased stress, which in turn can lead to cell death (Kassem et al., 2000). Additionally, there is some evidence that female sex hormones contribute to hyperinsulinemia and DC infiltration of NOD mouse islets through either modulation of DC function or effects mediated directly upon the islets (Rosmalen et al., 2001).

DC, as well as macrophages, within early pancreatic infiltrates produce significant amounts of the pro-inflammatory cytokine TNFα, prior to the arrival of lymphocytes. Notably, TNFα treatment of neonatal NOD mice induces DC maturation and enhances the frequency of $CD11c^+CD11b^+$ DC relative to $CD11c^+CD8\alpha^+$ DC in the islets. Development of diabetes is accelerated in TNFα-treated animals, whereas TNFα depletion completely inhibits diabetes development (Lee et al., 2005). These findings suggest a crucial role for DC and TNFα production in the development of destructive insulitis.

DC, it seems, are not only necessary for the development of insulitis, but also for maintenance of insulitic lesions throughout disease. For example, DC depletion correlates with reversal of lymphocytic infiltration of the islets (Nikolic et al., 2005), providing strong evidence that DC are essential for priming autoreactive lymphocytes within the target tissue and/or the pancreatic lymph node.

DC Defects Associated With T1D

DC are clearly involved in the pathogenesis of autoimmune diabetes at many stages of the disease. It is unlikely, however, that a single defect in DC biology is wholly responsible for the complicated processes required for the onset of T1D. Many differences in DC

development and activation (described below) have been observed in NOD mice and human diabetics.

DC of the NOD mouse

Studies detailing the DC populations of NOD mice have indicated phenotypic and functional differences in DC *in vivo* and in cultures of bone marrow-derived DC (BMDC). These findings have frequently been contradictory, possibly due to varying conditions of DC culture.

Several analyses of DC in NOD mice have focused upon culture of DC from bone marrow (BM)-derived precursor cells by addition of growth factors. It was found that BMDC cultures from NOD mice produced significantly increased yields of DC relative to non-autoimmune mice, when cultured with GM-CSF and IL-4 (Steptoe et al., 2002). In contrast, another group found that NOD BM cultures preferentially differentiate into MHC class II negative, $CD11c^+$ macrophage-like cells, with low yield of mature DC (Nikolic et al., 2004). These experiments utilized BM cells cultured with GM-CSF but not IL-4. Additionally, a study which utilized titrated doses of GM-CSF and IL-4 found that, under all conditions tested NOD BM differentiated mostly into granulocytes with decreased yields of DC (Morin et al., 2003). Finally, when recombinant murine GM-CSF and IL-4 were encoded by baculovirus infecting insect cells, the supernates induced DC differentiation, albeit at a lower frequency in NOD versus non-autoimmune mice (Prasad and Goodnow, 2002b). Despite variability in the results, it is clear that NOD DC develop aberrantly compared to DC cultured from non-autoimmune mice.

In addition to varying yields of DC from BM cultures, studies of NOD BMDC demonstrate alterations in function. Multiple studies have found that BMDC and *ex vivo*-derived DC from NOD mice demonstrate increased activity of the transcription factor NF-κB (Poligone et al., 2002; Weaver et al., 2001; Wheat et al., 2004). NF-κB hyper-activation induces increased CD40 and CD80 expression (Wheat et al., 2004), and elevated IL-12p70 secretion relative to non-autoimmune DC (Weaver et al., 2001). NF-κB hyperactivity in NOD DC also results in an enhanced capacity to stimulate T cells (Marleau and Singh, 2002; Poligone et al., 2002). In contrast, several studies have found that NOD DC demonstrate impaired maturation (Peng et al., 2003a), decreased MHC class II expression (Strid et al., 2001), poor stimulation of allogeneic lymphocytes (Nikolic et al., 2004), and ineffective presentation of soluble peptide antigen to T cells (Strid et al., 2001). One consistent finding among investigators is the relative decrease in frequency of $CD11c^{+}CD8\alpha^{+}$ DC and increased frequency of $CD11c^{+}CD11b^{+}$ DC (O'Keeffe et al., 2005; Prasad and Goodnow, 2002a). Together, these findings highlight the complexity of T1D, even when utilizing inbred strains of mice with essentially identical genetic backgrounds.

DC of at-risk and T1D patients

Investigation of DC populations in humans has provided similarly variable results. It has been reported that diabetic patients possess phenotypically similar DC, when compared to healthy individuals (Summers et al., 2003). Additionally, the maturation and T cell stimulating capacity of DC from recent-onset T1D patients was similar to that of healthy-donors (Zacher et al., 2002). Meanwhile, other findings indicate that DC of diabetics

demonstrate impaired maturation (Angelini et al., 2005), decreased inflammatory cytokine production (Skarsvik et al., 2004) and a decreased T cell stimulatory capacity relative to healthy individuals (Angelini et al., 2005; Skarsvik et al., 2004). Another study found increased DC numbers, increased MHC class II expression by DC and increased production of IFNγ by DC from at-risk and recent-onset T1D patients (Peng et al., 2003b). Clearly, the nature of DC maturation and function in diabetic patients requires further analysis. The important role of DC in insulitis and β cell destruction, as well as the observed DC variability in mice and humans, indicate that multiple functional differences in DC likely contribute to T1D development.

Immunoregulation of DC by Apoptotic Cells

DC activation is typically described in response to pathogens. Unperturbed environments foster maintenance of immature DC, while microbial pathogens induce activation and maturation of DC (Banchereau and Steinman, 1998). This representation of DC life cycle accurately represents a portion of immune regulation, but the model fails to address the activity of DC under homeostatic conditions. Multicellular organisms constantly harbor a mixed balance of viable, apoptotic and necrotic tissues. The continual exposure to self-antigen necessitates that DC maturation and effector function be regulated to maintain immunological tolerance (Wallet et al., 2005).

The clearance of apoptotic cells *in vivo* is carried out by multiple phagocytes including macrophages, neutrophils and DC. In the case of DC, it is only immature cells that are capable of phagocytosing apoptotic cells (Albert et al., 1998). Binding and ingestion of

apoptotic cells by immature DC is mediated by a group of receptors including phosphatidylserine receptor (PSR) (Li et al., 2003), $\alpha_V\beta_5$ integrin, CD36 (Albert et al., 1998) and the receptor tyrosine kinase MerTK (Cohen et al., 2002).

In general, when DC encounter apoptotic cells, a non-inflammatory and tolergenic phenotype typical of immature DC is induced (Steinman et al., 2000). For example DC which are pretreated with apoptotic cells exhibit a reduced capacity to produce IL-12p70 upon stimulation with bacterial endotoxin (Stuart et al., 2002). Immature DC treated with apoptotic cells also exhibit an impaired capacity to stimulate T cells. Notably, lipopolysaccharide (LPS) treatment is unable to overcome the "tolergenic" phenotype, indicating the effect of apoptotic cells is long-lasting and relatively stable (Takahashi and Kobayashi, 2003). As a result, activation of DC carrying the remnants of apoptotic cells is effectively prevented. In addition to inducing an immature DC phenotype, apoptotic cells have been reported to induce the expression of IL-6 and IL-12p40, and therefore further enhance the tolergenic function of the DC (Takahashi and Kobayashi, 2003). Apoptotic cell-induced IL-6 secretion by DC functions in an autocrine manner to block further maturation of DC (Figure 1.2). For example, activation of STAT3 following IL-6 pretreatment blocks LPS-induced up-regulation of CD80, CD86, CD40 and MHCII on DC, thereby reducing the T cell stimulatory capacity of the DC (Park et al., 2004). Additionally, secretion of high levels of IL-12p40 results in formation of IL-12p40 homodimers, which competitively inhibit IL-12p70 binding to the IL-12 receptor on T cells (Takahashi and Kobayashi, 2003; Wang et al., 1999). Consequently, differentiation of naïve $CD4^+$ T cells into type 1 cells is suppressed (Figure 1.2).

Phagocytosis of apoptotic cells by DC affects cross-presentation of self-antigens to $CD8^+$ T cells. Employing human DC and apoptotic versus necrotic 293 tumor cells, the importance of the mode of death of the phagocytosed cells was initially defined. Necrotic but not apoptotic 293 cells induced DC maturation based on up-regulation of CD83 and DC-LAMP. Furthermore, a robust mixed lymphocyte reaction (MLR) was induced by pulsing DC with necrotic but not apoptotic allogeneic splenocytes (Sauter et al., 2000). Furthermore, apoptotic cells modulate DC effector function *in vivo*. Injection of apoptotic splenocytes coupled with trinitrophenol into mice blocked *in vitro* recall responses to the hapten. Importantly, CD11c+CD8α+ DC were identified as the critical effectors for inducing the immune tolerance to apoptotic cell-derived antigens (Ferguson et al., 2002).

These data and work by others support a role for DC in maintaining immunological homeostasis (Basu et al., 2000; Stuart et al., 2002; Xu et al., 2004). In contrast, necrotic cells typical of microbial infections induce DC activation and maturation, and subsequent immunity. This balance in DC reactivity allows the peripheral immune system to continually monitor tissues for signs of death and mount the appropriate response. The importance of DC in clearance of apoptotic cells and the maintenance of immune homeostasis is highlighted in autoimmune diseases such as SLE. While not wholly causal for SLE, failure to effectively clear apoptotic cells contributes to the severity of disease (Gaipl et al., 2004).

Recent work by our group has been performed to describe the signaling mechanisms responsible for apoptotic cell-induced DC tolerance. Initial results show that apoptotic cells block pro-inflammatory stimuli, such as TLR and CD40 signaling through inhibition of NF-κB activation (unpublished data P.S., M.W. and R.T.).

Thymic Selection

Thymic selection is a complex process whereby thymocytes expressing high-avidity, self-specific T cell receptor (TCR) or non-functional TCR are removed. Much evidence exists to implicate failures of thymic selection in the development of T1D in NOD mice.

Positive selection

Typical thymic structure includes a well defined central medullary region and a surrounding cortex. Thymocyte populations include $CD4^-$ $CD8^-$ (double-negative, DN) cells, $CD4^+CD8^+$ (double-positive, DP) cells and single positive (SP) $CD4^+$ and $CD8^+$ T cells (Liu, 2006). Positive selection occurs when DP cells expressing a functional TCR encounter cortical thymic epithelial cells (CTE). CTE express MHC class I and MHC class II molecules which present self peptides. TCR which recognize self-MHC-peptide complexes mediate survival signals to DP cells. Meanwhile, DP cells expressing a TCR that fails to recognize self-MHC-peptide die by neglect (Hogquist et al., 2005; Starr et al., 2003; von Boehmer, 1994). Positively selected cells then proceed to the corticomedullary junction and medulla for negative selection.

Negative selection

Negative selection is the process whereby DP cells demonstrating relatively high-avidity recognition of self-MHC-peptide are deleted. Medullary thymic epithelial (MTE) cells express a multitude of peripheral tissue self-antigens under the control of the transcription

factor Aire (Anderson et al., 2002). Presentation of self antigen epitopes by MTE allows deletion of high-avidity self-specific DP cells.

Additionally, the murine thymus possesses three distinct populations of DC. pDC provide no observable role in thymic selection and instead, likely act as sentinels for infection within the thymus. $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ and $CD11c^+$ $CD11b^-$ $CD8\alpha^+$ cDC subsets are also found in the thymic medulla (Liu, 2006; Wu and Shortman, 2005). Currently, it is unclear which cDC mediate negative selection, however, evidence strongly suggests that one or both subsets play a direct role in self-tolerance through negative selection (Anderson et al., 1998; Brocker, 1997; Matzinger and Guerder, 1989).

Thymic selection in the NOD mouse

NOD defects in thymic positive and negative selection have been associated with the presence of pathogenic, high-avidity $CD4^+$ and $CD8^+$ T cells which mediate β cell destruction (Kwon et al., 2005). Two distinct failures of positive selection in NOD mice contribute to T1D progression. First, CTE of NOD mice appear to be inefficient in inducing $CD4^+CD25^+$ Treg development, which may in turn promote differentiation of autoreactive T effectors in the periphery (Thomas-Vaslin et al., 1997). Secondly, high expression of ERK and JNK by DP cells alters the outcome of low affinity TCR/MHC-peptide interactions in the thymus. Therefore, low affinity TCR-bearing DP cells which would typically die by neglect, go on to become pathogenic $CD8^+$ T cell effectors (Kwon et al., 2005).

It has been proposed that negative selection in NOD thymi is inefficient, in part due to abnormal medulla and cortex organization (Naquet et al., 1999; Rosmalen et al., 2002;

Savino et al., 1991). Additionally, expression of I-A^{g7} MHC class II in NOD mice and diabetogenic HLA-DR and HLA-DQ alleles in humans has been proposed to contribute to inefficient negative selection (Rosmalen et al., 2002). For example, studies suggest that I-A^{g7} binds self-peptides poorly resulting in decreased presentation of self-peptides required for deletion of high-avidity DP cells (Moustakas et al., 2000; Stratmann et al., 2000). Interestingly, it has been found that thymic expression of the β cell tissue antigen insulin is significantly decreased in NOD mice relative to other strains of mice (Brimnes et al., 2002), which may result in inefficient deletion of insulin-specific T cells.

MerTK

Background

MerTK is a 984 amino acid transmembrane protein (Figure 1.3) that is expressed by various cell types. MerTK (c-mer, NYK) belongs to a family of tyrosine kinases that includes Axl and Tyro3. All three receptors play roles in spermatogenesis (Chan et al., 2000; Lu et al., 1999), platelet function (Chen et al., 2004; Gould et al., 2005) and neuronal development (Prieto et al., 2000). Sequence analysis indicates that MerTK shares homology with the chicken proto-oncogene c-eyk and the chicken tumor retrovirus oncogene v-eyk (Graham et al., 1995). Similarly, human MerTK shares homology with v-eyk and thus both human and murine MerTK have been considered as putative proto-oncogenes (Graham et al., 1995; Graham et al., 1994). MerTK expression has been detected in neoplastic B and T cell lines (Graham et al., 1994), however, MerTK signaling in transfected tumor cells does not induce proliferation. Instead, cell cycle arrest and structural differentiation of tumor cells is induced

(Guttridge et al., 2002; Wu et al., 2004). Interestingly, transgene-induced overexpression of MerTK in hematopoetic cells of mice leads to lymphoblastic leukemia/lymphoma development (Keating et al., 2006).

The majority of MerTK studies in mice and humans have focused upon functional roles in phagocytosis of apoptotic cells and innate immunity. MerTK is expressed by retinal pigment epithelial cells (RPE) of humans, mice and rats (Duncan et al., 2003; Feng et al., 2002; Gal et al., 2000; Hall et al., 2003; Hall et al., 2005; Hall et al., 2001; McHenry et al., 2004; Zhao et al., 2006). MerTK expression by RPE is essential for efficient phagocytosis of apoptotic outer segment cells (OS) of photoreceptors in the eye (Hall et al., 2001). Lack of MerTK expression in mice and humans leads to accumulation of apoptotic debris, progressive retinal dystrophy and blindness (Gal et al., 2000{Duncan, 2003 #262; McHenry et al., 2004; Zhao et al., 2006).

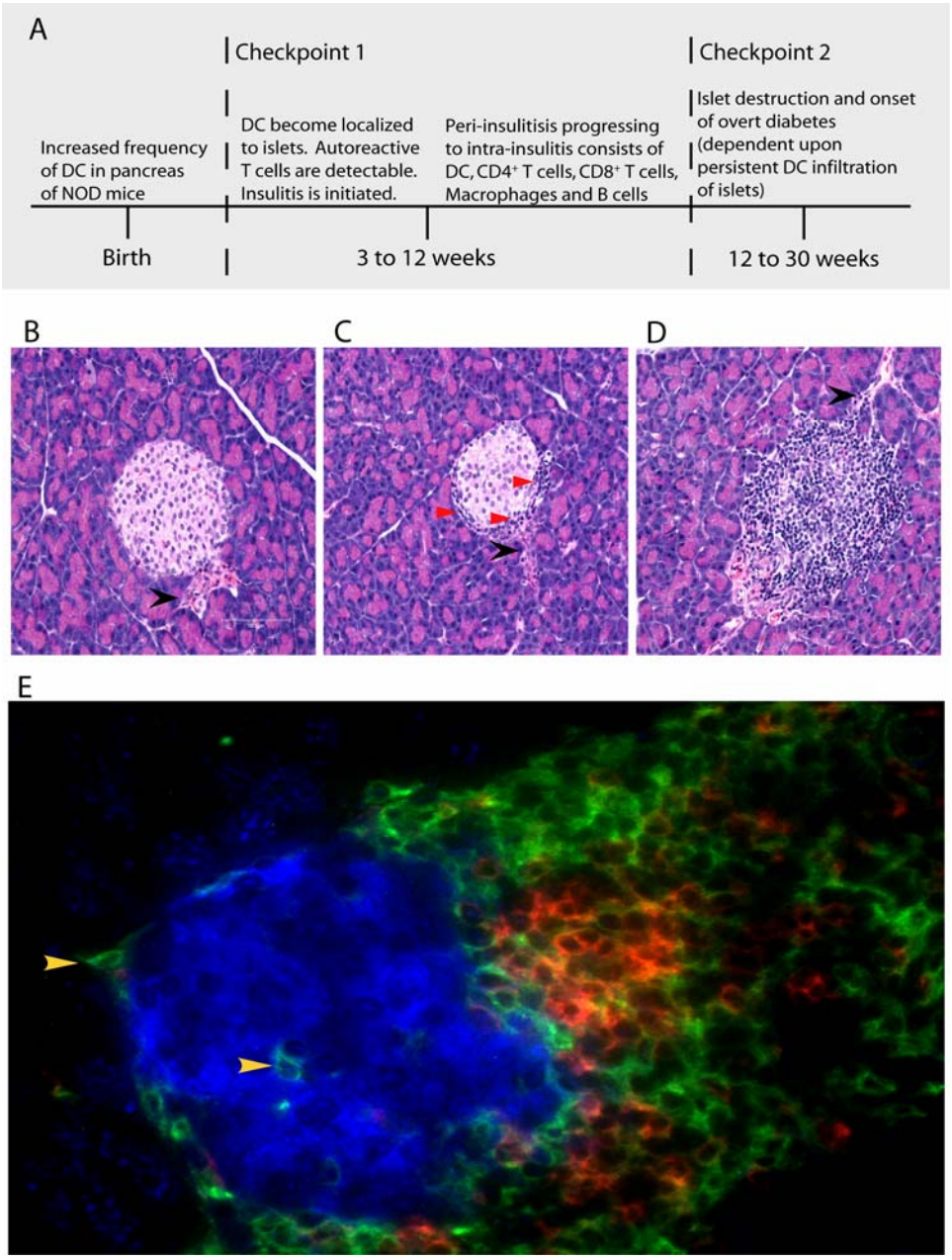
The role of MerTK in immune cells

Expression of MerTK among leukocytes is predominantly limited to cells of monocytic lineage (Graham et al., 1995). In particular, expression of MerTK by macrophages is essential for phagocytosis of apoptotic cells. (Cohen et al., 2002; Scott et al., 2001; Wu et al., 2005). The MerTK ligand growth arrest specific factor-6 (Gas6) (Chen et al., 1997; Nagata et al., 1996) is an opsonin of phosphatidylserine (PS). Apoptotic cells that expose PS on their outer cytoplasmic membrane become coated with Gas6. Notably, phagocytosis of apoptotic OS cells by RPE is dependent on recognition of Gas6 by MerTK (Hall et al., 2003; Hall et

al., 2001). Therefore, it is likely that Gas6 also mediates phagocytosis of apoptotic cells by macrophages.

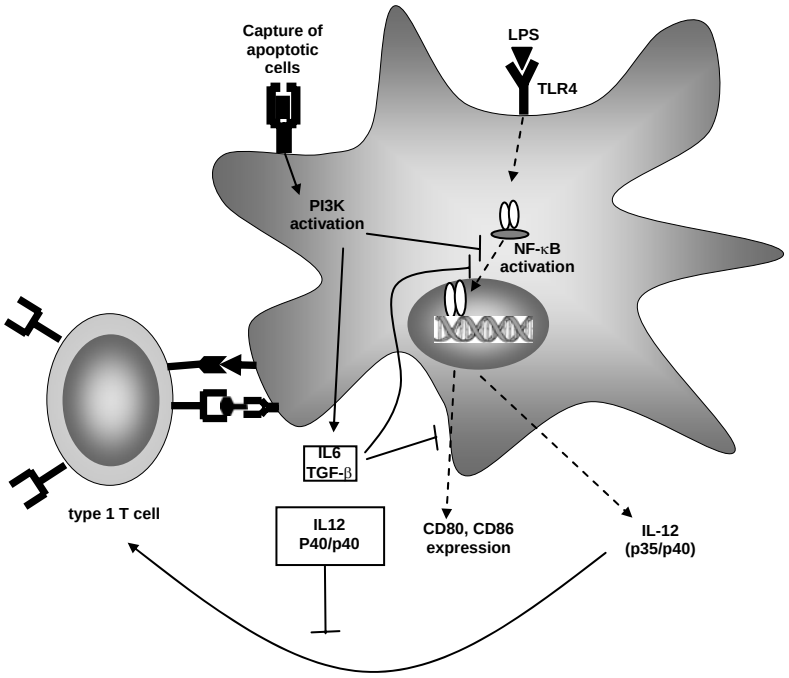
MerTK, Axl and Tyro3 play critical roles in peripheral immune regulation of mice. Macrophages lacking MerTK exhibit a hyperactive phenotype, marked by increased activation of NF-κB and secretion of TNFα (Camenisch et al., 1999; Li et al., 2006). Furthermore, mice lacking expression of MerTK, Axl and Tyro3 develop a severe lymphoproliferative disorder and systemic autoimmunity (Lu and Lemke, 2001). MerTK expression has been reported for DC, NK cells and NKT cells in mice. (Behrens et al., 2003). Unlike macrophages, the capacity of DC to phagocytose apoptotic cells is unaffected by the lack of MerTK expression (Behrens et al., 2003). In fact, the role of MerTK seems to be primarily to regulate DC activation and maturation. Recent results have indicated that apoptotic cells suppress activation of DC and inhibit NF-κB activation via MerTK (unpublished data P.S., M.W. and R.T.). The impact of MerTK on DC function will be addressed in Chapter 2 and Chapter 3.

Figure 1.1



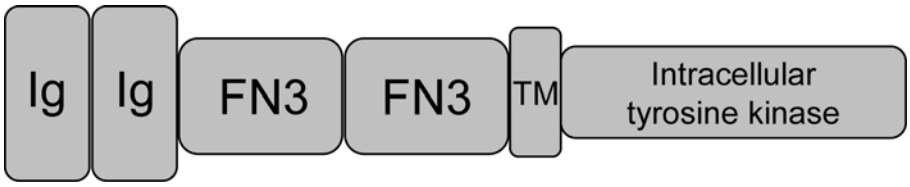
Insulitis of the NOD mouse. (A) Diabetes progression in the NOD mouse begins with the early presence of DC within the pancreas. Throughout insulitis, DC are essential to the autoimmune process. (B-D, black arrows) Mononuclear immune cells enter the islets via para-insular vessels. (C, red arrows) Early islet infiltration is marked by a ring of mononuclear cells which surround the islet, known as peri-insulitis. (D) As the disease progresses, intra-insulitis develops and islets are destroyed. (E) Insulitis consists of many cell types, however DC (E, green) and $CD4^+$ T cells (E, red) are critical to destruction of insulin-producing β cells (E, blue).

Figure 1.2



Apoptotic cells induce immunoregulation and tolerance induction through DC. DC scavenge tissues for microbes and dying tissue. Apoptotic cells induce phosphatidylinositol 3-kinase (PI3K)-dependent signaling resulting in inhibition of NF-κB translocation and co-stimulatory molecule (CD80/86) expression. Production of IL-6, TGF-β and IL-12p40 ensue. IL-12p40 homdimers inhibit the effects of IL-12p70 upon T lymphocytes, effectively inhibiting type 1 induction. Apoptotic cells induce a lasting inhibitory effect which renders DC refractory to LPS induced stimulation of type 1 lymphocytes. (Reproduced with permission from Wallet MA, Sen P, Tisch R. Immunoregulation of dendritic cells. Clin Med Res 2005;3:166-175. Copyright 2005 Marshfield Clinic. All rights reserved).

Figure 1.3



MerTK protein structure. MerTK is a 984 amino acid transmembrane protein. The extracellular domain consists of two immunoglobulin like domains (Ig) and two fibronectin type III domains (FN3). A transmembrane region spans the plasma membrane and the cytoplasmic tail consists of tyrosine kinase domains.

References

(1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med *329*, 977-986.

(1994). Effect of intensive diabetes treatment on the development and progression of long-term complications in adolescents with insulin-dependent diabetes mellitus: Diabetes Control and Complications Trial. Diabetes Control and Complications Trial Research Group. J Pediatr *125*, 177-188.

Abiru, N., Kawasaki, E., and Eguch, K. (2002). Current knowledge of Japanese type 1 diabetic syndrome. Diabetes Metab Res Rev *18*, 357-366.

Acha-Orbea, H., and McDevitt, H. O. (1987). The first external domain of the nonobese diabetic mouse class II I-A beta chain is unique. Proc Natl Acad Sci U S A *84*, 2435-2439.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med *188*, 1359-1368.

Anderson, B., Park, B. J., Verdaguer, J., Amrani, A., and Santamaria, P. (1999). Prevalent CD8(+) T cell response against one peptide/MHC complex in autoimmune diabetes. Proc Natl Acad Sci U S A *96*, 9311-9316.

Anderson, G., Partington, K. M., and Jenkinson, E. J. (1998). Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. J Immunol *161*, 6599-6603.

Anderson, M. S., and Bluestone, J. A. (2005). The NOD mouse: a model of immune dysregulation. Annu Rev Immunol *23*, 447-485.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Andre, I., Gonzalez, A., Wang, B., Katz, J., Benoist, C., and Mathis, D. (1996). Checkpoints in the progression of autoimmune disease: lessons from diabetes models. Proc Natl Acad Sci U S A *93*, 2260-2263.

Angelini, F., Del Duca, E., Piccinini, S., Pacciani, V., Rossi, P., and Manca Bitti, M. L. (2005). Altered phenotype and function of dendritic cells in children with type 1 diabetes. Clin Exp Immunol *142*, 341-346.

Anjos, S., and Polychronakos, C. (2004). Mechanisms of genetic susceptibility to type I diabetes: beyond HLA. Mol Genet Metab *81*, 187-195.

Baekkeskov, S., Aanstoot, H. J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., and De Camilli, P. (1990). Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature *347*, 151-156.

Bain, S. C., Prins, J. B., Hearne, C. M., Rodrigues, N. R., Rowe, B. R., Pritchard, L. E., Ritchie, R. J., Hall, J. R., Undlien, D. E., Ronningen, K. S., and et al. (1992). Insulin gene region-encoded susceptibility to type 1 diabetes is not restricted to HLA-DR4-positive individuals. Nat Genet *2*, 212-215.

Balamurugan, A. N., Bottino, R., Giannoukakis, N., and Smetanka, C. (2006). Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes. Pancreas *32*, 231-243.

Banchereau, J., Pascual, V., and Palucka, A. K. (2004). Autoimmunity through cytokine-induced dendritic cell activation. Immunity *20*, 539-550.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Basu, S., Binder, R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. (2000). Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol *12*, 1539-1546.

Behrens, E. M., Gadue, P., Gong, S. Y., Garrett, S., Stein, P. L., and Cohen, P. L. (2003). The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. Eur J Immunol *33*, 2160-2167.

Bergman, B., and Haskins, K. (1994). Islet-specific T-cell clones from the NOD mouse respond to beta-granule antigen. Diabetes *43*, 197-203.

Bonifacio, E., Atkinson, M., Eisenbarth, G., Serreze, D., Kay, T. W., Lee-Chan, E., and Singh, B. (2001). International Workshop on Lessons From Animal Models for Human Type 1 Diabetes: identification of insulin but not glutamic acid decarboxylase or IA-2 as specific autoantigens of humoral autoimmunity in nonobese diabetic mice. Diabetes *50*, 2451-2458.

Bottazzo, G. F., Florin-Christensen, A., and Doniach, D. (1974). Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet *2*, 1279-1283.

Brimnes, M. K., Jensen, T., Jorgensen, T. N., Michelsen, B. K., Troelsen, J., and Werdelin, O. (2002). Low expression of insulin in the thymus of non-obese diabetic mice. J Autoimmun *19*, 203-213.

Brocker, T. (1997). Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. J Exp Med *186*, 1223-1232.

Camenisch, T. D., Koller, B. H., Earp, H. S., and Matsushima, G. K. (1999). A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol *162*, 3498-3503.

Cantor, J., and Haskins, K. (2005). Effector function of diabetogenic CD4 Th1 T cell clones: a central role for TNF-alpha. J Immunol *175*, 7738-7745.

Chan, M. C., Mather, J. P., McCray, G., and Lee, W. M. (2000). Identification and regulation of receptor tyrosine kinases Rse and Mer and their ligand Gas6 in testicular somatic cells. J Androl *21*, 291-302.

Charre, S., Rosmalen, J. G., Pelegri, C., Alves, V., Leenen, P. J., Drexhage, H. A., and Homo-Delarche, F. (2002). Abnormalities in dendritic cell and macrophage accumulation in the pancreas of nonobese diabetic (NOD) mice during the early neonatal period. Histol Histopathol *17*, 393-401.

Chen, C., Li, Q., Darrow, A. L., Wang, Y., Derian, C. K., Yang, J., de Garavilla, L., Andrade-Gordon, P., and Damiano, B. P. (2004). Mer receptor tyrosine kinase signaling participates in platelet function. Arterioscler Thromb Vasc Biol *24*, 1118-1123.

Chen, J., Carey, K., and Godowski, P. J. (1997). Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. Oncogene *14*, 2033-2039.

Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A., Earp, H. S., Matsushima, G., and Reap, E. A. (2002). Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med *196*, 135-140.

Coulson, B. S., Witterick, P. D., Tan, Y., Hewish, M. J., Mountford, J. N., Harrison, L. C., and Honeyman, M. C. (2002). Growth of rotaviruses in primary pancreatic cells. J Virol *76*, 9537-9544.

Cravens, P. D., and Lipsky, P. E. (2002). Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. Immunol Cell Biol *80*, 497-505.

Daneman, D. (2006). Type 1 diabetes. Lancet *367*, 847-858.

Daniel, D., Gill, R. G., Schloot, N., and Wegmann, D. (1995). Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. Eur J Immunol *25*, 1056-1062.

Delovitch, T. L., and Singh, B. (1997). The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunity *7*, 727-738.

Duncan, J. L., Yang, H., Vollrath, D., Yasumura, D., Matthes, M. T., Trautmann, N., Chappelow, A. V., Feng, W., Earp, H. S., Matsushima, G. K., and LaVail, M. M. (2003). Inherited retinal dystrophy in Mer knockout mice. Adv Exp Med Biol *533*, 165-172.

Durant, S., Alves, V., Coulaud, J., and Homo-Delarche, F. (2002). Nonobese diabetic (NOD) mouse dendritic cells stimulate insulin secretion by prediabetic islets. Autoimmunity *35*, 449-455.

Feng, W., Yasumura, D., Matthes, M. T., LaVail, M. M., and Vollrath, D. (2002). Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J Biol Chem *277*, 17016-17022.

Ferguson, T. A., Herndon, J., Elzey, B., Griffith, T. S., Schoenberger, S., and Green, D. R. (2002). Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. J Immunol *168*, 5589-5595.

Filippi, C., and von Herrath, M. (2005). How viral infections affect the autoimmune process leading to type 1 diabetes. Cell Immunol *233*, 125-132.

Fong, D. S., Aiello, L. P., Ferris, F. L., 3rd, and Klein, R. (2004). Diabetic retinopathy. Diabetes Care *27*, 2540-2553.

Gaipl, U. S., Franz, S., Voll, R. E., Sheriff, A., Kalden, J. R., and Herrmann, M. (2004). Defects in the disposal of dying cells lead to autoimmunity. Curr Rheumatol Rep *6*, 401-407.

Gal, A., Li, Y., Thompson, D. A., Weir, J., Orth, U., Jacobson, S. G., Apfelstedt-Sylla, E., and Vollrath, D. (2000). Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat Genet *26*, 270-271.

Gould, W. R., Baxi, S. M., Schroeder, R., Peng, Y. W., Leadley, R. J., Peterson, J. T., and Perrin, L. A. (2005). Gas6 receptors Axl, Sky and Mer enhance platelet activation and regulate thrombotic responses. J Thromb Haemost *3*, 733-741.

Graham, D. K., Bowman, G. W., Dawson, T. L., Stanford, W. L., Earp, H. S., and Snodgrass, H. R. (1995). Cloning and developmental expression analysis of the murine c-mer tyrosine kinase. Oncogene *10*, 2349-2359.

Graham, D. K., Dawson, T. L., Mullaney, D. L., Snodgrass, H. R., and Earp, H. S. (1994). Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. Cell Growth Differ *5*, 647-657.

Guttridge, K. L., Luft, J. C., Dawson, T. L., Kozlowska, E., Mahajan, N. P., Varnum, B., and Earp, H. S. (2002). Mer receptor tyrosine kinase signaling: prevention of apoptosis and alteration of cytoskeletal architecture without stimulation or proliferation. J Biol Chem *277*, 24057-24066.

Haase, C., Skak, K., Michelsen, B. K., and Markholst, H. (2004). Local activation of dendritic cells leads to insulitis and development of insulin-dependent diabetes in transgenic mice expressing CD154 on the pancreatic beta-cells. Diabetes *53*, 2588-2595.

Hagopian, W. A., Michelsen, B., Karlsen, A. E., Larsen, F., Moody, A., Grubin, C. E., Rowe, R., Petersen, J., McEvoy, R., and Lernmark, A. (1993). Autoantibodies in IDDM primarily recognize the 65,000-M(r) rather than the 67,000-M(r) isoform of glutamic acid decarboxylase. Diabetes *42*, 631-636.

Hall, M. O., Agnew, B. J., Abrams, T. A., and Burgess, B. L. (2003). The phagocytosis of os is mediated by the PI3-kinase linked tyrosine kinase receptor, mer, and is stimulated by GAS6. Adv Exp Med Biol *533*, 331-336.

Hall, M. O., Obin, M. S., Heeb, M. J., Burgess, B. L., and Abrams, T. A. (2005). Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. Exp Eye Res *81*, 581-591.

Hall, M. O., Prieto, A. L., Obin, M. S., Abrams, T. A., Burgess, B. L., Heeb, M. J., and Agnew, B. J. (2001). Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. Exp Eye Res *73*, 509-520.

Hardin, J. A. (2005). Dendritic cells: potential triggers of autoimmunity and targets for therapy. Ann Rheum Dis *64 Suppl 4*, iv86-90.

Hogquist, K. A., Baldwin, T. A., and Jameson, S. C. (2005). Central tolerance: learning self-control in the thymus. Nat Rev Immunol *5*, 772-782.

Honeyman, M. C., Coulson, B. S., Stone, N. L., Gellert, S. A., Goldwater, P. N., Steele, C. E., Couper, J. J., Tait, B. D., Colman, P. G., and Harrison, L. C. (2000). Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. Diabetes *49*, 1319-1324.

Jansen, A., Homo-Delarche, F., Hooijkaas, H., Leenen, P. J., Dardenne, M., and Drexhage, H. A. (1994). Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice. Diabetes *43*, 667-675.

Julier, C., Hyer, R. N., Davies, J., Merlin, F., Soularue, P., Briant, L., Cathelineau, G., Deschamps, I., Rotter, J. I., Froguel, P., and et al. (1991). Insulin-IGF2 region on chromosome 11p encodes a gene implicated in HLA-DR4-dependent diabetes susceptibility. Nature *354*, 155-159.

Karsunky, H., Merad, M., Mende, I., Manz, M. G., Engleman, E. G., and Weissman, I. L. (2005). Developmental origin of interferon-alpha-producing dendritic cells from hematopoietic precursors. Exp Hematol *33*, 173-181.

Kassem, S. A., Ariel, I., Thornton, P. S., Scheimberg, I., and Glaser, B. (2000). Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes *49*, 1325-1333.

Kaufman, D. L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G. S., Robinson, P., Atkinson, M. A., Sercarz, E. E., Tobin, A. J., and Lehmann, P. V. (1993). Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature *366*, 69-72.

Kay, T. W., Chaplin, H. L., Parker, J. L., Stephens, L. A., and Thomas, H. E. (1997). CD4+ and CD8+ T lymphocytes: clarification of their pathogenic roles in diabetes in the NOD mouse. Res Immunol *148*, 320-327.

Keating, A. K., Salzberg, D. B., Sather, S., Liang, X., Nickoloff, S., Anwar, A., Deryckere, D., Hill, K., Joung, D., Sawczyn, K. K.*, et al.* (2006). Lymphoblastic leukemia/lymphoma in mice overexpressing the Mer (MerTK) receptor tyrosine kinase. Oncogene.

Kubach, J., Becker, C., Schmitt, E., Steinbrink, K., Huter, E., Tuettenberg, A., and Jonuleit, H. (2005). Dendritic cells: sentinels of immunity and tolerance. Int J Hematol *81*, 197-203.

Kwon, H., Jun, H. S., Yang, Y., Mora, C., Mariathasan, S., Ohashi, P. S., Flavell, R. A., and Yoon, J. W. (2005). Development of autoreactive diabetogenic T cells in the thymus of NOD mice. J Autoimmun *24*, 11-23.

Lampasona, V., Bearzatto, M., Genovese, S., Bosi, E., Ferrari, M., and Bonifacio, E. (1996). Autoantibodies in insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosine phosphatase-like IA-2 autoantigen. J Immunol *157*, 2707-2711.

Lan, M. S., Wasserfall, C., Maclaren, N. K., and Notkins, A. L. (1996). IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A *93*, 6367-6370.

Lee, L. F., Xu, B., Michie, S. A., Beilhack, G. F., Warganich, T., Turley, S., and McDevitt, H. O. (2005). The role of TNF-alpha in the pathogenesis of type 1 diabetes in the nonobese diabetic mouse: analysis of dendritic cell maturation. Proc Natl Acad Sci U S A *102*, 15995-16000.

Li, M. O., Sarkisian, M. R., Mehal, W. Z., Rakic, P., and Flavell, R. A. (2003). Phosphatidylserine receptor is required for clearance of apoptotic cells. Science *302*, 1560-1563.

Li, Y., Gerbod-Giannone, M. C., Seitz, H., Cui, D., Thorp, E., Tall, A. R., Matsushima, G. K., and Tabas, I. (2006). Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the Mer receptor. J Biol Chem *281*, 6707-6717.

Liblau, R. S., Singer, S. M., and McDevitt, H. O. (1995). Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today *16*, 34-38.

Lieberman, S. M., and DiLorenzo, T. P. (2003). A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens *62*, 359-377.

Lieberman, S. M., Evans, A. M., Han, B., Takaki, T., Vinnitskaya, Y., Caldwell, J. A., Serreze, D. V., Shabanowitz, J., Hunt, D. F., Nathenson, S. G.*, et al.* (2003). Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. Proc Natl Acad Sci U S A *100*, 8384-8388.

Liu, Y. J. (2006). A unified theory of central tolerance in the thymus. Trends Immunol *27*, 215-221.

Lu, Q., Gore, M., Zhang, Q., Camenisch, T., Boast, S., Casagranda, F., Lai, C., Skinner, M. K., Klein, R., Matsushima, G. K.*, et al.* (1999). Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature *398*, 723-728.

Lu, Q., and Lemke, G. (2001). Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science *293*, 306-311.

Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L., and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood *97*, 3333-3341.

Marleau, A. M., and Singh, B. (2002). Myeloid dendritic cells in non-obese diabetic mice have elevated costimulatory and T helper-1-inducing abilities. J Autoimmun *19*, 23-35.

Marron, M. P., Zeidler, A., Raffel, L. J., Eckenrode, S. E., Yang, J. J., Hopkins, D. I., Garchon, H. J., Jacob, C. O., Serrano-Rios, M., Martinez Larrad, M. T.*, et al.* (2000). Genetic and physical mapping of a type 1 diabetes susceptibility gene (IDDM12) to a 100-kb phagemid artificial chromosome clone containing D2S72-CTLA4-D2S105 on chromosome 2q33. Diabetes *49*, 492-499.

Matzinger, P., and Guerder, S. (1989). Does T-cell tolerance require a dedicated antigen-presenting cell? Nature *338*, 74-76.

McHenry, C. L., Liu, Y., Feng, W., Nair, A. R., Feathers, K. L., Ding, X., Gal, A., Vollrath, D., Sieving, P. A., and Thompson, D. A. (2004). MERTK arginine-844-cysteine in a patient with severe rod-cone dystrophy: loss of mutant protein function in transfected cells. Invest Ophthalmol Vis Sci *45*, 1456-1463.

Morin, J., Chimenes, A., Boitard, C., Berthier, R., and Boudaly, S. (2003). Granulocyte-dendritic cell unbalance in the non-obese diabetic mice. Cell Immunol *223*, 13-25.

Moustakas, A. K., Routsias, J., and Papadopoulos, G. K. (2000). Modelling of the MHC II allele I-A(g7) of NOD mouse: pH-dependent changes in specificity at pockets 9 and 6 explain several of the unique properties of this molecule. Diabetologia *43*, 609-624.

Nagata, K., Ohashi, K., Nakano, T., Arita, H., Zong, C., Hanafusa, H., and Mizuno, K. (1996). Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. J Biol Chem *271*, 30022-30027.

Naquet, P., Naspetti, M., and Boyd, R. (1999). Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. Semin Immunol *11*, 47-55.

Nikolic, T., Bunk, M., Drexhage, H. A., and Leenen, P. J. (2004). Bone marrow precursors of nonobese diabetic mice develop into defective macrophage-like dendritic cells in vitro. J Immunol *173*, 4342-4351.

Nikolic, T., Geutskens, S. B., van Rooijen, N., Drexhage, H. A., and Leenen, P. J. (2005). Dendritic cells and macrophages are essential for the retention of lymphocytes in (peri)-insulitis of the nonobese diabetic mouse: a phagocyte depletion study. Lab Invest *85*, 487-501.

O'Keeffe, M., Brodnicki, T. C., Fancke, B., Vremec, D., Morahan, G., Maraskovsky, E., Steptoe, R., Harrison, L. C., and Shortman, K. (2005). Fms-like tyrosine kinase 3 ligand administration overcomes a genetically determined dendritic cell deficiency in NOD mice and protects against diabetes development. Int Immunol *17*, 307-314.

Palmer, J. P., Asplin, C. M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P. K., and Paquette, T. L. (1983). Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science *222*, 1337-1339.

Park, S. J., Nakagawa, T., Kitamura, H., Atsumi, T., Kamon, H., Sawa, S., Kamimura, D., Ueda, N., Iwakura, Y., Ishihara, K.*, et al.* (2004). IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. J Immunol *173*, 3844-3854.

Peng, R., Bathjat, K., Li, Y., and Clare-Salzler, M. J. (2003a). Defective maturation of myeloid dendritic cell (DC) in NOD mice is controlled by IDD10/17/18. Ann N Y Acad Sci *1005*, 184-186.

Peng, R., Li, Y., Brezner, K., Litherland, S., and Clare-Salzler, M. J. (2003b). Abnormal peripheral blood dendritic cell populations in type 1 diabetes. Ann N Y Acad Sci *1005*, 222-225.

Pociot, F., and McDermott, M. F. (2002). Genetics of type 1 diabetes mellitus. Genes Immun *3*, 235-249.

Poligone, B., Weaver, D. J., Jr., Sen, P., Baldwin, A. S., Jr., and Tisch, R. (2002). Elevated NF-kappaB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. J Immunol *168*, 188-196.

Prasad, S. J., and Goodnow, C. C. (2002a). Cell-intrinsic effects of non-MHC NOD genes on dendritic cell generation in vivo. Int Immunol *14*, 677-684.

Prasad, S. J., and Goodnow, C. C. (2002b). Intrinsic in vitro abnormalities in dendritic cell generation caused by non-MHC non-obese diabetic genes. Immunol Cell Biol *80*, 198-206.

Prieto, A. L., Weber, J. L., and Lai, C. (2000). Expression of the receptor protein-tyrosine kinases Tyro-3, Axl, and mer in the developing rat central nervous system. J Comp Neurol *425*, 295-314.

Rabinovitch, A. (1994). Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? Diabetes *43*, 613-621.

Redondo, M. J., Rewers, M., Yu, L., Garg, S., Pilcher, C. C., Elliott, R. B., and Eisenbarth, G. S. (1999). Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. Bmj *318*, 698-702.

Rosmalen, J. G., Homo-Delarche, F., Durant, S., Kap, M., Leenen, P. J., and Drexhage, H. A. (2000). Islet abnormalities associated with an early influx of dendritic cells and macrophages in NOD and NODscid mice. Lab Invest *80*, 769-777.

Rosmalen, J. G., Pigmans, M. J., Kersseboom, R., Drexhage, H. A., Leenen, P. J., and Homo-Delarche, F. (2001). Sex steroids influence pancreatic islet hypertrophy and subsequent autoimmune infiltration in nonobese diabetic (NOD) and NODscid mice. Lab Invest *81*, 231-239.

Rosmalen, J. G., van Ewijk, W., and Leenen, P. J. (2002). T-cell education in autoimmune diabetes: teachers and students. Trends Immunol *23*, 40-46.

Rossi, M., and Young, J. W. (2005). Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J Immunol *175*, 1373-1381.

Sauter, B., Albert, M. L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med *191*, 423-434.

Savino, W., Boitard, C., Bach, J. F., and Dardenne, M. (1991). Studies on the thymus in nonobese diabetic mouse. I. Changes in the microenvironmental compartments. Lab Invest *64*, 405-417.

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Serreze, D. V., and Leiter, E. H. (2001). Genes and cellular requirements for autoimmune diabetes susceptibility in nonobese diabetic mice. Curr Dir Autoimmun *4*, 31-67.

Shinomiya, M., Nadano, S., Shinomiya, H., and Onji, M. (2000). In situ characterization of dendritic cells occurring in the islets of nonobese diabetic mice during the development of insulitis. Pancreas *20*, 290-296.

Shoda, L. K., Young, D. L., Ramanujan, S., Whiting, C. C., Atkinson, M. A., Bluestone, J. A., Eisenbarth, G. S., Mathis, D., Rossini, A. A., Campbell, S. E., *et al.* (2005). A comprehensive review of interventions in the NOD mouse and implications for translation. Immunity *23*, 115-126.

Skarsvik, S., Tiittanen, M., Lindstrom, A., Casas, R., Ludvigsson, J., and Vaarala, O. (2004). Poor in vitro maturation and pro-inflammatory cytokine response of dendritic cells in children at genetic risk of type 1 diabetes. Scand J Immunol *60*, 647-652.

Starr, T. K., Jameson, S. C., and Hogquist, K. A. (2003). Positive and negative selection of T cells. Annu Rev Immunol *21*, 139-176.

Steinman, R. M., Bonifaz, L., Fujii, S., Liu, K., Bonnyay, D., Yamazaki, S., Pack, M., Hawiger, D., Iyoda, T., Inaba, K., and Nussenzweig, M. C. (2005). The innate functions of dendritic cells in peripheral lymphoid tissues. Adv Exp Med Biol *560*, 83-97.

Steinman, R. M., Turley, S., Mellman, I., and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med *191*, 411-416.

Steptoe, R. J., Ritchie, J. M., and Harrison, L. C. (2002). Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. J Immunol *168*, 5032-5041.

Stratmann, T., Apostolopoulos, V., Mallet-Designe, V., Corper, A. L., Scott, C. A., Wilson, I. A., Kang, A. S., and Teyton, L. (2000). The I-Ag7 MHC class II molecule linked to murine diabetes is a promiscuous peptide binder. J Immunol *165*, 3214-3225.

Strid, J., Lopes, L., Marcinkiewicz, J., Petrovska, L., Nowak, B., Chain, B. M., and Lund, T. (2001). A defect in bone marrow derived dendritic cell maturation in the nonobesediabetic mouse. Clin Exp Immunol *123*, 375-381.

Stuart, L. M., Lucas, M., Simpson, C., Lamb, J., Savill, J., and Lacy-Hulbert, A. (2002). Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. J Immunol *168*, 1627-1635.

Summers, K. L., Behme, M. T., Mahon, J. L., and Singh, B. (2003). Characterization of dendritic cells in humans with type 1 diabetes. Ann N Y Acad Sci *1005*, 226-229.

Sutherland, D. E. (2003). Current status of beta-cell replacement therapy (pancreas and islet transplantation) for treatment of diabetes mellitus. Transplant Proc *35*, 1625-1627.

Sutherland, D. E., Gruessner, R. W., Dunn, D. L., Matas, A. J., Humar, A., Kandaswamy, R., Mauer, S. M., Kennedy, W. R., Goetz, F. C., Robertson, R. P., *et al.* (2001). Lessons learned from more than 1,000 pancreas transplants at a single institution. Ann Surg *233*, 463-501.

Takahashi, M., and Kobayashi, Y. (2003). Cytokine production in association with phagocytosis of apoptotic cells by immature dendritic cells. Cell Immunol *226*, 105-115.

Thomas-Vaslin, V., Damotte, D., Coltey, M., Le Douarin, N. M., Coutinho, A., and Salaun, J. (1997). Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. Proc Natl Acad Sci U S A *94*, 4598-4603.

Tisch, R., and McDevitt, H. (1996). Insulin-dependent diabetes mellitus. Cell *85*, 291-297.

Tisch, R., Yang, X. D., Singer, S. M., Liblau, R. S., Fugger, L., and McDevitt, H. O. (1993). Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature *366*, 72-75.

Todd, J. A., Bell, J. I., and McDevitt, H. O. (1987). HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature *329*, 599-604.

Trembleau, S., Penna, G., Bosi, E., Mortara, A., Gately, M. K., and Adorini, L. (1995). Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice. J Exp Med *181*, 817-821.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol *3*, 133-146.

Trinchieri, G., Kubin, M., Bellone, G., and Cassatella, M. A. (1993). Cytokine cross-talk between phagocytic cells and lymphocytes: relevance for differentiation/activation of phagocytic cells and regulation of adaptive immunity. J Cell Biochem *53*, 301-308.

Trinchieri, G., Wysocka, M., D'Andrea, A., Rengaraju, M., Aste-Amezaga, M., Kubin, M., Valiante, N. M., and Chehimi, J. (1992). Natural killer cell stimulatory factor (NKSF) or interleukin-12 is a key regulator of immune response and inflammation. Prog Growth Factor Res *4*, 355-368.

Trudeau, J. D., Dutz, J. P., Arany, E., Hill, D. J., Fieldus, W. E., and Finegood, D. T. (2000). Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes *49*, 1-7.

Turley, S., Poirot, L., Hattori, M., Benoist, C., and Mathis, D. (2003). Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. J Exp Med *198*, 1527-1537.

Verge, C. F., Gianani, R., Kawasaki, E., Yu, L., Pietropaolo, M., Jackson, R. A., Chase, H. P., and Eisenbarth, G. S. (1996). Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes *45*, 926-933.
von Boehmer, H. (1994). Positive selection of lymphocytes. Cell *76*, 219-228.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L., and Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. J Exp Med *176*, 47-58.

Wallet, M. A., Sen, P., and Tisch, R. (2005). Immunoregulation of dendritic cells. Clin Med Res *3*, 166-175.

Wang, X., Wilkinson, V. L., Podlaski, F. J., Wu, C., Stern, A. S., Presky, D. H., and Magram, J. (1999). Characterization of mouse interleukin-12 p40 homodimer binding to the interleukin-12 receptor subunits. Eur J Immunol *29*, 2007-2013.

Wang, Y. H., and Liu, Y. J. (2004). Mysterious origin of plasmacytoid dendritic cell precursors. Immunity *21*, 1-2.

Weaver, D. J., Jr., Poligone, B., Bui, T., Abdel-Motal, U. M., Baldwin, A. S., Jr., and Tisch, R. (2001). Dendritic cells from nonobese diabetic mice exhibit a defect in NF-kappa B regulation due to a hyperactive I kappa B kinase. J Immunol *167*, 1461-1468.

Wheat, W., Kupfer, R., Gutches, D. G., Rayat, G. R., Beilke, J., Scheinman, R. I., and Wegmann, D. R. (2004). Increased NF-kappa B activity in B cells and bone marrow-derived dendritic cells from NOD mice. Eur J Immunol *34*, 1395-1404.

Wicker, L. S., Todd, J. A., and Peterson, L. B. (1995). Genetic control of autoimmune diabetes in the NOD mouse. Annu Rev Immunol *13*, 179-200.

Wong, F. S., and Janeway, C. A., Jr. (1999). The role of CD4 vs. CD8 T cells in IDDM. J Autoimmun *13*, 290-295.

Wu, L., and Shortman, K. (2005). Heterogeneity of thymic dendritic cells. Semin Immunol *17*, 304-312.

Wu, Y., Singh, S., Georgescu, M. M., and Birge, R. B. (2005). A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. J Cell Sci *118*, 539-553.

Wu, Y. M., Robinson, D. R., and Kung, H. J. (2004). Signal pathways in up-regulation of chemokines by tyrosine kinase MER/NYK in prostate cancer cells. Cancer Res *64*, 7311-7320.

Xu, D. L., Liu, Y., Tan, J. M., Li, B., Zhong, C. P., Zhang, X. H., Wu, C. Q., and Tang, X. D. (2004). Marked prolongation of murine cardiac allograft survival using recipient immature dendritic cells loaded with donor-derived apoptotic cells. Scand J Immunol *59*, 536-544.

Yoon, J. W., and Jun, H. S. (2005). Autoimmune destruction of pancreatic beta cells. Am J Ther *12*, 580-591.

Yu, L., Robles, D. T., Abiru, N., Kaur, P., Rewers, M., Kelemen, K., and Eisenbarth, G. S. (2000). Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. Proc Natl Acad Sci U S A *97*, 1701-1706.

Zacher, T., Knerr, I., Rascher, W., Kalden, J. R., and Wassmuth, R. (2002). Characterization of monocyte-derived dendritic cells in recent-onset diabetes mellitus type 1. Clin Immunol *105*, 17-24.

Zhao, C., Lu, S., Zhou, X., Zhang, X., Zhao, K., and Larsson, C. (2006). A novel locus (RP33) for autosomal dominant retinitis pigmentosa mapping to chromosomal region 2cen-q12.1. Hum Genet *119*, 617-623.

CHAPTER II

THE ROLE OF MERTK IN APOPTOTIC CELL-INDUCED SELF-TOLERANCE

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Abstract

Self-antigens expressed by apoptotic cells may become targets for autoimmunity. Tolerance to these self-antigens is established partly by an ill-defined capacity of apoptotic cells to inhibit the activation and function of antigen presenting cells such as dendritic cells (DC). We present evidence that the receptor tyrosine kinase Mer (MerTK) has a key role in mediating apoptotic cell-induced inhibition of DC. Pretreatment of DC prepared from nonobese diabetic (NOD) mice with apoptotic cells blocked IL-12p70 secretion and up-regulation of co-stimulatory molecule expression following stimulation. In contrast, NOD DC treated with a blocking anti-MerTK antibody or lacking MerTK expression (NOD.MerTK$^{KD/KD}$) were resistant to apoptotic cell-induced inhibition. Apoptotic cell pretreatment failed to inhibit the capacity of NOD.MerTK$^{KD/KD}$ but not NOD DC to stimulate CD4$^+$ and CD8$^+$ T cells *in vitro*. Similarly, diabetogenic CD4$^+$ T cells adoptively transferred into NOD.MerTK$^{KD/KD}$ mice in which β cell apoptosis was induced, exhibited increased expansion and differentiation into type 1 T effectors. Finally, the development of

autoimmune diabetes was exacerbated in NOD.MerTK$^{KD/KD}$ versus NOD mice expressing a diabetogenic transgenic T cell receptor. These findings demonstrate that MerTK plays a critical role in apoptotic cell-induced self-tolerance.

Introduction

The processes of tissue remodeling, wound healing and removal of damaged cells rely on cell death by apoptosis. Cells undergoing apoptosis undergo a highly ordered process characterized by cell shrinkage, retention of organelles, nuclear chromatin condensation and fragmentation, and redistribution of phosphatidylserine (PS) on the plasma membrane (Fadok et al., 1992; Kerr et al., 1972). Under homeostatic conditions, APC such as macrophages and DC recognize and ingest apoptotic cells using a variety of receptors including the PS receptor, scavenger receptor-A (SR-A), CD36 and $\alpha_v\beta_5$ integrin (Wallet et al., 2005). In this way, the breakdown and release of potentially immunogenic self-antigens by apoptotic cells is avoided (Savill et al., 1993). Indeed, inefficient clearance of apoptotic cells has been linked to autoimmunity (Gaipl et al., 2004).

The binding and ingestion of apoptotic cells also results in immunoregulation of APC to further promote self-tolerance. For instance, activation of macrophages is blocked and TGF-β secretion induced by apoptotic cell pretreatment (Huynh et al., 2002; Reidy and Wright, 2003). Similarly, immature DC become refractory to subsequent stimulation upon encounter with apoptotic cells, and maintain a "tolergenic" phenotype characterized by low levels of MHC, CD40, CD80 and CD86 expression and the lack of proinflammatory cytokine secretion (Morelli et al., 2003; Voll et al., 1997). Consequently, these DC are unable to

effectively stimulate naïve T cells or promote type 1 $CD4^+$ T cell differentiation. In the non-obese diabetic (NOD) mouse model of autoimmune diabetes, induction of limited apoptosis of pancreatic β cells prevents progression of the diabetogenic response. This correlates with the establishment of tolergenic DC, which in turn elicit immunoregulatory T cells (Hugues et al., 2002). The key receptors expressed by DC and the relative contribution of these receptors in mediating the inhibitory effects of apoptotic cells, however, remain ill defined.

A family of receptor tyrosine kinases (RTKs) consisting of Axl, Tyro3 and Mer (MerTK) has been reported to regulate homeostatic activation of macrophages and DC. Mice lacking all three RTKs exhibit systemic autoimmunity marked by highly activated APC *in vivo* (Lu and Lemke, 2001). The mechanism(s) by which Axl, Tyro3 and MerTK regulate APC activation is unclear. MerTK, which also is expressed by NKT cells, certain epithelial cell types and reproductive tissues, is required for efficient phagocytosis of apoptotic cells by peritoneal macrophages (Cohen et al., 2002; Scott et al., 2001). Lupus-like symptoms detected in aging C57BL/6 mice in which MerTK expression is blocked due to insertion of a neomycin cassette in the kinase domain of the *mertk* gene ($MerTK^{KD}$) are believed to be, in part, the result of inefficient clearance of apoptotic cells by macrophages. Investigation of retinal pigment epithelial cells (RPE) has also shown that engulfment of apoptotic outer segments shed from the photoreceptor is mediated by MerTK via recognition of growth arrest-specific protein-6 (Gas6) and protein S (Behrens et al., 2003) (Nandrot et al., 2004). Gas6 is ubiquitously expressed and in addition to binding MerTK, also serves as a PS opsonin mediating phagocytosis of apoptotic cells (Ishimoto et al., 2000).

In view of its function as a receptor for apoptotic cells by macrophages and RPE, we investigated a role for MerTK in immunoregulation of DC by apoptotic cells. Data is

presented demonstrating that MerTK$^{KD/KD}$ DC are no longer sensitive to the inhibitory effects of apoptotic cells, and that T1D is exacerbated in MerTK$^{KD/KD}$ NOD mice (NOD.MerTK$^{KD/KD}$) expressing a β cell-specific transgenic TCR.

Results

Apoptotic cells inhibit DC activation and maturation via MerTK

Previous studies have demonstrated that apoptotic cells regulate DC activation and maturation (Chen et al., 2004b). Furthermore, MerTK has been shown to mediate apoptotic cell phagocytosis by macrophages and RPE. Accordingly, a role for MerTK in apoptotic cell-induced inhibition of DC was investigated using NOD.MerTK$^{KD/KD}$ mice as a source of DC lacking MerTK expression. The NOD.MerTK$^{KD/KD}$ line of mice used in this study contained a 17 cM segment of 129/Ola chromosome 2 harboring the MerTK^{KD} mutation that was introgressed 11 backcross generations (see Materials and Methods).

Pretreatment of NOD mouse bone marrow-derived DC (BMDC) with apoptotic cells for 3 hrs completely inhibited LPS-stimulated IL-12p70 secretion that was readily detected in LPS-only treated BMDC (Figure 2.1A). In contrast, NOD.MerTK$^{KD/KD}$ BMDC secreted similar levels of IL-12p70 in response to LPS, regardless of apoptotic cell pretreatment (Figure 2.1A). Apoptotic cells also blocked LPS-stimulated IL-12p70 secretion by splenic DC (sDC) prepared from NOD but not NOD.MerTK$^{KD/KD}$ mice (Figure 2.1B). Notably, in MerTK-expressing CD11c^{+}CD8α^{+} and CD11c^{+}CD11b^{+} NOD sDC (Figure 2.2B, C), LPS-stimulated expression of intracellular IL-12 was inhibited by apoptotic cell pretreatment (Figure 2.1C).

In order to rule out strain-specific variations in the NOD mouse that could be contributing to the effects of apoptotic cells upon DC, non-autoimmune BALB/c DC were analyzed. As demonstrated in Figure 2.6A, apoptotic cells also induced inhibition of IL-12p70 production in BALB/c BMDC. In addition, BMDC from B6.MerTK$^{KD/KD}$ mice, like NOD.MerTK$^{KD/KD}$ DC, were no longer sensitive to the inhibitory effects of apoptotic cells (unpublished data P.S., M.W. and R.T.).

Analogous to IL-12 production, up-regulation of CD40, CD80, and CD86 expression was inhibited by apoptotic cell pretreatment of NOD BMDC stimulated with LPS (Figure 2.1D). In contrast, apoptotic cell pretreatment had no significant effect on the induction of co-stimulatory molecule expression by LPS-stimulated NOD.MerTK$^{KD/KD}$ BMDC (Figure 2.1D).

To rule out the possibility that apoptotic cell-induced inhibition was selective to LPS stimulation, NOD versus NOD.MerTK$^{KD/KD}$ BMDC treated with anti-CD40 antibody (Ab) were compared. Crosslinking with anti-CD40 Ab induced IL-12p70 secretion by NOD BMDC that was significantly reduced by apoptotic cell pretreatment ($p<10^{-3}$) (Figure 2.1E). On the other hand, anti-CD40 Ab-induced IL-12p70 secretion was unaffected by apoptotic cell pretreatment of NOD.MerTK$^{KD/KD}$ BMDC (Figure 2.1E). Stimulation of NOD BMDC with anti-CD40 Ab also induced up regulation of CD40 expression. CD40 induction was inhibited by co-culture of DC with apoptotic cells before CD40 crosslinking (Figure 2.1F). CD40 expression by NOD.MerTK$^{KD/KD}$ BMDC was not inhibited by apoptotic cells (Figure 2.1F).

The effect of apoptotic cell pretreatment on the capacity of NOD and NOD.MerTK$^{KD/KD}$ BMDC to stimulate CD4$^+$ and CD8$^+$ T cells *in vitro* was investigated. For this purpose, CD4$^+$

and $CD8^+$ T cells were isolated from the spleens of NOD.BDC2.5 (Haskins et al., 1989; Katz et al., 1993) and NOD.CL4 (Morgan et al., 1996) TCR transgenic mice, respectively. In the absence of apoptotic cells, peptide-pulsed NOD and NOD.MerTK$^{KD/KD}$ BMDC stimulated BDC2.5 $CD4^+$ and CL4 $CD8^+$ T cells to similar levels based on IL-2 secretion (Figure 2.3A, B). However, apoptotic cell pretreatment significantly inhibited the capacity of peptide-pulsed NOD BMDC to stimulate BDC2.5 $CD4^+$ ($p<10^{-3}$) and CL4 $CD8^+$ ($p<10^{-3}$) T cells (Figure 2.3A, B). In contrast, apoptotic cell pretreatment failed to inhibit peptide-pulsed NOD.MerTK$^{KD/KD}$ BMDC stimulation of BDC2.5 $CD4^+$ and CL4 $CD8^+$ T cells (Figure 2.3A, B).

Finally, to ensure that the lack of sensitivity of NOD.MerTK$^{KD/KD}$ DC to apoptotic cell pretreatment was not due to an artifact of DC developing in the absence of MerTK expression, the effect of a blocking anti-MerTK polyclonal Ab on NOD BMDC was assessed. Importantly, the blocking function of the polyclonal Ab was verified by demonstrating that phosphorylation of MerTK induced by apoptotic cells was effectively inhibited by treating NOD DC with anti-MerTK Ab but not an isotype control Ab (Figure 2.4). Despite apoptotic cell pretreatment, LPS-stimulated IL-12p70 secretion (Figure 2.5A) and up-regulation of co-stimulatory molecule expression (Figure 2.5B) were readily detected in NOD BMDC incubated with anti-MerTK Ab. Furthermore, apoptotic cells failed to inhibit stimulation of BDC2.5 $CD4^+$ (Figure 2.5C) and CL4 $CD8^+$ (Figure 2.5D) T cells by NOD BMDC incubated with anti-MerTK but not the isotype control Ab. Together these findings demonstrate that apoptotic cell-induced inhibition of activation, maturation and effector function of BMDC or sDC is dependent on MerTK expression/activation.

The MerTK ligand Gas6 is expressed by apoptotic cells and is necessary for apoptotic cell-induced inhibition of DC

An effort was made to define the ligand recognized by MerTK on the surface of AC. Gas6 and Protein S are two known ligands of MerTK (Hall et al., 2005), although other, yet to be identified, ligands have been proposed. Initial work indicated that Protein S, which is found in serum, was not associated with apoptotic cells used in our experiments. RTPCR analysis was performed and did not detect Protein S gene expression in apoptotic cells. Additionally, the inhibitory properties of apoptotic cells on NOD BMDC were not affected when serum-free medium was used in the experiments (unpublished data P.S., M.W. and R.T.). Next, whether Gas6 functions as a MerTK ligand was determined. Gas6 has been reported to bind PS (Wu et al., 2006) and therefore should be exposed on the outer leaflet of apoptotic cells. A VAD-FMK-FITC conjugate was used to identify apoptotic cells in cultures of γ-irradiated thymocytes, in addition to apoptotic thymocytes prepared directly from the thymus. Flow cytometric analysis demonstrated that Gas6 was detected on the surface of apoptotic thymocytes induced by γ-irradiation, and apoptotic thymocytes found normally within the thymus (Figure 2.7A). Furthermore, Gas6 protein expression was induced, as determined by Western Blot, in irradiated thymocytes cultured under serum-free conditions (Figure 2.7C)

A direct role for Gas6 in AC-mediated inhibition of DC was determined by using a polyclonal anti-Gas6 Ab. Pretreatment of apoptotic cells with anti-Gas6 but not an isotype control Ab effectively blocked the capacity of apoptotic cells to inhibit LPS-stimulated IL-12p70 secretion by NOD BMDC (Figure 2.7C). These results demonstrate that Gas6 surface expression is induced upon apoptotic cells, and is necessary for MerTK-mediated inhibition of DC.

MerTK-mediated recognition of apoptotic cells induces *in vivo* tolerance to self antigens

The lack of sensitivity of peripheral MerTK$^{KD/KD}$ DC to AC-induced inhibition *in vivo* would be expected to promote autoimmunity. To initially test this hypothesis, NOD.BDC2.5 mice expressing the β cell-specific BDC2.5 TCR transgene were bred with NOD.MerTK$^{KD/KD}$ mice, and the development of diabetes monitored in female mice that expressed wild-type levels of MerTK (NOD.BDC2.5) or no MerTK (NOD.MerTK$^{KD/KD}$.BDC2.5). Only 10% (1/10) of NOD.BDC2.5 female mice developed diabetes (Figure 2.8), consistent with frequencies reported for other NOD.BDC2.5 colonies. In contrast, the age of diabetes onset was earlier (11 weeks vs. 21 weeks) and the frequency of diabetes (65% vs. 10%) was markedly increased in NOD.MerTK$^{KD/KD}$.BDC2.5 versus NOD.BDC2.5 female mice (Figure 2.8).

The above results suggested that in NOD.MerTK$^{KD/KD}$.BDC2.5 mice, apoptotic β cells failed to inhibit DC activation and/or maturation leading to enhanced activation of BDC2.5 CD4$^+$ T cells, and exacerbation of disease progression. To further investigate this hypothesis, a model system was established examining the reactivity of naïve BDC2.5 CD4$^+$ T cells transferred into 4 week-old NOD versus NOD.MerTK$^{KD/KD}$ recipients in which β cell apoptosis was synchronized by treatment with a single dose of streptozotocin (STZ). This approach was necessary since analysis of NOD.BDC2.5 and NOD.MerTK$^{KD/KD}$.BDC2.5 mice is problematic due to heavy islet infiltration at an early age (i.e. by 3 wks of age) and the fact that β cell apoptosis is not synchronized. STZ has been used extensively to induce β cell apoptosis, and treatment of 4 wk old NOD female mice with a single low dose of STZ prevents diabetes due in part to induction of tolergenic DC (Hugues et al., 2002). NOD or NOD.MerTK$^{KD/KD}$ mice received a single i.p. injection of 120 mg/kg of STZ or citrate saline

as a vehicle control. Gas6 was readily detected on the surface of apoptotic β cells following STZ treatment (Figure 2.9). CFSE labeled BDC2.5 $CD4^+$ T cells were adoptively transferred, and 3 days later proliferation was measured in the draining pancreatic lymph nodes. Only limited BDC2.5 $CD4^+$ T cell proliferation was detected in NOD recipients treated with STZ or citrate saline (Figure 2.10). Similarly, no significant BDC2.5 $CD4^+$ T cell proliferation was observed in citrate saline treated NOD.MerTK$^{KD/KD}$ recipients (Figure 2.10). In marked contrast, BDC2.5 $CD4^+$ T cells exhibited significant proliferation in NOD.MerTK$^{KD/KD}$ mice treated with STZ (Figure 2.10).

The total number of $CFSE^+$ BDC2.5 $CD4^+$ T cells in the pancreatic lymph nodes of citrate saline-treated NOD and NOD.MerTK$^{KD/KD}$ mice was similar (Table 2.1). In contrast, significant increases in the total number (11-fold) of $CFSE^+$ BDC2.5 $CD4^+$ T cells and the frequency (16-fold) of $IFN\gamma^+$ $CFSE^+$ BDC2.5 $CD4^+$ T cells were detected in STZ-treated NOD.MerTK$^{KD/KD}$ versus NOD recipients (Table 2.1). These data suggest that MerTK is essential for *in vivo* tolerance to apoptotic cells and that apoptotic cell death in the absence of MerTK expression on DC leads to aberrant T cell stimulation. Experiments are ongoing to address the specific cell types which are responsible for excessive T cell priming in MerTK-deficient mice.

Discussion

Apoptotic cells provide a continuous source of self-antigen which can be ingested, processed and presented by DC. Mechanisms are in place to ensure that DC do not initiate immunity to these self-antigens. This study was undertaken to gain further insight into

apoptotic cell-induced immunoregulation of DC. MerTK has an established role in phagocytosis of apoptotic cells by macrophages (Cohen et al., 2002; Scott et al., 2001; Wu et al., 2005) and RPE (Hall et al., 2001). In addition, MerTK and its family members, Axl and Tyro3, mediate immunoregulation in mice (Camenisch et al., 1999; Lu and Lemke, 2001). In light of these facts, a potential role for MerTK in immunoregulation of DC by apoptotic cells was investigated. Analysis of BMDC as well as *ex vivo*-derived $CD11c^+$ $CD11b^+$ and $CD11c^+$ $CD8\alpha^+$ sDC indicated an essential role for MerTK in apoptotic cell-induced immunoregulation. Pretreatment of BMDC or sDC with apoptotic cells induced inhibition of DC activation in response to LPS or CD40 ligation. Typically, LPS and CD40 ligation induce IL-12p70 secretion and expression of co-stimulatory molecules. Apoptotic cells inhibited LPS- or CD40-induced IL-12p70 secretion and co-stimulatory molecule expression by NOD DC but not by MerTK-deficient NOD DC (Figure 2.1). The defect observed in NOD.MerTK$^{KD/KD}$ DC was not due to alterations in DC development as demonstrated by blockade of MerTK upon NOD DC with a specific polyclonal Ab. Pre-treatment of NOD DC with anti-MerTK Ab prevented the inhibitory effects of apoptotic cells similar to MerTK-deficient DC (Figure 2.5).

Apoptotic cell-induced immunoregulation of DC also inhibited the capacity of DC to stimulate $CD4^+$ and $CD8^+$ T cells. Again, MerTK-deficient DC were not inhibited by apoptotic cells and continued to stimulate T cells (Figure 2.3). This is not surprising, as DC mediated T cell stimulation requires co-stimulatory molecule expression and positive feedback activation of DC through CD40, both of which are inhibited by apoptotic cells.

Importantly, DC from NOD or NOD.MerTK$^{KD/KD}$ mice functioned similarly in the absence of apoptotic cells. Levels of IL-12 production, co-stimulatory molecule expression

and T cell stimulation were similar regardless of MerTK expression (Figure 2.1). Only when pre-treated with apoptotic cells was there a marked difference in activation and maturation between MerTK-expressing versus MerTK-deficient DC. Thus, the primary role of MerTK in DC is recognition of apoptotic cells and subsequent transduction of inhibitory signals. For example, MerTK was dispensable for efficient phagocytosis of apoptotic cells by DC (Figure 2.11) but not for macrophages and RPE.

Importantly, the effects observed in NOD DC are not strain-specific. DC from BALB/c mice were similarly immunoregulated by apoptotic cells via MerTK (Figure 2.6). In addition, MerTK-deficient C57BL/6 DC were resistant to apoptotic cell-induced inhibition similar to NOD.MerTK$^{KD/KD}$ mice (unpublished data P.S., M.W. and R.T.).

Previous studies have shown that Gas6 is a ligand bound by MerTK (Chen et al., 1997; Nagata et al., 1996) and Gas6 is essential for MerTK-mediated phagocytosis of apoptotic OS cells by RPE (Gould et al., 2005; Hall et al., 2003; Hall et al., 2001; Naquet et al., 1999). Additionally, Gas6 induces platelet activation via MerTK (Chen et al., 2004a; Gould et al., 2005). These findings, along with a well-established role for Gas6 as a PS opsonin (Nakano et al., 1997), led to investigation of Gas6 as the ligand for MerTK in apoptotic cell-induced inhibition of DC. Gas6 was expressed on the surface of apoptotic thymocytes (Figure 2.7A) as well as apoptotic β cells (Figure 2.9). Antibody blockade of Gas6 prevented the immunoregulatory effects of apoptotic cells upon DC (Figure 2.7C). These findings clearly demonstrate that MerTK binds Gas6 on the surface of apoptotic cells triggering signaling events which inhibit subsequent DC activation.

The impact of MerTK-deficiency on DC immunoregulation led to the prediction that peripheral T cell-tolerance would be defective in mice lacking MerTK. The NOD.BDC2.5

mouse provides an ideal model to test this hypothesis *in vivo*. Although NOD.BDC2.5 mice exhibit significant insulitis, only a relatively low frequency (10-20%) of mice progress beyond Checkpoint 1 and develop overt diabetes (Andre et al., 1996; Katz et al., 1995; Ohashi et al., 1993). Notably, NOD.MerTK$^{KD/KD}$.BDC2.5 mice developed accelerated onset and increased frequency of diabetes compared to NOD.BDC2.5 mice (Figure 2.8). This finding supports a role for MerTK in regulating T cell-mediated autoimmunity *in vivo.* However, the data only provides indirect evidence that MerTK-mediated immunoregulation *in vivo* is induced by apoptotic cells. To directly address this issue, a model of apoptotic cell-induced immune tolerance was utilized. Previous work had demonstrated that induction of β cell apoptosis with STZ results in DC-mediated immune tolerance and protection from T1D (Hugues et al., 2002). In this model, tolergenic DC are established which, in turn, promote differentiation of immunoregulatory T cells. Our findings suggest that MerTK plays a key role in establishing these tolergenic DC. For example, STZ treatment of NOD.MerTK$^{KD/KD}$ mice induced increased expansion and type 1 differentiation of BDC2.5 CD4$^+$ T compared to similarly treated NOD mice (Figure 2.10, Table 2.1). Importantly, differences in BDC2.5 CD4$^+$ T cell proliferation and differentiation were not the result of aberrant induction of β cell apoptosis or clearance of apoptotic β cells, which were similar between STZ-treated NOD and NOD.MerTK$^{KD/KD}$ mice (data not shown).

We propose that DC lacking MerTK expression become activated upon encountering apoptotic β cells and, in turn, promote a pro-inflammatory response. The latter is characterized by differentiation of pathogenic T effectors which drive the progression of β cell destruction and the development of diabetes.

One highly intriguing issue centers on the location of *mertk* within a T1D-susceptibility locus in mice and humans. It is possible that allelic variants of *mertk* encode proteins with differing immunoregulatory properties. Thus, activation status of DC may be altered, resulting in different levels of autoimmune susceptibility. Analysis of *mertk* alleles in mice is discussed further in Chapter 3.

Interpretation of MerTK-deficiency and its impact upon autoimmunity is not a simple task. Dysregulation of DC function impacts not just peripheral immunity but also thymic selection, due to the presence of DC within the thymus. Interestingly, MerTK-deficient NOD mice are protected from spontaneous diabetes due to enhanced thymic negative selection and deletion of pathogenic autoreactive T cells (Chapter 3). This result contrasts with the findings in NOD.MerTK$^{KD/KD}$.BDC2.5 mice, which are prone to T1D, illustrating that alterations in DC function in the periphery and thymus can have dramatically different outcomes.

Together, the data presented here support a novel mechanism of self-tolerance. Apoptotic cells expressing surface Gas6 induce phosphorylation of MerTK on DC. MerTK phosphorylation leads to a signaling cascade which inhibits NF-κB-mediated DC activation (unpublished data, P.S., M.W. and R.T.). MerTK signaling induces functional inhibition of DC and blocks secretion of IL-12p70, up-regulation of co-stimulatory molecules and T cell stimulation. *In vivo* apoptotic β cells induce MerTK-dependent immunoregulation which prevents expansion and differentiation of type 1 CD4$^+$ T cells.

Materials and methods

Mice

NOD/LtJ "NOD", NOD.Cg-Tg(TcraBDC2.5)1Doi Tg(TcrbBDC2.5)2Doi "NOD.BDC2.5", NOD-Tg(TcraCl4, TcrbCl4) "NOD.CL4" and BALB/c mice were maintained and bred under specific-pathogen free conditions. Establishment of MerTK^{KD} mice has been described (Camenisch et al., 1999). Briefly, the tyrosine kinase domain of *Mertk* was replaced with a neomycin resistance gene, and B6.MerTK^{KD} mice established. NOD MerTK^{KD} mice were generated by breeding B6.MerTK^{KD} and NOD mice, and then backcrossing the *Mertk^{KD}* gene onto the NOD genome for an additional 11 generations. At N11, Mouse MapPairs™ distinguishing B6, 129/Ola, and NOD/LtJ [Chr. 2 (D2Mit378, D2Mit94, D2Mit14, D2Mit393, D2Mit395, D2Mit190, D2Mit164, D2Mit256, D2Mit304, D2Mit224, D2Mit338, D2Mit307, D2Mit260, D2Mit309, D2Mit493, D2Mit451, D2Mit496, D2Mit287, D2Mit456, D2Mit265) (Invitrogen, Carlsbad, CA) were used in PCR according to the supplier's directions to define a 17 cM segment derived from 129/Ola and containing *Mertk^{KD}*. Use of mice was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Preparation of BMDC and sDC

BMDC and sDC were prepared from male or female mice between 8-12 weeks as described (Bhattacharyya et al., 2004).

Flow cytometry

The following monoclonal Abs used for fluorescence staining were purchased from BD PharMingen (San Diego, CA): FITC-anti-CD40, FITC-anti-CD86, FITC-anti-CD80, PE-anti-CD11c, APC-anti-CD11c and PE-anti-IFNγ. Goat polyclonal affinity purified anti-MerTK, anti-Gas6 and normal goat IgG were purchased from R&D Systems (Minneapolis, MN). Biotinylated anti-goat IgG was obtained from Vector Laboratories (Burlingame, CA). anti-CD16/32 for Fc blocking, PerCP-streptavidin and PE-streptavidin were purchased from BD Pharmingen. Stained cells were analyzed on a FACScan or FACScalibur (BD Biosciences, San Jose, CA) using Summit Software (Cytomation, Ft. Collins, CO).

Pretreatment of DC with apoptotic cells

Thymocytes prepared from 4-6 week old mice, were adhered to plastic for 2 hr to remove DC and macrophages, irradiated at 600 rad and then cultured in base medium for 12 hr. Flow cytometry demonstrated >95% apoptotic and <1% necrotic thymocytes based on Annexin-V and propidium iodide staining. Apoptosis was confirmed via DNA fragmentation analysis. DC were co-cultured with apoptotic cells at a ratio of 1:10 (DC:apoptotic cell) for 3hr. In some experiments, DC were treated with anti-MerTK Ab prior to apoptotic cell incubation. Briefly, DC ($5x10^6$/well) were incubated with anti-mouse FcγIII/II (BD PharMingen) in 6-well ultra-low cluster plates for 0.5 hr at 37^{o}C to block Fc receptor binding. DC were then treated for 1 hr at 37^{o}C with 20 μg of either goat anti-MerTK Ab (AF591, R&D Systems, Inc., Minneapolis, MN) or goat IgG (R&D Systems), an isotype control. Alternatively, some apoptotic cell samples were pretreated with polyclonal anti-Gas6 or goat IgG (R&D) for 30 min. prior to co-culture with DC.

Phagocytosis of apoptotic cells

DC were tested for phagocytosis of apoptotic cells. Apoptotic thymocytes were stained with 2.5 μM Cell Tracker Green (Molecular Probes, Eugene, OR) and co-cultured with DC at a ratio of 5:1 for 6 hr at 37°C or 4°C. Cells were then cultured for 10 min in PBS with 2 mM EDTA and washed vigorously to remove non-ingested but bound apoptotic cells which was confirmed by confocal microscopy. DC were stained with APC-anti-CD11c and analyzed for ingestion of fluorescent apoptotic cells by flow cytometry.

Measurement of IL-12 production from DC

DC ($9x10^5$/well) were pretreated with or without apoptotic cells for 3 hr, washed and then stimulated with LPS for 72 hr. Supernatants were collected and assayed for IL-12p70 in triplicate using an ELISA kit (BD PharMingen) following the manufacturer's instructions. Intracellular IL-12 staining was performed by using the Cytofix/Cytoperm cell permeabilization kit (BD Biosciences, San Diego, CA) and PE-labeled anti-mouse IL-12p40/70. Alternatively, DC were stimulated by crosslinking CD40. DC were cultured for 30 min with 10μg/ml anti-CD40 IgM (HM40-3, BD Pharmingen). Crosslinking was induced by addition of anti-IgM antibody (G188-2, BD Pharmingen).

DC:T cell stimulation assay

DC were pretreated (or not) with apoptotic cells for 3 hr, washed and plated in 24-well ultra-low cluster plates at $3x10^4$ DC/well. BDC2.5 $CD4^+$ or CL4 $CD8^+$ T cells ($3x10^5$/well) plus varying concentrations (0.1-100 μg/ml) of the I-A^{g7}-restricted BDC2.5 mimotope (RTRPLWVRME) or H2K^d-restricted *influenza* virus hemagglutinin peptide (IYSTVASSL)

were added, and cultures incubated for 72 hr. Supernatants were harvested and IL-2 concentration measured in triplicate by a sandwich ELISA using the JES6-1A12 (eBioscience, San Diego, CA), and biotinylated-JES6-EH4 anti-IL-2 Abs, and streptavidin-HRP (BD PharMingen). Concentration of IL-2 was determined using a standard curve.

Splenic BDC2.5 $CD4^+$ and CL4 $CD8^+$ T cells were prepared by depleting DC, Mϕ and B cells via magnetic cell sorting using anti-CD11c, anti-CD11b and anti-CD19 Ab-magnetic microbeads (Miltenyi Biotec, Auburn, CA), respectively. BDC2.5 $CD4^+$ and CL4 $CD8^+$ T cells were then positively selected using anti-CD4 or anti-CD8 Ab-magnetic microbeads (Miltenyi Biotec). Purity of BDC2.5 $CD4^+$ and CL4 $CD8^+$ T cells was routinely >98%.

Streptozotocin treatment

Four week old NOD or NOD.MerTK^{KD} mice were treated with either 120 mg/kg STZ (Sigma) or citrate saline buffer alone. Four hours later $5x10^6$ NOD.BDC2.5 $CD4^+$ CFSE-labeled T cells were injected intravenously. Five days later, pancreatic lymph nodes and pancreatic islets were harvested, dissociated and stained for FACS analysis of T cell proliferation and IFNγ production.

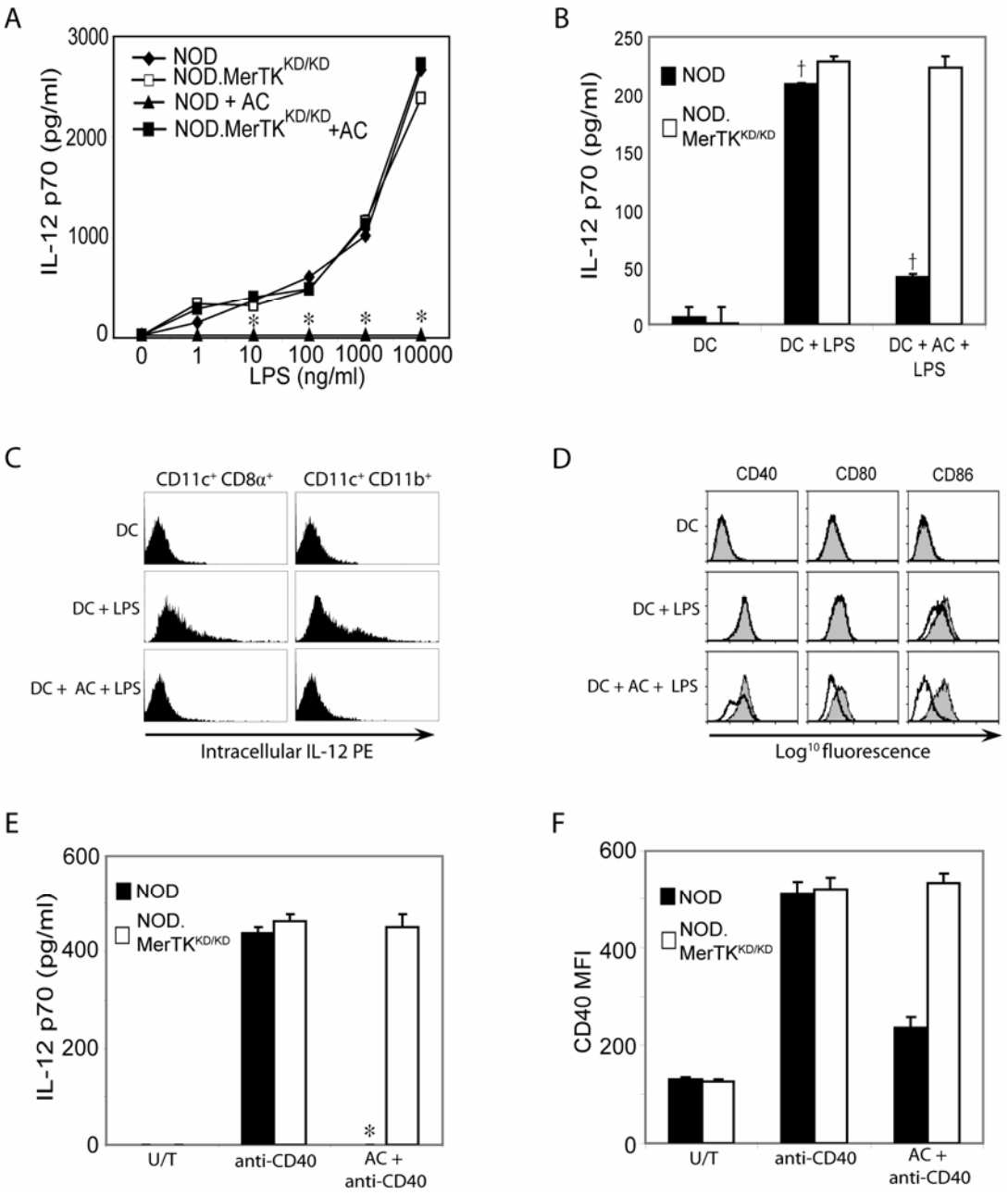
Table 2.1

Analysis of type 1 $CD4^+$ T cell expansion in STZ-treated mice

	Cells per pancreatic lymph node			
	NOD		NOD.MerTK$^{KD/KD}$	
	Cit Sal	STZ	Cit Sal	STZ
$CFSE^+$ BDC2.5 $CD4^+$ T Cells	5,695	4,950	3300	54,560
$CFSE^+$ $IFN\gamma^+$ BDC2.5 $CD4^+$ T Cells	670	275	275	4,400

Cit Sal = 100μl citrate saline pH4.5
STZ = 120mg/kg streptozotocin in citrate saline

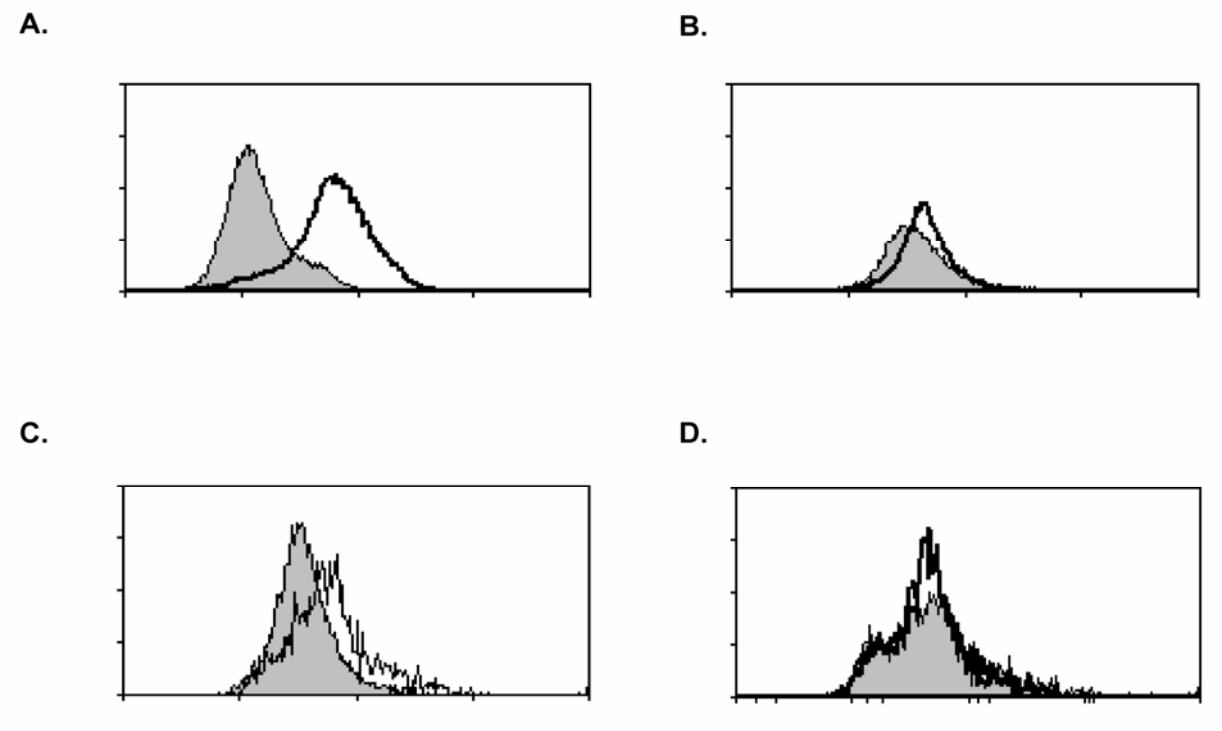
Figure 2.1



MerTK deficient DC are not immunoregulated by apoptotic cells when stimulated with LPS or CD40 ligation. (A) BMDC from NOD or NOD.MerTK$^{KD/KD}$ mice were untreated or co-cultured with apoptotic cells (AC) followed by stimulation with titrated doses of LPS for 72hr. IL-12p70 production was determined by ELISA. (B) *Ex vivo* derived sDC from NOD and NOD.MerTK$^{KD/KD}$ mice were untreated or co-cultured with AC the treated with 1μg/ml LPS for 72hr and IL-12p70 levels were measured. (C) *Ex vivo* NOD sDC were untreated or co-cultured with AC. After 24hr culture with 1μg/ml LPS, sDC were stained for surface

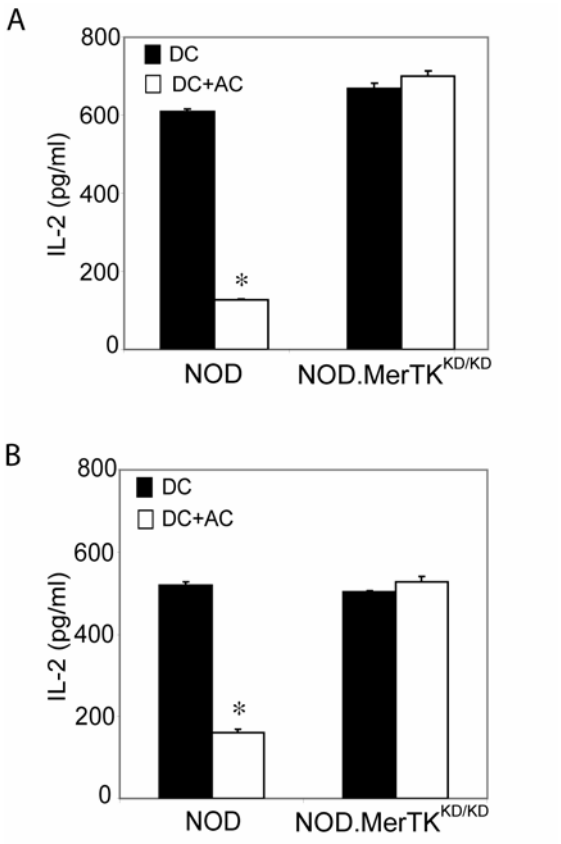
markers and intracellular IL-12. (D) NOD (open histograms) and NOD.MerTK$^{KD/KD}$ (shaded histograms) BMDC were untreated or co-cultured with AC followed by stimulation with 100 ng/ml LPS for 24hr. DC were stained for co-stimulatory molecules and gated on CD11c^{+} cells for analysis. (E) NOD and NOD.MerTK$^{KD/KD}$ BMDC were untreated or co-cultured with AC and the stimulated by crosslinking CD40. Production of IL-12p70 was measured, and (F) expression of CD40 was assessed by FACS as determined by mean fluorescence intensity (MFI). Student's t test *$p<0.001$, †$p=0.011$

Figure 2.2



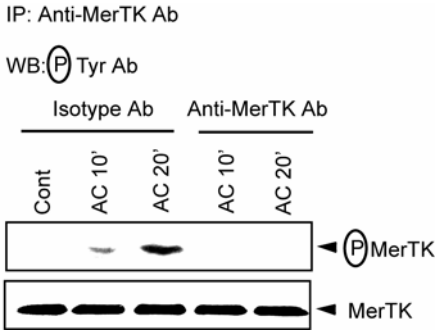
MerTK is expressed by conventional DC. FACS analysis was performed to determine expression levels of MerTK on (A) BMDC, (B) $CD11c^{+}CD11b^{+}$ DC, (C) $CD11c^{+}CD8\alpha^{+}$ DC and (D) $CD11c^{+}B220^{+}$ plasmacytoid DC. Shaded histograms represent background staining of NOD.$MerTK^{KD/KD}$ DC and open histograms represent MerTK staining on NOD DC.

Figure 2.3



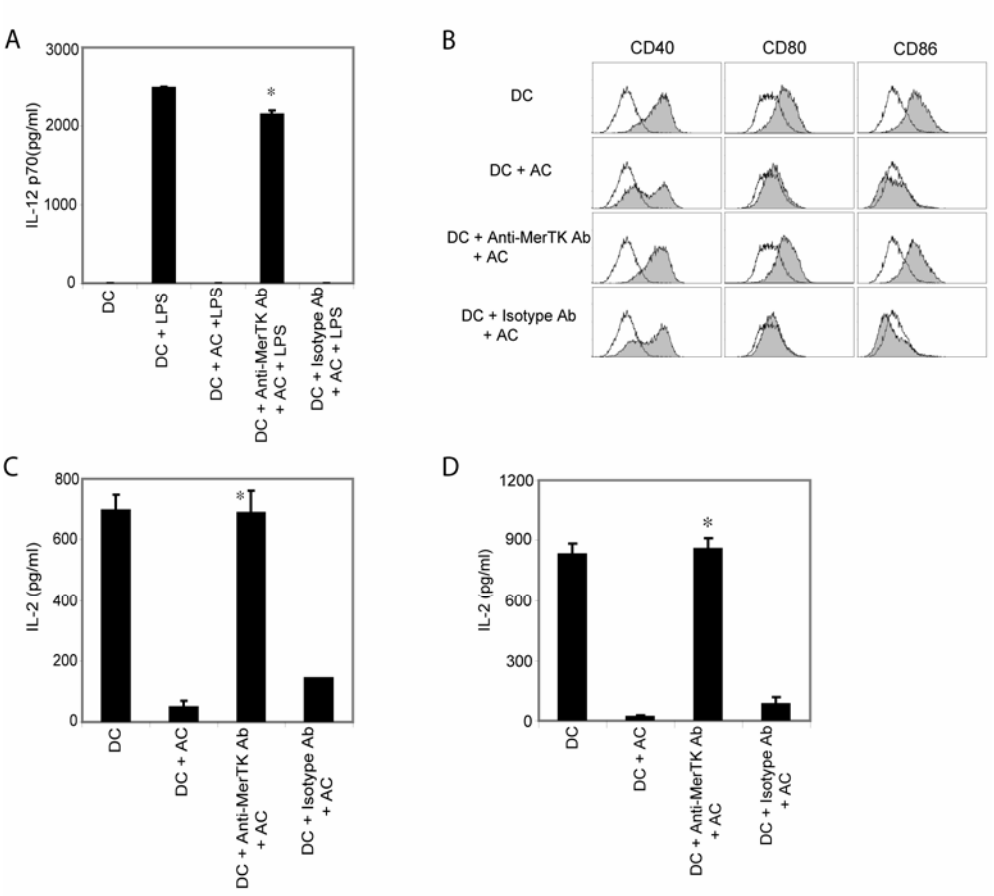
T cell stimulation by MerTK deficient DC is not inhibited by apoptotic cells.
MerTK$^{KD/KD}$ BMDC were left untreated or co-cultured with apoptotic cells (AC). (A) DC were pulsed with BDC2.5 peptide and mixed with BDC2.5 CD4$^+$ T cells and cultured for 72hr. IL-2 was measured by ELISA. (B) DC or AC-treated DC were pulsed with HA peptide, cultured with CL4 CD8$^+$ T cells for 72hr and IL-2 levels were determined. Student's t test *$p<0.001$

Figure 2.4



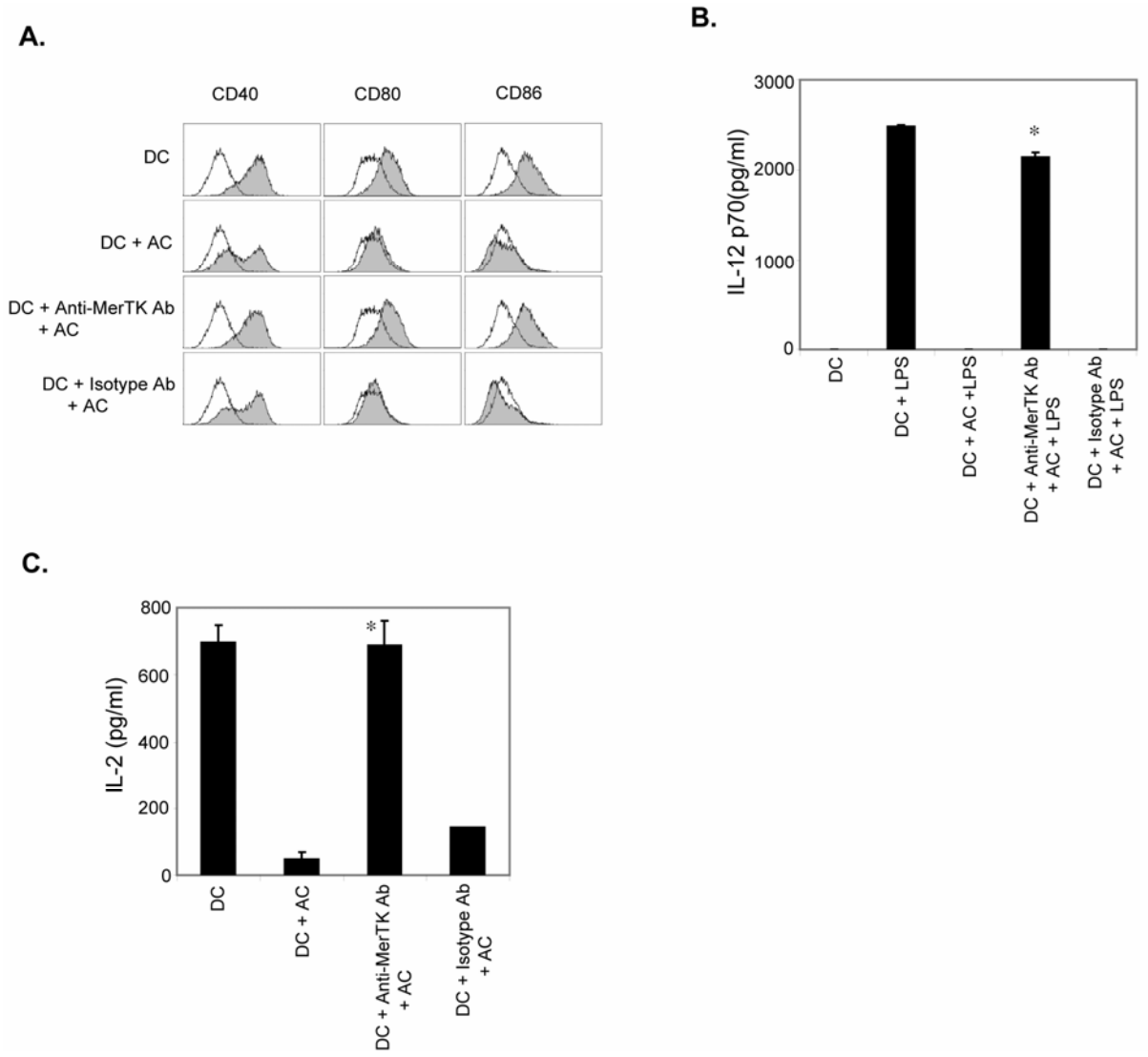
MerTK is phosphorylated upon apoptotic cell incubation. NOD BMDC were pre-blocked with either normal goat IgG isotype control Ab or goat polyclonal IgG anti-MerTK Ab for 30min. Preblocked DC were left untreated or co-cultured with apoptotic cells (AC) for 10 or 20 min at 37^{o}C. Cell lysates were immunoprecipitated with anti-MerTK and Sepharose-Protein G. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-phosphotyrosine Ab (upper blot). Blots were stripped and re-probed with anti-MerTK Ab (lower blot).

Figure 2.5



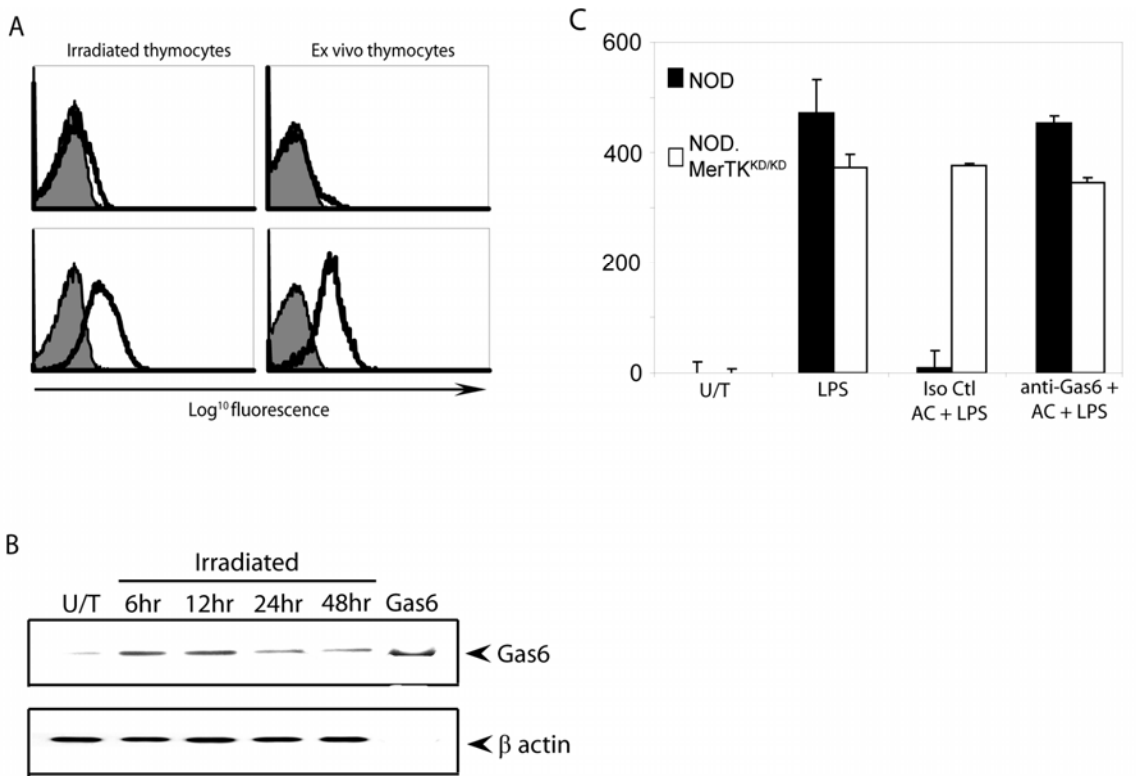
Blockade of MerTK prevents the inhibitory effects of apoptotic cells upon DC. (A-D) NOD BMDC were incubated with either isotype control Ab or anti-MerTK Ab prior to co-culture with apoptotic cells (AC). (A) 72hr after stimulation with 100 ng/ml LPS, IL-12p70 production was measured by ELISA. (B) Expression of co-stimulatory molecules was determined after 24hr of 100 ng/ml LPS treatment. Stimulation of (C) BDC2.5 $CD4^+$ and (D) CL4 $CD8^+$ for 72hr was determined by IL-2 ELISA. Student's t test *$p<0.001$

Figure 2.6



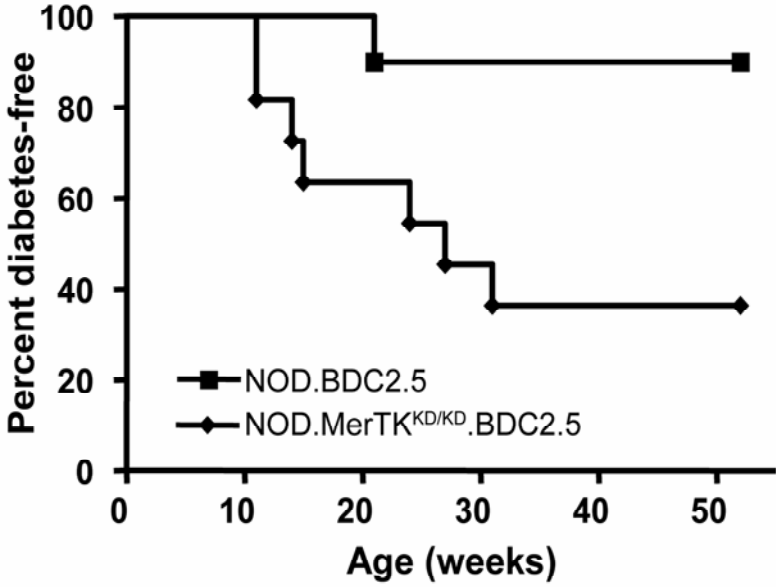
Blockade of MerTK prevents the inhibitory effects of apoptotic cells upon DC in BALB/c mice. (A-C) BALB/c BMDC were incubated with either isotype control Ab or anti-MerTK Ab prior to co-culture with apoptotic cells (AC). (A) Expression of co-stimulatory molecules was determined 24hr after treatment with 100 ng/ml LPS. (B) 72hr after stimulation with 100 ng/ml LPS, IL-12p70 production was measured by ELISA. (C) Stimulation of CL4 $CD8^+$ was determined by IL-2 ELISA. Student's t test *$p<0.001$

Figure 2.7



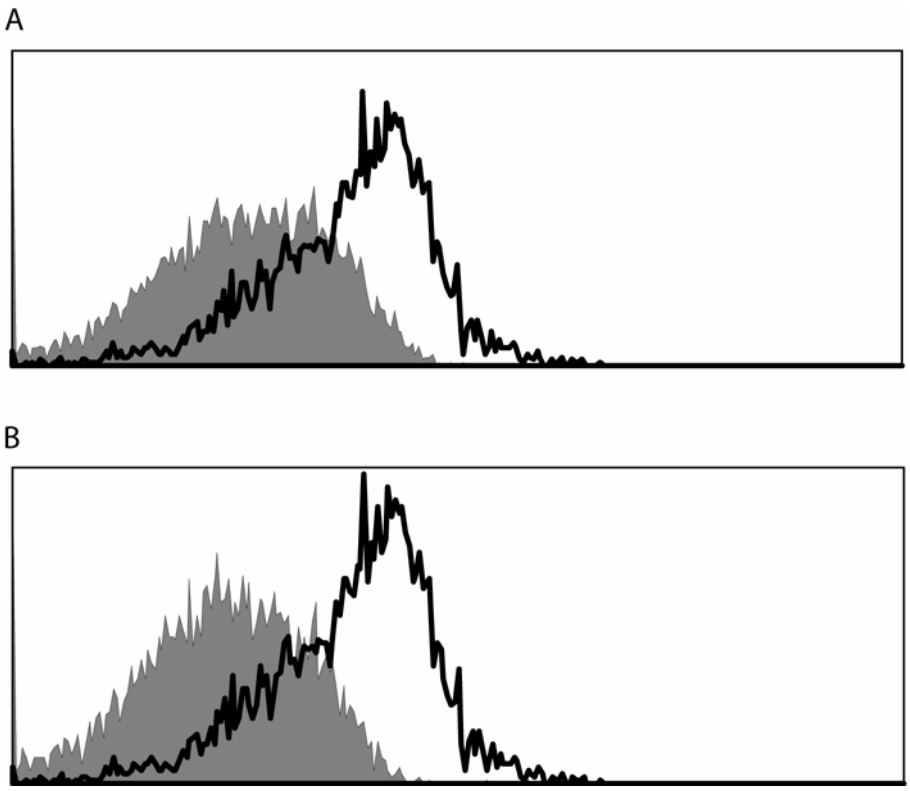
Gas6 expression on the surface of apoptotic cells is essential for the inhibitory effects upon DC. (A) FITC-VAD-FMK negative (non-apoptotic) thymocytes (shaded histograms) and FITC-VAD-FMK positive (apoptotic) thymocytes (open-histograms) were stained with isotype control Ab (upper panels) or anti-Gas6 Ab (lower panels). (B) Thymocyte lysates were prepared from fresh untreated (U/T) thymocytes or at various time points after induction of apoptosis by exposure to 600 rad of gamma irradiation. Western blot for Gas6 protein was performed (upper panel). 10ng of recombinant Gas6 protein was loaded as a positive control. (C) Gas6 was blocked by pre-incubation of apoptotic cells (AC) with polyclonal anti-Gas6 Ab. The effects of Gas6 blockade upon the inhibitory properties of AC on DC was determined by analysis of LPS-induced IL-12p70 production.

Figure 2.8



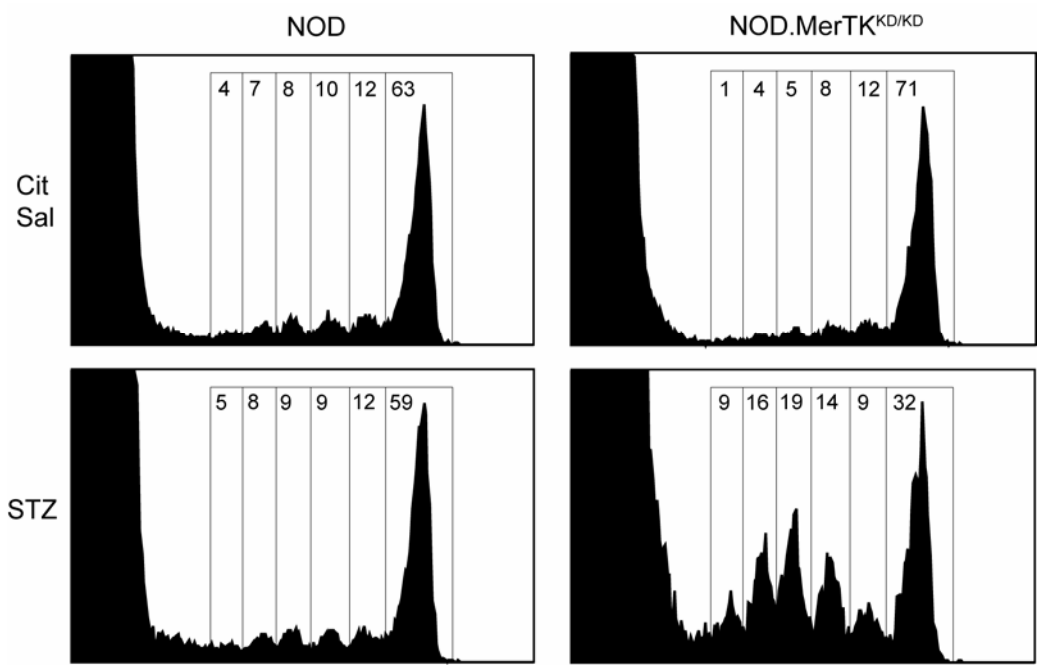
MerTK deficient mice expressing a diabetogenic TCR transgene exhibit exacerbated T1D. NOD.BDC2.5 (n=10) and NOD.MerTK$^{KD/KD}$.BDC2.5 (n=11) mice were monitored for diabetes based on urine glucose measurement for 52 weeks. Upon two consecutive readings above 250 mg/dL, mice were considered diabetic. Kaplan-Meyer log rank comparison p=0.013.

Figure 2.9



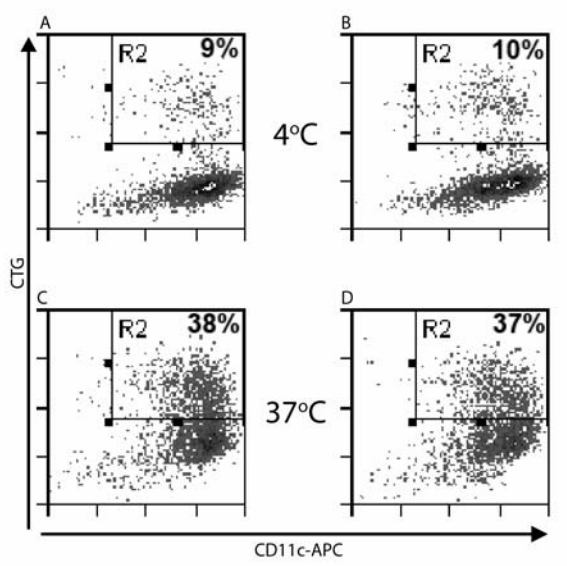
Apoptotic β cell express Gas6 on their surface. Four week old NOD mice were injected i.p. with 120 mg/kg STZ. After 8hr, islets were purified and dissociated. Apoptosis was determined by staining with VAD-FMK-FITC. (A) Anti-Gas6 staining on non-apoptotic (shaded histogram) and apoptotic (open histogram) β cells. (B) Anti-Gas6 staining (open histogram) or isotype control staining (shaded histogram) of apoptotic β cells.

Figure 2.10



MerTK deficiency causes enhanced T cell proliferation *in vivo*. NOD or NOD.MerTK$^{KD/KD}$ mice were treated with citrate saline control (Cit Sal) or 120 mg/kg STZ. 2.5x10^6 CFSE-labeled BDC2.5 CD4$^+$ T cells were adoptively transferred to each mouse. 72 hr later pancreatic lymph nodes were harvested, dissociated and BDC2.5 CD4$^+$ T cell proliferation was determined by FACS analysis. Numbers represent the percentage of total CFSE$^+$ cells within each gate. Each descending peak in CFSE intensity represents one cell division.

Figure 2.11



MerTK expression is not essential for phagocytosis of apoptotic cells by DC. (A and C) NOD BMDC or (B and D) NOD.MerTK$^{KD/KD}$ BMDC were co-cultured with Cell Tracker Green (CTG)-labeled apoptotic thymocytes for 6hr at 4^{o}C or 37^{o}C. Phagocytosis was measured by FACS analysis for the presence of CD11c^{+} CTG^{+} DC.

References

Andre, I., Gonzalez, A., Wang, B., Katz, J., Benoist, C., and Mathis, D. (1996). Checkpoints in the progression of autoimmune disease: lessons from diabetes models. Proc Natl Acad Sci U S A *93*, 2260-2263.

Behrens, E. M., Gadue, P., Gong, S. Y., Garrett, S., Stein, P. L., and Cohen, P. L. (2003). The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. Eur J Immunol *33*, 2160-2167.

Camenisch, T. D., Koller, B. H., Earp, H. S., and Matsushima, G. K. (1999). A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol *162*, 3498-3503.

Chen, C., Li, Q., Darrow, A. L., Wang, Y., Derian, C. K., Yang, J., de Garavilla, L., Andrade-Gordon, P., and Damiano, B. P. (2004a). Mer receptor tyrosine kinase signaling participates in platelet function. Arterioscler Thromb Vasc Biol *24*, 1118-1123.

Chen, J., Carey, K., and Godowski, P. J. (1997). Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. Oncogene *14*, 2033-2039.

Chen, X., Doffek, K., Sugg, S. L., and Shilyansky, J. (2004b). Phosphatidylserine regulates the maturation of human dendritic cells. J Immunol *173*, 2985-2994.

Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A., Earp, H. S., Matsushima, G., and Reap, E. A. (2002). Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med *196*, 135-140.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol *148*, 2207-2216.

Gaipl, U. S., Franz, S., Voll, R. E., Sheriff, A., Kalden, J. R., and Herrmann, M. (2004). Defects in the disposal of dying cells lead to autoimmunity. Curr Rheumatol Rep *6*, 401-407.

Gould, W. R., Baxi, S. M., Schroeder, R., Peng, Y. W., Leadley, R. J., Peterson, J. T., and Perrin, L. A. (2005). Gas6 receptors Axl, Sky and Mer enhance platelet activation and regulate thrombotic responses. J Thromb Haemost *3*, 733-741.

Hall, M. O., Agnew, B. J., Abrams, T. A., and Burgess, B. L. (2003). The phagocytosis of os is mediated by the PI3-kinase linked tyrosine kinase receptor, mer, and is stimulated by GAS6. Adv Exp Med Biol *533*, 331-336.

Hall, M. O., Obin, M. S., Heeb, M. J., Burgess, B. L., and Abrams, T. A. (2005). Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. Exp Eye Res *81*, 581-591.

Hall, M. O., Prieto, A. L., Obin, M. S., Abrams, T. A., Burgess, B. L., Heeb, M. J., and Agnew, B. J. (2001). Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. Exp Eye Res *73*, 509-520.

Haskins, K., Portas, M., Bergman, B., Lafferty, K., and Bradley, B. (1989). Pancreatic islet-specific T-cell clones from nonobese diabetic mice. Proc Natl Acad Sci U S A *86*, 8000-8004.

Hugues, S., Mougneau, E., Ferlin, W., Jeske, D., Hofman, P., Homann, D., Beaudoin, L., Schrike, C., Von Herrath, M., Lehuen, A., and Glaichenhaus, N. (2002). Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. Immunity *16*, 169-181.

Huynh, M. L., Fadok, V. A., and Henson, P. M. (2002). Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest *109*, 41-50.

Ishimoto, Y., Ohashi, K., Mizuno, K., and Nakano, T. (2000). Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. J Biochem (Tokyo) *127*, 411-417.

Katz, J. D., Benoist, C., and Mathis, D. (1995). T helper cell subsets in insulin-dependent diabetes. Science *268*, 1185-1188.

Katz, J. D., Wang, B., Haskins, K., Benoist, C., and Mathis, D. (1993). Following a diabetogenic T cell from genesis through pathogenesis. Cell *74*, 1089-1100.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Lu, Q., and Lemke, G. (2001). Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science *293*, 306-311.

Morelli, A. E., Larregina, A. T., Shufesky, W. J., Zahorchak, A. F., Logar, A. J., Papworth, G. D., Wang, Z., Watkins, S. C., Falo, L. D., Jr., and Thomson, A. W. (2003). Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. Blood *101*, 611-620.

Morgan, D. J., Liblau, R., Scott, B., Fleck, S., McDevitt, H. O., Sarvetnick, N., Lo, D., and Sherman, L. A. (1996). CD8(+) T cell-mediated spontaneous diabetes in neonatal mice. J Immunol *157*, 978-983.

Nagata, K., Ohashi, K., Nakano, T., Arita, H., Zong, C., Hanafusa, H., and Mizuno, K. (1996). Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. J Biol Chem *271*, 30022-30027.

Nakano, T., Ishimoto, Y., Kishino, J., Umeda, M., Inoue, K., Nagata, K., Ohashi, K., Mizuno, K., and Arita, H. (1997). Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. J Biol Chem *272*, 29411-29414.

Nandrot, E. F., Kim, Y., Brodie, S. E., Huang, X., Sheppard, D., and Finnemann, S. C. (2004). Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking alphavbeta5 integrin. J Exp Med *200*, 1539-1545.

Naquet, P., Naspetti, M., and Boyd, R. (1999). Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. Semin Immunol *11*, 47-55.

Ohashi, P. S., Oehen, S., Aichele, P., Pircher, H., Odermatt, B., Herrera, P., Higuchi, Y., Buerki, K., Hengartner, H., and Zinkernagel, R. M. (1993). Induction of diabetes is influenced by the infectious virus and local expression of MHC class I and tumor necrosis factor-alpha. J Immunol *150*, 5185-5194.

Reidy, M. F., and Wright, J. R. (2003). Surfactant protein A enhances apoptotic cell uptake and TGF-beta1 release by inflammatory alveolar macrophages. Am J Physiol Lung Cell Mol Physiol *285*, L854-861.

Savill, J., Fadok, V., Henson, P., and Haslett, C. (1993). Phagocyte recognition of cells undergoing apoptosis. Immunol Today *14*, 131-136.

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R., and Girkontaite, I. (1997). Immunosuppressive effects of apoptotic cells. Nature *390*, 350-351.

Wallet, M. A., Sen, P., and Tisch, R. (2005). Immunoregulation of dendritic cells. Clin Med Res *3*, 166-175.

Wu, Y., Singh, S., Georgescu, M. M., and Birge, R. B. (2005). A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. J Cell Sci *118*, 539-553.

Wu, Y., Tibrewal, N., and Birge, R. B. (2006). Phosphatidylserine recognition by phagocytes: a view to a kill. Trends Cell Biol *16*, 189-197.

CHAPTER III

MERTK-DEFICIENT NOD MICE ARE PROTECTED FROM DIABETES THROUGH ENHANCED THYMIC NEGATIVE SELECTION

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Abstract

Nonobese diabetic (NOD) mice exhibit several defects in the induction and/or maintenance of immune self-tolerance. As a result, autoreactive T cells mediate destruction of the insulin-producing β cells within pancreatic islets and type 1 diabetes (T1D) develops. Dendritic cells (DC) play critical roles in the induction of immune responses to foreign pathogens and tolerance to self antigens. Defects in immune regulation of DC may lead to a breakdown of self-tolerance and contribute to autoimmunity. The receptor tyrosine kinase Mer (MerTK) regulates DC activation/maturation. Here we show that MerTK-deficient NOD mice (NOD.MerTK$^{KD/KD}$) are protected from autoimmune diabetes. Mononuclear cell infiltration of pancreatic islets (insulitis) is less severe in NOD.MerTK$^{KD/KD}$ mice. Additionally, the frequency of IFNγ-producing, β cell-specific CD4^{+} and CD8^{+} T cells within the islets of NOD.MerTK$^{KD/KD}$ mice is reduced relative to NOD mice which express MerTK. Notably, protection from T1D is due to enhanced thymic negative selection in MerTK-deficient NOD mice, which results in depletion of autoreactive CD4^{+} and CD8^{+} T cell precursors.

Introduction

The nonobese diabetic (NOD) mouse model of type 1 diabetes (T1D) closely parallels human T1D inheritance and pathology (Tisch and McDevitt, 1996). Several genetic susceptibility loci are shared between NOD mice and humans and similar β cell-specific antigens are targeted (Lieberman and DiLorenzo, 2003; Wicker et al., 1995). In NOD mice, progressive autoimmune-mediated destruction of pancreatic β cells occurs between 3 and 30 weeks of age. Complete β cell destruction requires cooperation of several immune cell types including macrophages, dendritic cells (DC), B cells, CD4 and $CD8^+$ T cells (Jansen et al., 1994; Shoda et al., 2005; Wong and Janeway, 1999). Of particular importance are IL-12-producing DC and type 1 $CD4^+$ and $CD8^+$ T cells (Anderson and Bluestone, 2005; Delovitch and Singh, 1997; Rabinovitch, 1994; Toyoda and Formby, 1998).

Comparisons between NOD and non-diabetic, congenic strains such as NOR have helped to identify critical checkpoints in the autoimmune process (Andre et al., 1996). The first checkpoint, occurring at approximately 3 weeks of age, (Checkpoint 1) involves the initial pathology of T1D marked by a peripheral mononuclear cell infiltrate of the islets (peri-insulitis) (Bergman and Haskins, 1994; Kaufman et al., 1993). From 3 weeks to about 12 weeks of age, insulitis progresses and invades the islet structure (intra-insulitis). Despite the presence of islet infiltrating $CD4^+$ and $CD8^+$ T cells during this phase, the majority of β cell mass remains intact (Andre et al., 1996). At approximately 12 weeks of age, however, a shift toward more "aggressive" insulitis occurs resulting in massive β cell destruction and overt diabetes. T1D development following Checkpoint 2 requires expansion of type 1 $CD4^+$ and $CD8^+$ T cells within the islets (Andre et al., 1996; Liblau et al., 1995; Rabinovitch, 1994; Rothe et al., 2001)

The NOD mouse is characterized by several defects in peripheral and central tolerance. NOD mice demonstrate defects in regulatory NKT cells (Cardell, 2006), a general skewing towards type 1 T cell effectors (Raz et al., 2005) and aberrant development and function of natural immunoregulatory $CD4^+$ $CD25^+$ T cells (Treg) (Pop et al., 2005; Wu et al., 2002). In addition to peripheral immune defects, alterations in thymic selection contribute to survival and thymic emigration of high-avidity β cell-specific $CD4^+$ and $CD8^+$ T cells. The events contributing to inefficient negative selection in NOD mice are poorly understood, however, the diabetogenic I-A^{g7} major histocompatibility complex (MHC) class II allele likely plays a role. Inefficient binding of self-peptides by I-A^{g7} expressed by medullary thymic epithelial cells is believed to lead to an increased frequency of β cell-specific T cell precursors in NOD mice. (Rosmalen et al., 2002). Furthermore, defects in T cell signaling promote survival of low-avidity $CD4^+$ $CD8^+$ double-positive (DP) thymocytes which would typically die by neglect further enhancing the development of pathogenic β cell-specific $CD8^+$ T cells (Kwon et al., 2005).

T1D in humans and NOD mice is controlled by many genetic loci and poorly understood environmental factors. Studies have identified as many as 25 T1D susceptibility or resistance loci (*Idd1* to *Idd25*) in NOD mice (Barker, 2006; Kim and Polychronakos, 2005; Wicker et al., 1995). Approximately 50% of T1D inheritance is encoded by the *Idd1* locus which encodes I-A^{g7} MHC class II as well as MHC class I molecules $H2K^d$ and $H2D^b$. (Tisch and McDevitt, 1996; Wicker et al., 1995). A host of non-MHC genes also contribute to T1D susceptibility. The *Idd13* locus, located on murine chromosome 2 (syntenic on human chromosome 2) contributes to T1D susceptibility via at least two genes. The first encodes the MHC class I-associated protein β_2-microglobulin (β2m) (Serreze et al., 1998). A diabetes-

permissive *b2m-a* allele is carried by NOD and other strains. On the other hand, C57BL/6 mice carry a diabetes-resistant *b2m-b* allele which, when introgressed onto the NOD genetic background, reduced the frequency of diabetes (Serreze et al., 1998). In addition to *b2m*, a second unidentified gene within *Idd13* confers additional susceptibility to T1D. Evidence suggests that the *il1* gene cluster, located in *Idd13* once a likely candidate gene, has only a marginal impact on T1D development (Thomas et al., 2004). The *mertk* gene is also located within *Idd13.* Currently, no evidence suggests that *mertk* is involved in T1D inheritance, but we have identified at least three different *mertk* alleles in mice (Table 3.1).

The MerTK tyrosine kinase receptor and related family members Axl and Tyro3 contribute to peripheral immune regulation in mice (Lu and Lemke, 2001). MerTK is a 984 amino acid transmembrane protein with distinct extracellular ligand-binding domains and intracellular tyrosine kinase domain (Figure 1.3). MerTK deficient mice were created by targeted deletion of the tyrosine kinase domain resulting in a MerTK "kinase-dead" ($MerTK^{KD}$) molecule (Camenisch et al., 1999) that is no longer expressed. MerTK deficiency results in impaired phagocytosis of apoptotic cells by macrophages and retinal pigment epithelial (RPE) cells (Cohen et al., 2002; Feng et al., 2002; Scott et al., 2001). Additionally, lack of MerTK, Axl and Tyro3 expression results in profound immune dysregulation and lymphoproliferative disease (Lu and Lemke, 2001). Based on these findings, it has been proposed that this family of receptor tyrosine kinases plays a key role in regulation of peripheral immune homeostasis.

MerTK mediates the inhibitory effects of apoptotic cells on DC via the phosphatidylserine opsonin Gas6 (Chapter 2). In the periphery, AC-induced inhibition of DC activation likely contributes to self-tolerance. For example, C57BL/6 mice lacking MerTK expression develop systemic lupus-like autoimmunity (Cohen et al., 2002). Furthermore, lack of MerTK

expression leads to exacerbated T1D development in NOD mice expressing the diabetogenic BDC2.5 TCR transgene (Chapter 2) supporting the idea that MerTK is essential for peripheral immune regulation.

Data is presented here supporting a novel role for MerTK in regulation of thymic selection. NOD mice lacking MerTK expression (NOD.MerTK$^{KD/KD}$) demonstrate enhanced deletion of autoreactive T cell precursors within the thymus and protection from T1D. We propose that thymic DC hyper-activation in NOD.MerTK$^{KD/KD}$ thymi compensates for defects in negative selection of NOD mice.

Results

NOD.MerTK$^{KD/KD}$ mice are protected from diabetes

Female NOD mice develop overt diabetes beginning at approximately 12 weeks of age and by 30 weeks, approximately 80% of female mice are diabetic (Kikutani and Makino, 1992; Wicker et al., 1995). The NOD.MerTK$^{KD/KD}$ line of mice used in this study contained a 17 cM segment of 129/Ola chromosome 2 harboring the MerTK^{KD} mutation that was introgressed 11 backcross generations (see Materials and Methods). Female NOD.MerTK$^{KD/KD}$, heterozygous NOD.MerTK$^{+/KD}$ mice and wildtype littermates were followed 52 weeks for diabetes incidence. No significant difference in the onset or frequency of diabetes was detected between NOD.MerTK$^{+/+}$ (20/24) and NOD.MerTK$^{+/KD}$ (35/39) mice (Figure 3.1). In contrast, none of the NOD.MerTK$^{KD/KD}$ mice (0/30) developed diabetes (Figure 3.1).

Analysis of insulitis revealed further differences between the respective groups of mice. NOD.MerTK$^{+/KD}$ pancreata had moderate insulitis at 12 weeks of age with approximately 50% of islets showing no signs of insulitis (Figure 3.2A). Noteworthy is that the frequency and severity of insulitis in 12 week-old NOD.MerTK$^{KD/KD}$ mice was similar to that of NOD.MerTK$^{+/KD}$ mice (Figure 3.2A). By 17 weeks of age, islets prepared from NOD.MerTK$^{+/KD}$ mice were heavily infiltrated with only 20% remaining free of insulitis. On the other hand, 60% of islets from NOD.MerTK$^{KD/KD}$ mice remained free from insulitis (Figure 3.2A).

Next, the lymphoid composition of the islet infiltrates of the respective groups was examined. In 12 week-old NOD.MerTK$^{+/KD}$ mice, CD4^{+} T cells were dominant, consisting of 58% of total lymphocytes within islet infiltrates, whereas CD8^{+} T cells and B cells made up 26% and 16% respectively (Figure 3.2B). In contrast, B cells (47%) dominated the infiltrates of NOD.MerTK$^{KD/KD}$ mice and the frequency of CD4^{+} T cells (34%) and CD8^{+} T cells (19%) were markedly reduced (Figure 3.2B). Together, these results demonstrate that NOD.MerTK$^{KD/KD}$ mice remain diabetes-free and that this protection correlates with reduced insulitis and an altered composition of the islet infiltrate.

NOD.MerTK$^{KD/KD}$ mice lack diabetogenic T cell effectors

IFNγ-producing CD4^{+} and CD8^{+} T cells, specific for β cell antigens, mediate destructive insulitis in NOD mice (Lieberman and DiLorenzo, 2003; Toyoda and Formby, 1998). CD4^{+} and CD8^{+} T cells from islet infiltrates of 12 week old NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ mice were analyzed for reactivity to a panel of known β cell antigens and

mimetic peptides. An I-A^{g7}-restricted peptide corresponding to amino acids 9-23 of insulin B chain (InsB9-23) (Pankewycz et al., 1991) stimulated significantly more IFNγ-producing $CD4^+$ T cells from NOD.$MerTK^{+/KD}$ versus NOD.$MerTK^{KD/KD}$ islets ($p<10^{-3}$) (Figure 3.3). Similarly, the frequency of type 1 effectors, responding to full-length GAD65 or the BDC2.5 mimetic peptide, were reduced in NOD.$MerTK^{KD/KD}$ versus NOD.$MerTK^{+/KD}$ mice (Figure 3.3). Compared to NOD.$MerTK^{KD/KD}$ islets, NOD.$MerTK^{+/KD}$ islets also had a greater frequency of IFNγ-producing $H2K^d$-restricted $CD8^+$ T cells specific for a peptide epitope of islet-specific glucose-6-phosphatase associated protein (IGRP) ($p=0.005$), an IGRP mimetic peptide (NRPV7) ($p<10^{-3}$) and a peptide corresponding to amino acids 15-23 of insulin B chain (InsB15-23) ($p=0.042$) (Figure 3.3). No IL-4- or IL-10-producing $CD4^+$ or $CD8^+$ T cells were detected in NOD.$MerTK^{+/KD}$ or NOD.$MerTK^{KD/KD}$ islets when stimulated with the panel of autoantigens (data not shown).

To further assess the frequency of β cell-specific $CD8^+$ T cells within the islets, $H2K^d$ tetramers, complexed with NRPV7 mimetic peptide were utilized. The frequency of NRPV7-specific $CD8^+$ T cells infiltrating the islets of NOD.$MerTK^{+/KD}$ mice (9.4%) was increased relative to NOD.$MerTK^{KD/KD}$ mice (2.3%) (Figure 3.4). Together, these data demonstrate that NOD.$MerTK^{KD/KD}$ mice have a significantly reduced frequency of β cell-specific type 1 $CD4^+$ and $CD8^+$ T cells.

Negative selection is enhanced in NOD.$MerTK^{KD/KD}$ mice

The reduced frequency of β cell-specific $CD4^+$ and $CD8^+$ T cells in NOD.$MerTK^{KD/KD}$ mice suggested that thymic selection was altered. Fetal thymic organ cultures (FTOC) were

prepared to examine thymic selection *in vitro*. Addition of BDC2.5 peptide to NOD.MerTK$^{+/KD}$.BDC2.5 FTOC induced depletion of DP thymocytes and a concomitant increase in SP CD8^{+} thymocytes (Figure 3.5). Strikingly, depletion of DP thymocytes ($p=0.003$) and the increase in SP CD8^{+} thymocytes ($p=0.015$) were significantly enhanced in NOD.MerTK$^{KD/KD}$.BDC2.5 FTOC (Figure 3.5).

To assess thymic selection *in vivo*, NOD.MerTK$^{+/KD}$.BDC2.5 and NOD.MerTK$^{KD/KD}$.BDC2.5 mice were injected i.v. with 400 μg of BDC2.5 mimetic peptide and apoptosis in DP thymocytes was measured 8 hours later. In NOD.MerTK$^{/KD}$.BDC2.5 mice, a 3.2-fold increase in apoptotic DP thymocytes was detected, relative to control ovalbumin (Ova) peptide-treated mice (Figure 3.6). In contrast, a 10-fold increase in apoptotic DP thymocytes was detected in BDC2.5 peptide-treated versus Ova peptide-treated NOD.MerTK$^{KD/KD}$.BDC2.5 mice (Figure 3.6). The increased frequency of apoptotic DP cells in MerTK-deficient thymi was not due to inefficient phagocytosis of apoptotic cells, as induction of thymocyte apoptosis with dexamethasone indicated no difference in apoptotic cell clearance at the same 8 hour timepoint analyzed above (data not shown). These data demonstrate that NOD mice lacking MerTK expression have an increased capacity to delete autoreactive T cell precursors within the thymus.

Bone marrow-derived precursors promote diabetes protection in NOD.MerTK$^{KD/KD}$ mice

Both medullary thymic epithelial cells and DC play roles in negative selection (Anderson et al., 2002; Liu, 2006; Wu and Shortman, 2005) and thus it was essential to determine which

population contributes to the phenotype observed in MerTK-deficient mice. Bone marrow chimera mice were established whereby NOD.MerTK$^{+/KD}$ or NOD.MerTK$^{KD/KD}$ irradiated recipient mice were reconstituted with bone marrow prepared from NOD mice transgenic for green fluorescent protein (GFP) (NOD.GFP). Reconstituted animals were characterized by GFP^{+} leukocyte populations as well as GFP^{+} thymic DC. Diabetes developed in both NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ recipient groups a similar time of onset and frequency (Figure 3.7A). Additionally, irradiated NOD mice were reconstituted with NOD.MerTK$^{+/KD}$.GFP or NOD.MerTK$^{KD/KD}$.GFP bone marrow. Reconstitution was successful among peripheral leukocytes and thymic DC. Several recipients of NOD.MerTK$^{+/KD}$.GFP bone marrow (5/9) developed diabetes by 16 weeks of age. Meanwhile, recipients of NOD.MerTK$^{KD/KD}$.GFP bone marrow (8/8) remained diabetes-free for 16 weeks (Figure 3.7B). Analysis of this experimental group is ongoing, however, the delay in T1D progression in recipients of MerTK-deficient bone marrow is statistically significant ($p=0.015$). The effects of MerTK-deficiency upon protection from diabetes are clearly bone marrow-derived and thus it is likely that T1D prevention in NOD.MerTK$^{KD/KD}$ is mediated by thymic DC.

MerTK-deficiency alone mediates protection from T1D

In view of the fact that the *mertk* gene is located within the *Idd13* diabetes susceptibility locus, a concerted effort was made to determine whether the null allele was directly linked to the phenotype observed in NOD.MerTK$^{KD/KD}$ mice. Genetic mapping by microsatellite marker analysis showed that a 17cM region of parental 129/Ola genome flanked the *mertk*

gene in NOD.MerTK$^{KD/KD}$ mice (Figure 3.9). 129/Ola mice express the diabetogenic *b2m-a* allele, which was confirmed in NOD.MerTK$^{KD/KD}$ mice by restriction fragment length polymorphism analysis (data not shown). To determine the potential impact of other 129/Ola-derived diabetes resistance genes within *Idd13*, a control congenic mouse was established. Specifically, the 129/Ola *Idd13* gene region was introgressed onto the NOD genetic background for 5 generations using a speed congenic approach. Importantly, NOD$^{129.Idd13}$ mice developed diabetes at a similar time of onset and frequency compared to NOD littermates (Figure 3.9). These results support our conclusion that MerTK-deficiency is directly involved in T1D protection in NOD.MerTK$^{KD/KD}$ mice.

Discussion

NOD mice present with pathogenic autoreactive CD4$^+$ and CD8$^+$ T cells which mediate β cell destruction and T1D (Toyoda and Formby, 1998). Defects in both central (Brimnes et al., 2002; Kwon et al., 2005; Moustakas et al., 2000; Naquet et al., 1999; Rosmalen et al., 2002; Savino et al., 1991; Stratmann et al., 2000; Thomas-Vaslin et al., 1997) and peripheral (Colucci et al., 1997; Pop et al., 2005; Wu et al., 2002) tolerance contribute to T1D pathogenesis. MerTK mediates immune regulation of DC function in the periphery and thymus which, in its absence, significantly impacts the autoimmune phenotype of NOD mice.

Lack of diabetes in NOD.MerTK$^{KD/KD}$ mice correlated with a reduced frequency of insulitis and type 1 CD4$^+$ and CD8$^+$ T cell effectors. Noteworthy is that the frequency of insulitis was equivalent among the respective groups of mice up 12 weeks of age. Beyond this age, however, the progression of insulitis was effectively blocked in NOD.MerTK$^{KD/KD}$

mice. This is consistent with studies indicating that the transition from Checkpoint 1 to Checkpoint 2 involves TCR affinity/avidity maturation and expansion of type 1 T cell effectors (Andre et al., 1996; Hoglund et al., 1999; Lieberman and DiLorenzo, 2003). In NOD.MerTK$^{KD/KD}$ mice, this process is prevented at the level of precursor deletion in the thymus, thus effectively altering the lymphoid composition of the islet infiltrate and subsequent β cell destruction.

The previously-described role of MerTK in regulating the activational status of APC (Chapter 2) provides an alternative explanation for protection from diabetes in MerTK-deficient NOD mice. Namely, macrophage and DC which typically express MerTK could be dysregulated in MerTK-deficient animals leading to defective expansion of pathogenic effector T cells. Extensive analysis of NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ macrophage and DC functions was performed and no differences were observed in macrophage or DC function with regard to activation, maturation, cytokine production or T cell stimulation (Table 3.2) Furthermore, adoptive transfer of diabetogenic BDC2.5 CD4^{+} T cells induced diabetes at a similar rate in both NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ recipients (data not shown). The only observed differences between NOD and NOD.MerTK$^{KD/KD}$ APC function were seen when DC were pretreated with apoptotic cells (Table 3.2), as described in Chapter 2. These data indicate that peripheral APC function is sufficient to induce effector T cell differentiation and β cell destruction in the absence of MerTK expression. Additionally, NOD.MerTK$^{KD/KD}$ islets are not intrinsically resistant to immune-mediated destruction as adoptive transfer of BDC2.5 CD4^{+} T cells induces T1D efficiently.

NOD.BDC2.5 TCR transgenic mice develop a uniform population of CD4^{+} T cells which recognize an unknown β cell antigen (Stratmann et al., 2000). BDC2.5 T cells are not

negatively selected within the thymus as evidenced by unfettered emigration from the thymus (Dobbs and Haskins, 2001). Analysis of FTOC demonstrated that cognate TCR/MHC-peptide interaction in BDC2.5 thymi induced negative selection and the efficiency of negative selection in NOD.MerTK$^{KD.KD}$.BDC2.5 thymic lobes was significantly enhanced (Figure 3.5). This data was corroborated by *in vivo* analyses demonstrating similarly enhanced negative selection and induction of apoptosis in DP thymocytes in NOD.MerTK$^{KD/KD}$.BDC2.5 mice (Figure 3.6) Negative selection is believed to be mediated by both medullary thymic epithelium and thymic DC (Anderson et al., 1998; Anderson et al., 2002; Brocker, 1997; Matzinger and Guerder, 1989; Wu and Shortman, 2005). Importantly, protection from diabetes in MerTK-deficient mice was found to be associated with bone marrow-derived cells, strongly arguing that DC were promoting enhanced negative selection. (Figure 3.8).

MerTK has a direct role in regulation of DC activation/maturation (Chapter 2). Accordingly, thymic DC in MerTK-deficient mice may exhibit an enhanced activational status and thus, induce more efficient deletion of autoreactive T cell precursors. Changes in expression of MHC molecules, co-stimulatory molecules or cytokines may all contribute to alterations in thymic selection efficiency. As we have shown, apoptotic cells inhibit DC activation *in vitro* and *in vivo* (Chapter 2). With this in mind, we propose that the activational status of thymic DC is regulated by apoptotic thymocytes. In this way, thymic DC cannot obtain an enhanced state of activation and in turn promote "purging" of the TCR repertoire. In the absence of MerTK expression, thymic DC may obtain increased activational status that results in enhanced negative selection. Notably, preliminary results indicate that differences in thymic DC activation and maturation appear to be subtle but nevertheless sufficient to

effectively delete β cell-specific precursors. Studies have been initiated to better understand the specific role of thymic DC in negative selection and the impact of MerTK deficiency upon this process.

T1D inheritance in mice and humans is complex with numerous susceptibility and resistance loci scattered throughout the genomes (Kim and Polychronakos, 2005; Wicker et al., 1995). Among these loci is *Idd13* which contains *b2m* and at least one additional T1D susceptibility gene (Serreze et al., 1998). The *mertk* gene is located within *Idd13* and sequence analysis has identified 3 distinct *mertk* alleles (Table 3.1). Perhaps allelic variants of *mertk* encode different levels of protein or proteins which exhibit different functions. As evidenced by the important role of MerTK in immunoregulation of DC by apoptotic cells (Chapter 2) and maintenance of homeostatic regulation of thymic selection, alterations in MerTK's function among allelic variants could influence autoimmunity and immunity to pathogens. It is unclear if *mertk* allelic variations contribute to T1D inheritance, however investigations are underway to assess this possibility.

Materials and methods

Mice

NOD/LtJ, NOD.BDC2.5, NOD.CL4, NOD.MerTK$^{+/KD}$.BDC2.5, NOD.MerTK$^{KD/KD}$.BDC2.5 and NOD.GFP mice were maintained and bred under specific-pathogen free conditions. Establishment of MerTK^{KD} mice has been described (Camenisch et al., 1999). Briefly, the tyrosine kinase domain of *mertk* was replaced with a neomycin resistance gene, and B6.MerTK^{KD} mice established. NOD.MerTK$^{KD/KD}$ mice were generated

by breeding B6.MerTK^{KD} and NOD mice, and then backcrossing the *mertk^{KD}* gene onto the NOD genome for an additional 11 generations. At N11, Mouse MapPairs™ distinguishing B6, 129/Ola, and NOD/LtJ [Chr. 2 (D2Mit378, D2Mit94, D2Mit14, D2Mit393, D2Mit395, D2Mit190, D2Mit164, D2Mit256, D2Mit304, D2Mit224, D2Mit338, D2Mit307, D2Mit260, D2Mit309, D2Mit493, D2Mit451, D2Mit496, D2Mit287, D2Mit456, D2Mit265) (Invitrogen, Carlsbad, CA) were used in PCR according to the supplier's directions to define a 17 cM segment derived from 129/Ola and containing *Mertk^{KD}*. Use of mice was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Islet Histology

Pancreata from NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ mice were formalin fixed and embedded in paraffin for sectioning. 5μm sections were de-parafinized and stained with hematoxylin and eosin. Three sections, 100 μm apart, from each pancreas were analyzed. The total number of islets as well as islets demonstrating peri-insulitis and intra-insulitis were counted by light microscopy.

Islet purification

Pancreata were perfused via the bile duct with collagenase P (Roche Diagnostics Corporation, Indianapolis, IN), dissociated and digested at 37^{o}C. Islets were enriched by separation over a Ficoll gradient. Individual islets were picked with a siliconized Pasteur pipette under a dissecting microscope. Islets were dissociated in enzyme-free dissociation buffer (Gibco, Gran Island, NY). Where necessary, T cells were purified by magnetic bead

selection with microbeads coated with anti-CD3, anti-CD4 or anti-CD8 (Miltenyi Biotec, Auburn, CA).

ELISPOT

Opaque 96-well immuspot plates were coated with 2 μg/ml purified anti-IFNγ antibody (BD Pharmingen, San Diego, CA). $5x10^6$ irradiated splenocytes were added to each well as antigen presenting cells. $1x10^4$ purified $CD3^+$ lymphocytes from islets were added to each well. A panel of autoantigen peptides was added to individual wells at a concentration of 10μg/ml. After 48hrs, $IFN\gamma^+$ spots were identified by using 2 μg/ml biotinylated anti-IFNγ, streptavidin-HRP (BD Pharmingen) and 3-amino-9-ethyl carbazole substrate (Sigma-Aldrich, St. Louis, MO). Spots were counted using an Immuspot plate reader and software (Cellular Technology Ltd., Cleveland, OH).

Tetramer staining

$H2K^d$ MHC class I tetramers were prepared as described previously (Wong et al., 2006). Purified islets were cultured for 12 hrs in RPMI complete media with 2 ng/ml IL-2. Islets were dissociated and individual cells were stained with anti-CD3-FITC, anti-CD8-PerCP and PE-labeled tetramer loaded with either influenza nucleoprotein (NP) or NRPV7. Cells were analyzed by FACS using a BD FACSCalibur.

FTOC

$NOD.MerTK^{+/KD}$.BDC2.5 male and female mice were bred and timed pregnancies were established. On day 13 of gestation, pregnant females were sacrificed and fetuses were

removed. Fetal thymic lobes were individually removed under a dissecting microscope. Fetal liver samples were also taken for genotyping. Thymic lobes from individual mice were separated and placed on different Millicell culture plate insert discs (Millipore, Bedford, MA). Millicell discs were overlaid upon a small volume such that media contacted the membrane, however thymic lobes remained exposed to air. FTOC were allowed to grow for 3 days without treatment. After 3 days, media was replaced with fresh media containing either 1μg/ml Ova peptide (ISQAVHAAHAEINEAGR) or 1μg/ml BDC2.5 peptide (RTRPLWVRME). Fresh media and peptide were replaced daily for 4 days. Thymic lobes were dissociated and stained for FACS with anti-CD3-FITC, anti-CD4-APC, anti-CD8-PerCP and anti-Vβ4-PE (clonotypic TCR β chain encoded by BDC2.5 transgene).

***In vivo* thymic selection**

Four week-old female NOD.MerTK$^{+/KD}$.BDC2.5 or NOD.MerTK$^{KD/KD}$.BDC2.5 mice were treated with either 400 μg Ova or BDC2.5 peptide by i.v. injection. Eight hours later mice were sacrificed and thymi were removed and dissociated. Thymocytes were stained with anti-CD4-APC, anti-CD8-PerCP, anti-Vβ4-PE and VAD-FMK-FITC and then analyzed by FACS.

Bone marrow chimera mice

Bone marrow was isolated from tibias and femurs of NOD.GFP or NOD.MerTK$^{KD/KD}$.GFP mice. CD4$^+$ and CD8$^+$ T cells were depleted with specific antibodies (GK1.5 and HO2.2 respectively) and rabbit serum complement mediated lysis. 2x10^6 bone marrow cells were transferred intravenously to 4 week old NOD.MerTK$^{+/KD}$ or NOD.MerTK$^{KD/KD}$ mice that

received 950 rad of gamma irradiation. Chimerism was confirmed by GFP^+ lymphocytes in peripheral blood samples. Chimeric mice were monitored for urine glucose for 30 weeks. Thymi were harvested and dissociated. Thymic DC were stained with anti-CD11c-APC and analyzed for GFP expression by FACS.

Table 3.1

Allelic variants of *mertk*

Strain (allele):	NOD(a)	Balb/c(b)	C57BL/6(a)	CTS(c)	ALR(a)
Nuc 526					
AA 176					
Codon	AAT	**GAT**	AAT	AAT	AAT
AA	N	**D**	N	N	N
Nuc 1321					
AA 441					
Codon	GCT	**ACT**	GCT	GCT	GCT
AA	A	**T**	A	A	A
Nuc 1536					
AA 512					
Codon	ATT	ATT	ATT	**GTT**	ATT
AA	I	I	I	**V**	I
Nuc 2461					
AA 821					
Codon	TTG	**CTG**	TTG	**CTG**	TTG
AA	L	L	L	L	L
Nuc 2851					
AA 951					
Codon	GTC	**ATC**	GTC	GTC	GTC
AA	V	**I**	V	V	V

Table 3.2

Analysis of APC function in NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ mice

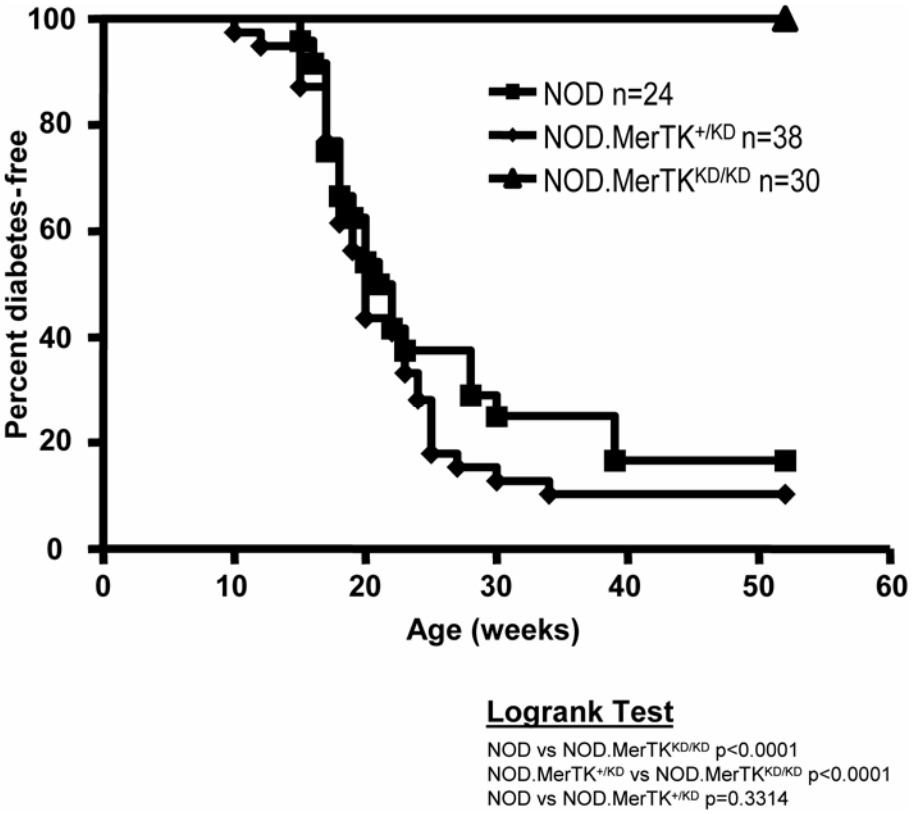
Cell types	Experiment	Assay	Results: NOD.MerTK$^{+/KD}$	Results: NOD.MerTK$^{KD/KD}$
pMacs	LPS or anti-CD40 Ab stimulation	TNFα	++++	++++
sMacs		IFNγ	+++	+++
BM macs		IL-12	++	++
sDC		IL-1α	+++	+++
BMDC		IL-1β	++	++
		CD40	↑	↑
		CD80	↑	↑
		CD86	↑	↑
	CD4$^+$ and CD8$^+$ T cell stimulation	IL-2	+++	+++
sDC	Pre-treatment with apoptotic cells then LPS or anti-CD40 Ab stimulation	TNFα	-	++++*
BMDC		IL-12	-	++*
		CD40	n.c.	↑
		CD80	n.c.	↑
		CD86	n.c.	↑
	Pre-treatment with apoptotic cells then CD4$^+$ and CD8$^+$ T cell stimulation	IL-2	-	+++*

peritoneal macrophages (p-Macs); splenic macrophages (sMacs); bone marrow-derive macrophages (BM Macs); splenic dendritic cells (sDC); bone marrow-derived dendritic cells (BMDC)

n.c. = no change in comparison to unstimulated cells; ↑= increased surface expression determined by FACS

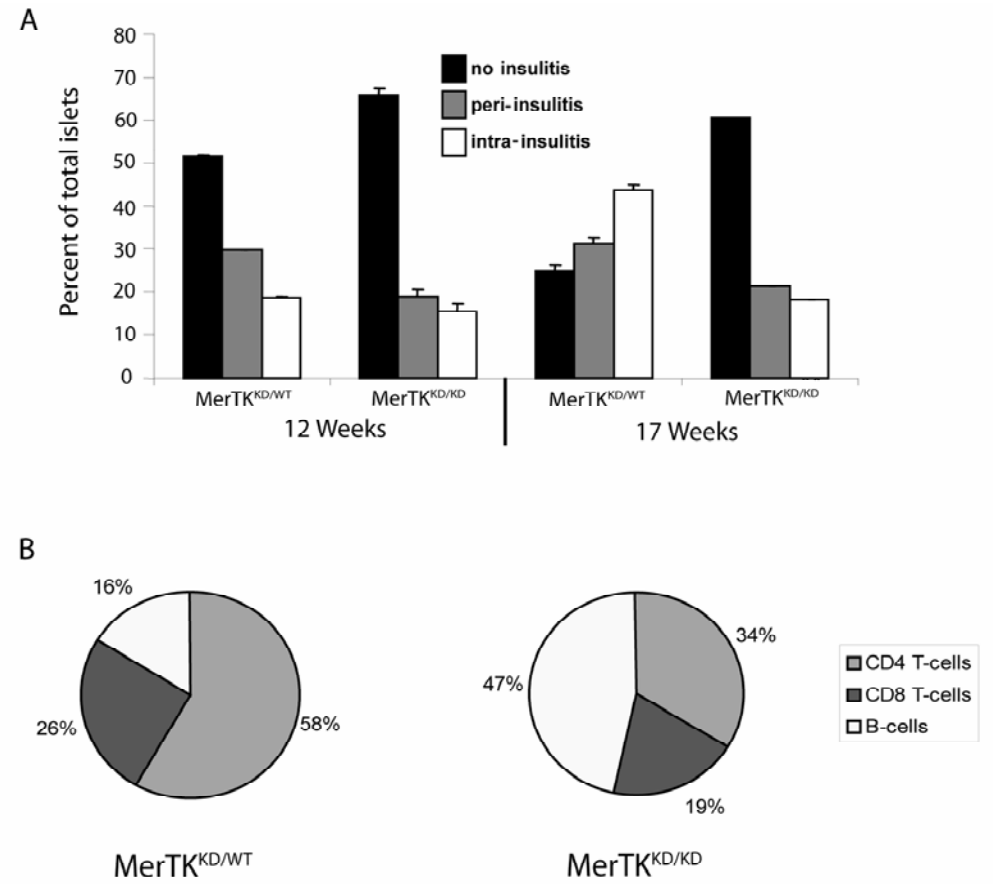
*$p<10^{-3}$

Figure 3.1



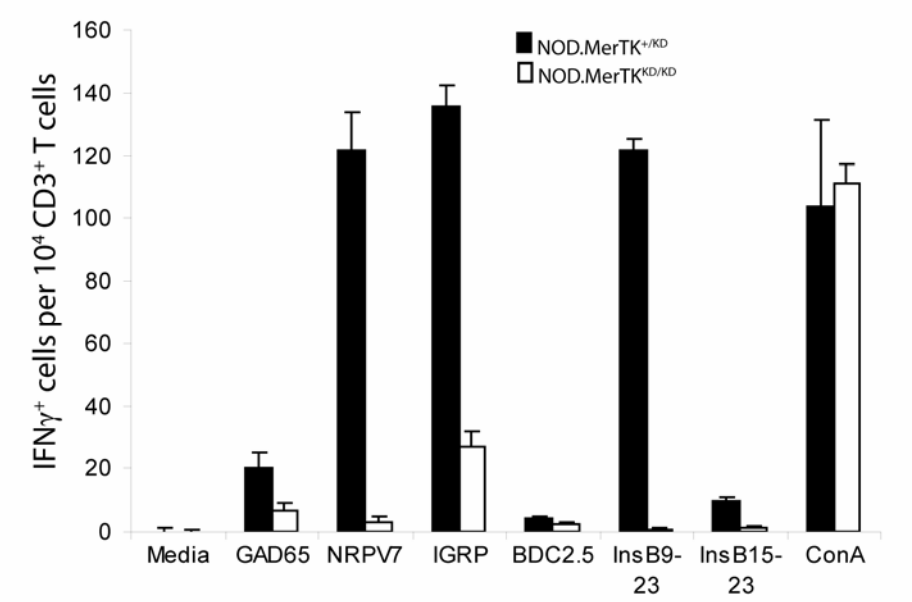
MerTK-deficient NOD mice are protected from diabetes. Three groups of littermates were established after 11 generations of backcrosses to NOD. Animals were followed for 52 weeks with weekly urine glucose monitoring.

Figure 3.2



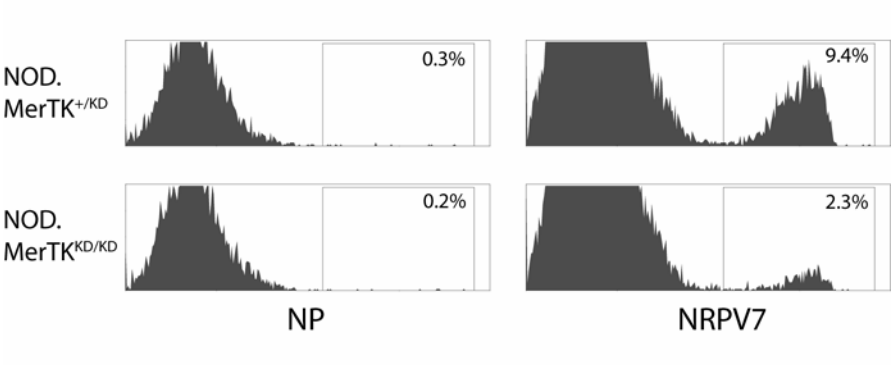
MerTK deficient NOD mice develop less severe insulitis. (A) 12 week old NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ were sacrificed insulitis was scored by hematoxylin and eosin staining. (B) Islets were purified from 12 week old NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ mice and lymphocytic infiltrates were characterized by FACS.

Figure 3.3



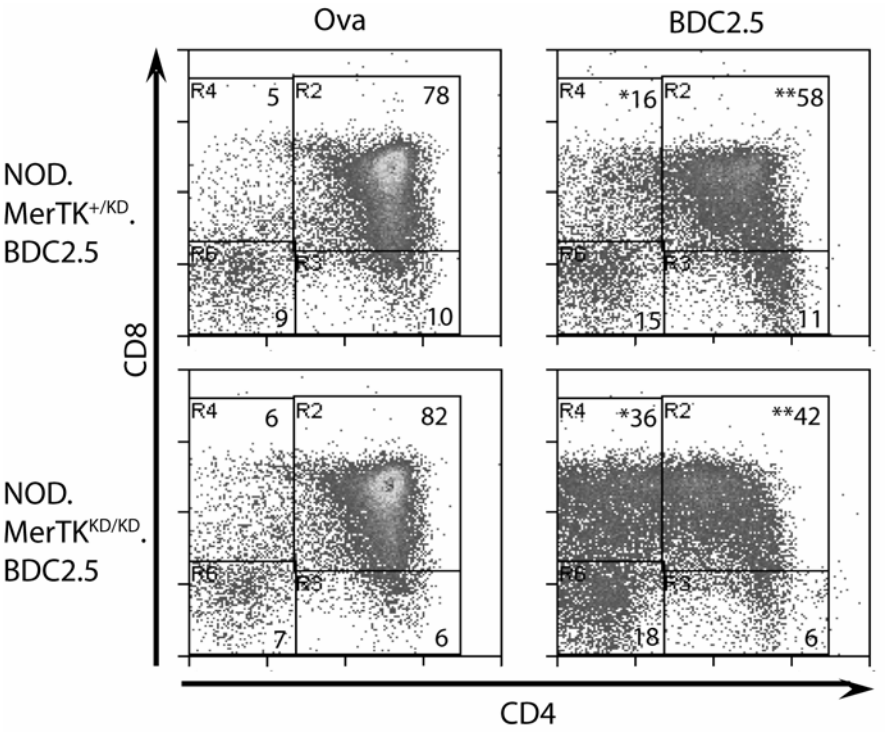
MerTK-deficient NOD mice lack diabetogenic effector T cells in islet infiltrates. ELISPOT analysis of T cells from 12 week old NOD.MerTK$^{+/KD}$ and NOD.MerTK$^{KD/KD}$ mice was performed to determine the frequency of β cell-specific IFNγ^{+} T cells. Concavilin A (ConA) was use as a non-specific positive control. ConA results are plotted at 1/10 the recorded values for scale. (Student T test NRPV7 $p<0.001$, IGRP $p=0.005$, InsB9-23 $p<0.001$, InsB15-23 $p=0.042$)

Figure 3.4



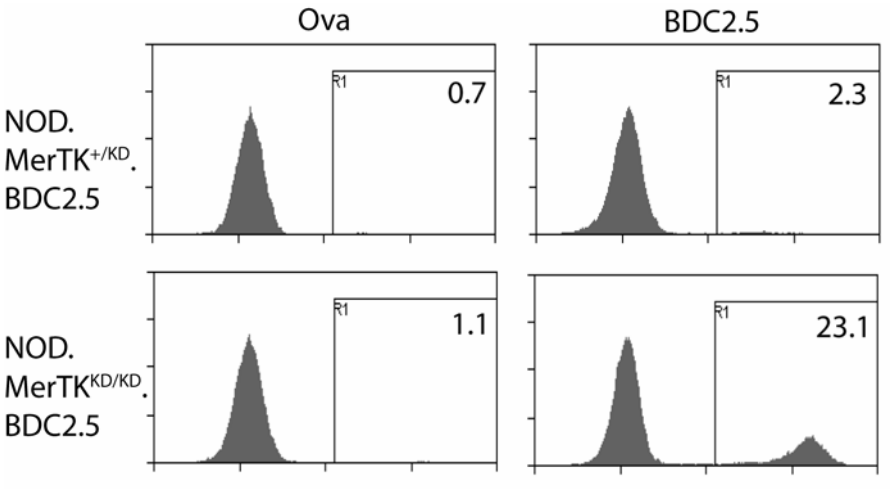
The islets of NOD mice lacking MerTK expression have fewer β cell-specific $CD8^+$ T cells. The frequency of $CD8^+$ T cells expressing a diabetogenic NRPV7-specific TCR within the islets was determined by staining with NRPV7 tetramer and FACS analysis. Data are representative of 3 separate experiments which included 2-3 mice per group.

Figure 3.5



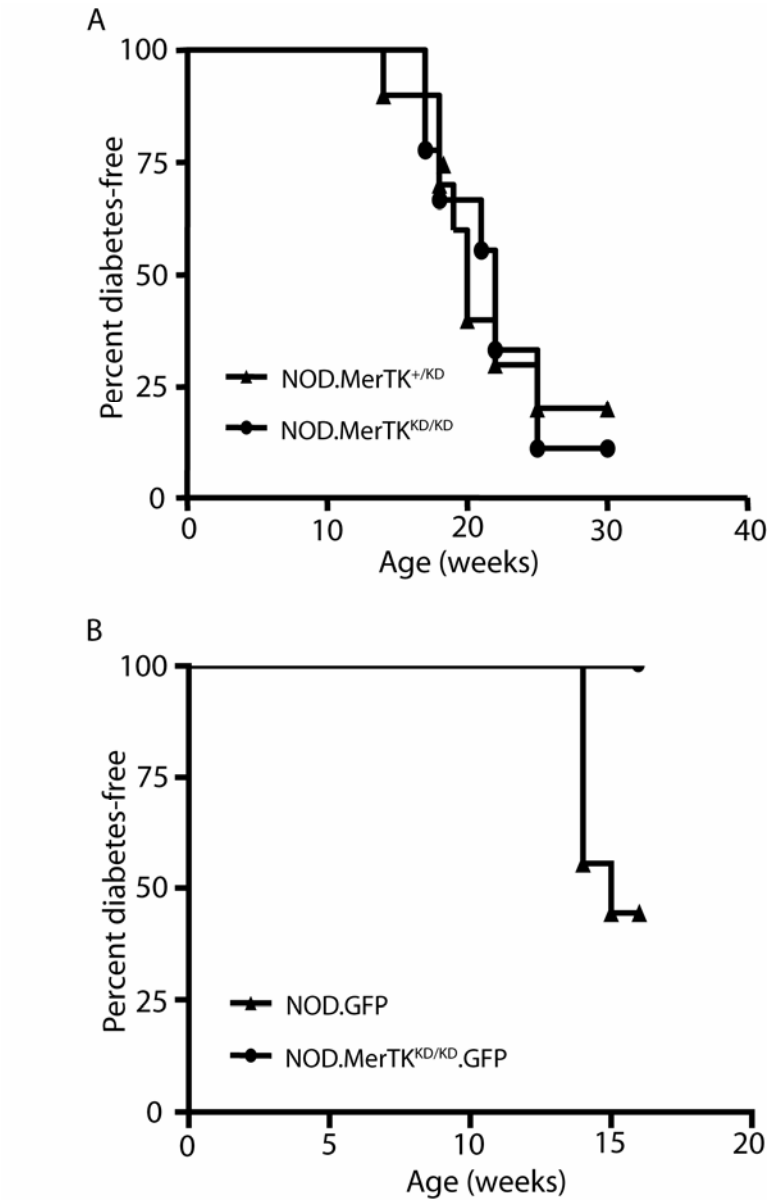
Thymic negative selection is enhanced in MerTK-deficient NOD mice. Negative selection efficiency was determined by FACS analysis of FTOC. FTOC cultures were treated for 4 days with either irrelevant Ova peptide or BDC2.5 peptide. Percentages are reflective of 3 independent experiments. (Student's T test *p=0.015, **p=0.003)

Figure 3.6



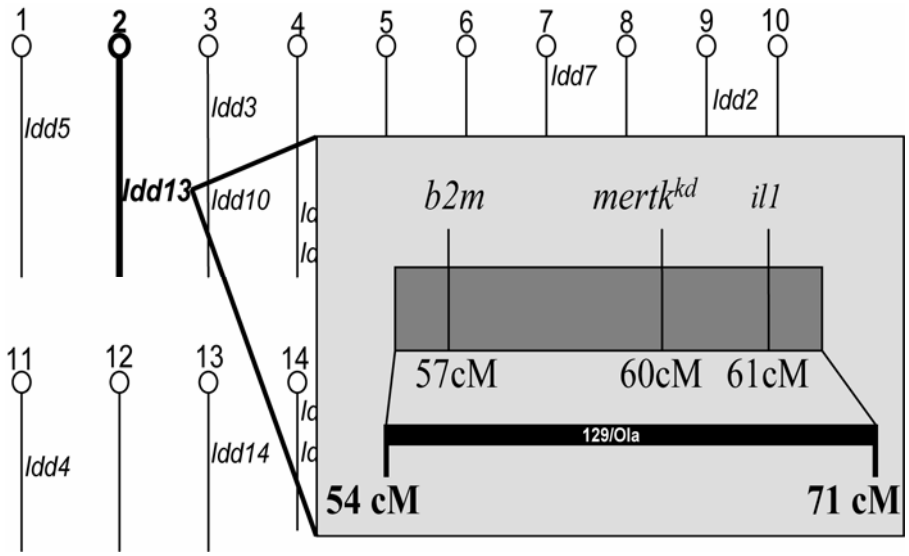
Thymic selection is altered *in vivo* in MerTK-deficient NOD mice. 12 week old NOD.MerTK$^{+/KD}$ or NOD.MerTK$^{KD/KD}$ mice were intravenously injected with either 400µg Ova peptide or 400µg BDC2.5 peptide. Thymocyte apoptosis was determined by FACS analysis of VAD-FMK-FITC staining.

Figure 3.7



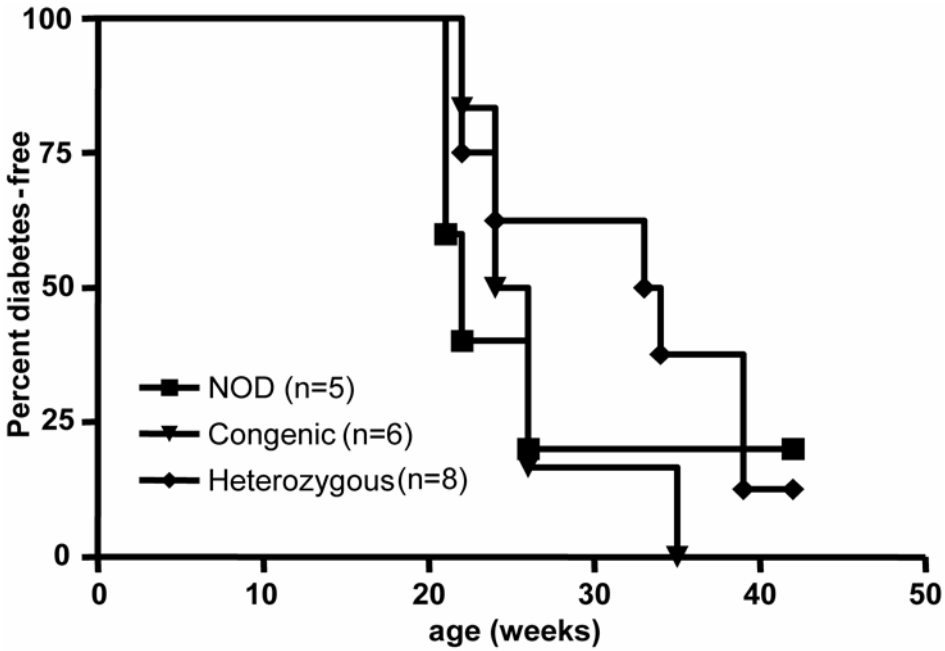
Protection from diabetes in MerTK-deficient mice is a bone marrow-derived trait. (A) Bone marrow chimera mice were created by administration of NOD.GFP bone marrow to irradiated NOD.MerTK$^{+/KD}$ or NOD.MerTK$^{KD/KD}$ recipients. Animals were followed for 30 weeks and urine glucose was measured weekly. (B) NOD mice were irradiated and reconstituted with either NOD.GFP bone marrow or NOD.MerTK$^{KD/KD}$ bone marrow. Diabetes was monitored weekly (Kaplan-Meyer log rank test p=0.015).

Figure 3.8



The *mertk* gene is located within the diabetes susceptibility locus *Idd13.* Several *Idd* loci are distributed across many chromosomes of the mouse genome (Wicker et al., 1995).The $MerTK^{KD}$ mutation was introgressed for 11 generations to the NOD genetic background. A 17cM segment of parental 129/Ola genomic DNA, including *b2m* and *il1* remained flanking MerTK.

Figure 3.9



Protection from diabetes is mediated by MerTK-deficiency and not by diabetes resistance alleles within 129/Ola *Idd13.* A congenic mouse which carries the *Idd13* gene segment of 129/Ola on NOD background was analyzed for diabetes development. Urine glucose was monitored weekly. No significant difference was observed between NOD, congenic or heterozygous animals.

References

Anderson, G., Partington, K. M., and Jenkinson, E. J. (1998). Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. J Immunol *161*, 6599-6603.

Anderson, M. S., and Bluestone, J. A. (2005). The NOD mouse: a model of immune dysregulation. Annu Rev Immunol *23*, 447-485.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Andre, I., Gonzalez, A., Wang, B., Katz, J., Benoist, C., and Mathis, D. (1996). Checkpoints in the progression of autoimmune disease: lessons from diabetes models. Proc Natl Acad Sci U S A *93*, 2260-2263.

Barker, J. M. (2006). Clinical review: Type 1 diabetes-associated autoimmunity: natural history, genetic associations, and screening. J Clin Endocrinol Metab *91*, 1210-1217.

Bergman, B., and Haskins, K. (1994). Islet-specific T-cell clones from the NOD mouse respond to beta-granule antigen. Diabetes *43*, 197-203.

Brimnes, M. K., Jensen, T., Jorgensen, T. N., Michelsen, B. K., Troelsen, J., and Werdelin, O. (2002). Low expression of insulin in the thymus of non-obese diabetic mice. J Autoimmun *19*, 203-213.

Brocker, T. (1997). Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. J Exp Med *186*, 1223-1232.

Camenisch, T. D., Koller, B. H., Earp, H. S., and Matsushima, G. K. (1999). A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol *162*, 3498-3503.

Cardell, S. L. (2006). The natural killer T lymphocyte: a player in the complex regulation of autoimmune diabetes in non-obese diabetic mice. Clin Exp Immunol *143*, 194-202.

Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A., Earp, H. S., Matsushima, G., and Reap, E. A. (2002). Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med *196*, 135-140.

Colucci, F., Bergman, M. L., Penha-Goncalves, C., Cilio, C. M., and Holmberg, D. (1997). Apoptosis resistance of nonobese diabetic peripheral lymphocytes linked to the Idd5 diabetes susceptibility region. Proc Natl Acad Sci U S A *94*, 8670-8674.

Delovitch, T. L., and Singh, B. (1997). The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunity *7*, 727-738.

Dobbs, C., and Haskins, K. (2001). Comparison of a T cell clone and of T cells from a TCR transgenic mouse: TCR transgenic T cells specific for self-antigen are atypical. J Immunol *166*, 2495-2504.

Feng, W., Yasumura, D., Matthes, M. T., LaVail, M. M., and Vollrath, D. (2002). Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J Biol Chem *277*, 17016-17022.

Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C., and Mathis, D. (1999). Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med *189*, 331-339.

Jansen, A., Homo-Delarche, F., Hooijkaas, H., Leenen, P. J., Dardenne, M., and Drexhage, H. A. (1994). Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice. Diabetes *43*, 667-675.

Kaufman, D. L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G. S., Robinson, P., Atkinson, M. A., Sercarz, E. E., Tobin, A. J., and Lehmann, P. V. (1993). Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature *366*, 69-72.

Kikutani, H., and Makino, S. (1992). The murine autoimmune diabetes model: NOD and related strains. Adv Immunol *51*, 285-322.

Kim, M. S., and Polychronakos, C. (2005). Immunogenetics of type 1 diabetes. Horm Res *64*, 180-188.

Kwon, H., Jun, H. S., Yang, Y., Mora, C., Mariathasan, S., Ohashi, P. S., Flavell, R. A., and Yoon, J. W. (2005). Development of autoreactive diabetogenic T cells in the thymus of NOD mice. J Autoimmun *24*, 11-23.

Liblau, R. S., Singer, S. M., and McDevitt, H. O. (1995). Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today *16*, 34-38.

Lieberman, S. M., and DiLorenzo, T. P. (2003). A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens *62*, 359-377.
Liu, Y. J. (2006). A unified theory of central tolerance in the thymus. Trends Immunol *27*, 215-221.

Lu, Q., and Lemke, G. (2001). Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science *293*, 306-311.
Matzinger, P., and Guerder, S. (1989). Does T-cell tolerance require a dedicated antigen-presenting cell? Nature *338*, 74-76.

Moustakas, A. K., Routsias, J., and Papadopoulos, G. K. (2000). Modelling of the MHC II allele I-A(g7) of NOD mouse: pH-dependent changes in specificity at pockets 9 and 6 explain several of the unique properties of this molecule. Diabetologia *43*, 609-624.

Naquet, P., Naspetti, M., and Boyd, R. (1999). Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. Semin Immunol *11*, 47-55.

Pankewycz, O., Strom, T. B., and Rubin-Kelley, V. E. (1991). Islet-infiltrating T cell clones from non-obese diabetic mice that promote or prevent accelerated onset diabetes. Eur J Immunol *21*, 873-879.

Pop, S. M., Wong, C. P., Culton, D. A., Clarke, S. H., and Tisch, R. (2005). Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. J Exp Med *201*, 1333-1346.

Rabinovitch, A. (1994). Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? Diabetes *43*, 613-621.

Raz, I., Eldor, R., and Naparstek, Y. (2005). Immune modulation for prevention of type 1 diabetes mellitus. Trends Biotechnol *23*, 128-134.

Rosmalen, J. G., van Ewijk, W., and Leenen, P. J. (2002). T-cell education in autoimmune diabetes: teachers and students. Trends Immunol *23*, 40-46.

Rothe, H., Ito, Y., and Kolb, H. (2001). Disease resistant, NOD-related strains reveal checkpoints of immunoregulation in the pancreas. J Mol Med *79*, 190-197.

Savino, W., Boitard, C., Bach, J. F., and Dardenne, M. (1991). Studies on the thymus in nonobese diabetic mouse. I. Changes in the microenvironmental compartments. Lab Invest *64*, 405-417.

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Serreze, D. V., Bridgett, M., Chapman, H. D., Chen, E., Richard, S. D., and Leiter, E. H. (1998). Subcongenic analysis of the Idd13 locus in NOD/Lt mice: evidence for several susceptibility genes including a possible diabetogenic role for beta 2-microglobulin. J Immunol *160*, 1472-1478.

Shoda, L. K., Young, D. L., Ramanujan, S., Whiting, C. C., Atkinson, M. A., Bluestone, J. A., Eisenbarth, G. S., Mathis, D., Rossini, A. A., Campbell, S. E., *et al.* (2005). A comprehensive review of interventions in the NOD mouse and implications for translation. Immunity *23*, 115-126.

Stratmann, T., Apostolopoulos, V., Mallet-Designe, V., Corper, A. L., Scott, C. A., Wilson, I. A., Kang, A. S., and Teyton, L. (2000). The I-Ag7 MHC class II molecule linked to murine diabetes is a promiscuous peptide binder. J Immunol *165*, 3214-3225.

Thomas, H. E., Irawaty, W., Darwiche, R., Brodnicki, T. C., Santamaria, P., Allison, J., and Kay, T. W. (2004). IL-1 receptor deficiency slows progression to diabetes in the NOD mouse. Diabetes *53*, 113-121.

Thomas-Vaslin, V., Damotte, D., Coltey, M., Le Douarin, N. M., Coutinho, A., and Salaun, J. (1997). Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. Proc Natl Acad Sci U S A *94*, 4598-4603.

Tisch, R., and McDevitt, H. (1996). Insulin-dependent diabetes mellitus. Cell *85*, 291-297.

Toyoda, H., and Formby, B. (1998). Contribution of T cells to the development of autoimmune diabetes in the NOD mouse model. Bioessays *20*, 750-757.

Wicker, L. S., Todd, J. A., and Peterson, L. B. (1995). Genetic control of autoimmune diabetes in the NOD mouse. Annu Rev Immunol *13*, 179-200.

Wong, C. P., Li, L., Frelinger, J. A., and Tisch, R. (2006). Early autoimmune destruction of islet grafts is associated with a restricted repertoire of IGRP-specific CD8+ T cells in diabetic nonobese diabetic mice. J Immunol *176*, 1637-1644.

Wong, F. S., and Janeway, C. A., Jr. (1999). The role of CD4 vs. CD8 T cells in IDDM. J Autoimmun *13*, 290-295.

Wu, A. J., Hua, H., Munson, S. H., and McDevitt, H. O. (2002). Tumor necrosis factor-alpha regulation of CD4+CD25+ T cell levels in NOD mice. Proc Natl Acad Sci U S A *99*, 12287-12292.

Wu, L., and Shortman, K. (2005). Heterogeneity of thymic dendritic cells. Semin Immunol *17*, 304-312.

CHAPTER IV

SUMMARY

The immune system must strike a constant balance between immunity and tolerance. The primary lymphoid tissues, bone marrow and the thymus, foster development of a diverse repertoire of B and T cells respectively. This diversity provides a broad spectrum of protection against a multitude of infectious pathogens including viruses, bacteria and eukaryotic parasites. In conjunction with development of "protective" B and T cells, the immune system must remove lymphocytes which would otherwise target self-antigens. Deletion of autoreactive B and T cells is the primary mechanism by which autoimmunity is prevented. T cell depletion occurs mainly within the thymus where medullary thymic epithelial cells and dendritic cells (DC) mediate negative selection.

Despite the efficiency of negative selection, deletion of all T cells, which may target countless self-antigens, is not possible. Thus, peripheral tolerance mechanisms provide and additional barrier against autoimmunity. For example, regulatory T cells (Treg) suppress the function of otherwise destructive, self-specific $CD4^+$ and $CD8^+$ T cells (Dejaco et al., 2006). Furthermore, peripheral DC prevent autoimmunity through various mechanisms (Wallet et al., 2005). One critical regulatory function of DC is recognition and phagocytosis of cells that die by apoptosis. Apoptotic cells induce functional changes in DC resulting in a "tolerized," non-immunogenic phenotype. Thus, DC cannot induce

differentiation of pathogenic type 1 $CD4^+$ and $CD8^+$ T cells that target apoptotic cell-derived self-antigens (Steinman et al., 2000; Stuart et al., 2002).

Failures in both central and peripheral tolerance contribute to development of T cell-mediated autoimmune diseases such as type 1 diabetes (T1D). The findings discussed in Chapters 2 and 3 highlight the important roles of DC in peripheral and central tolerance respectively. Interestingly, a common defect, lack of MerTK expression, results in contrasting effects in the periphery versus the thymus. In the pancreatic lymph nodes (PLN), MerTK-deficient DC induce differentiation and expansion of pathogenic, β cell-specific type 1 $CD4^+$ T cells in response to β cell apoptosis. Within the thymus, however, MerTK-deficient DC mediate enhanced deletion of β cell-specific T cell precursors.

MerTK plays a role in immune tolerance as evidenced by mouse models of MerTK deficiency. Mice lacking MerTK expression ($MerTK^{KD/KD}$) develop lupus-like autoimmunity with age (Camenisch et al., 1999; Cohen et al., 2002; Li et al., 2006). Furthermore, mice lacking MerTK expression, as well as related receptor tyrosine kinases Axl and Tyro3, develop severe autoimmunity and lymphoproliferative disorders (Lu and Lemke, 2001). The potential role for MerTK in DC was heretofore unknown, although previous works have implicated MerTK in phagocytosis of apoptotic cells by macrophages (Cohen et al., 2002; Scott et al., 2001; Wu et al., 2005) and retinal pigment epithelial cells (Hall et al., 2003; Hall et al., 2005; Hall et al., 2001; Wong and Janeway, 1999). Thus, we have investigated the potential role of MerTK in immunoregulation of DC by apoptotic cells.

Non-obese diabetic (NOD) mice were utilized to assess the impact of MerTK-deficiency *in vitro* and *in vivo*. DC from NOD.$MerTK^{KD/KD}$ mice were first used to

determine the role of MerTK in apoptotic cell-induced immunoregulation of DC (Chapter 2). Findings are presented demonstrating that MerTK expression is essential to the inhibitory effects of apoptotic cells upon DC. Pretreatment of NOD, but not NOD.MerTK$^{KD/KD}$ DC prevented subsequent activation of the "tolerized" DC (Figure 2.1) as well as stimulation of T cells by DC (Figure 2.3).

The effects observed *in vitro* predictably correlated with immune dysregulation *in vivo*. Induction of β cell apoptosis in NOD mice results in a "regulatory" DC phenotype within the PLN. As such, differentiation and expansion of β cell-specific type 1 CD4$^+$ T cells within the PLN is inhibited (Hugues et al., 2002). In contrast, β cell apoptosis in NOD.MerTK$^{KD/KD}$ mice resulted in significant expansion of β cell-specific type 1 effectors (Figure 2.10, Table 2.1). Furthermore, NOD.MerTK$^{KD/KD}$ mice expressing a diabetogenic β cell specific TCR transgene (NOD.MerTK$^{KD/KD}$.BDC2.5) developed accelerated diabetes (Figure 2.8). We conclude that MerTK expression by DC is essential for maintenance of peripheral self-tolerance to apoptotic cell-derived antigens.

The findings from Chapter 2 may lead to the prediction that NOD.MerTK$^{KD/KD}$ mice would develop accelerated diabetes similar to NOD.MerTK$^{KD/KD}$.BDC2.5 mice. In fact, we found that MerTK deficiency prevented spontaneous diabetes development in NOD.MerTK$^{KD/KD}$ mice (Figure 3.1). As shown in Chapter 3, NOD.MerTK$^{KD/KD}$ mice lack diabetogenic CD4$^+$ and CD8$^+$ T cells in the periphery (Figures 3.2-3.4. Consistent with the findings of Chapter 2, the deficit in β cell-specific typ1 1 T cells was not due to impaired expansion of effectors. Instead, we found that thymic negative selection was enhanced in NOD.MerTK$^{KD/KD}$ mice (Figures 3.5 and 3.6). This trait was conferred by bone marrow-derived antigen presenting cells (Figure 3.7), which we concluded were

thymic DC and not medullary thymic epithelial cells. It is likely that MerTK mediates regulation of thymic DC activation, thus allowing negative selection while preventing overzealous "purging" of the T cell repertoire.

Together, the findings of Chapters 2 and 3 support an essential role for MerTK in immune homeostasis. In the periphery MerTK mediates immunoregulation of DC by apoptotic cells. In the thymus, MerTK regulates DC activation and therefore negative selection efficiency. Interestingly, we found that in T cell-mediated autoimmunity, thymic selection can exhibit dominant tolerance. This is evident when comparing data from NOD.MerTK$^{KD/KD}$ mice with NOD.MerTK$^{KD/KD}$.BDC2.5 mice. BDC2.5 CD4^{+} T cells are not deleted within the thymus, like due to lack of the cognate antigen. In NOD.MerTK$^{KD/KD}$.BDC2.5 mice, immune dysregulation associated with MerTK-deficiency was evident as diabetes was exacerbated. In contrast, NOD.MerTK$^{KD/KD}$ mice, in which the diverse T cell repertoire is subject to negative selection, are protected from diabetes due to MerTK-deficiency. This protection was dominant over the decidedly pro-inflammatory peripheral environment in NOD.MerTK$^{KD/KD}$ mice. These findings highlight the important role of thymic selection in prevention of T cell-mediated autoimmunity. We will continue to investigate the specific role of DC in thymic negative selection and the phenotypic changes within NOD.MerTK$^{KD/KD}$ thymic DC which contribute to altered thymic selection.

It is interesting to note that *mertk* is located within the diabetes susceptibility locus *Idd13* in mice and humans. As described in Chapter 3, we have identified three allelic variants of *mertk* among five mouse strains analyzed (Table 3.1). These alleles will be cloned and expressed in MerTK$^{KD/KD}$ DC to assess potential functional differences

between the molecules. Perhaps alterations in ligand-binding or signaling will result in differential DC function in the thymus or periphery. Such differences could impact susceptibility to type 1 diabetes or other autoimmune diseases.

References

Camenisch, T. D., Koller, B. H., Earp, H. S., and Matsushima, G. K. (1999). A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol *162*, 3498-3503.

Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A., Earp, H. S., Matsushima, G., and Reap, E. A. (2002). Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med *196*, 135-140.

Dejaco, C., Duftner, C., Grubeck-Loebenstein, B., and Schirmer, M. (2006). Imbalance of regulatory T cells in human autoimmune diseases. Immunology *117*, 289-300.

Hall, M. O., Agnew, B. J., Abrams, T. A., and Burgess, B. L. (2003). The phagocytosis of os is mediated by the PI3-kinase linked tyrosine kinase receptor, mer, and is stimulated by GAS6. Adv Exp Med Biol *533*, 331-336.

Hall, M. O., Obin, M. S., Heeb, M. J., Burgess, B. L., and Abrams, T. A. (2005). Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. Exp Eye Res *81*, 581-591.

Hall, M. O., Prieto, A. L., Obin, M. S., Abrams, T. A., Burgess, B. L., Heeb, M. J., and Agnew, B. J. (2001). Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. Exp Eye Res *73*, 509-520.

Hugues, S., Mougneau, E., Ferlin, W., Jeske, D., Hofman, P., Homann, D., Beaudoin, L., Schrike, C., Von Herrath, M., Lehuen, A., and Glaichenhaus, N. (2002). Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. Immunity *16*, 169-181.

Li, Y., Gerbod-Giannone, M. C., Seitz, H., Cui, D., Thorp, E., Tall, A. R., Matsushima, G. K., and Tabas, I. (2006). Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the Mer receptor. J Biol Chem *281*, 6707-6717.

Lu, Q., and Lemke, G. (2001). Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science *293*, 306-311.

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Steinman, R. M., Turley, S., Mellman, I., and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med *191*, 411-416.

Stuart, L. M., Lucas, M., Simpson, C., Lamb, J., Savill, J., and Lacy-Hulbert, A. (2002). Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. J Immunol *168*, 1627-1635.

Wallet, M. A., Sen, P., and Tisch, R. (2005). Immunoregulation of dendritic cells. Clin Med Res *3*, 166-175.

Wong, F. S., and Janeway, C. A., Jr. (1999). The role of CD4 vs. CD8 T cells in IDDM. J Autoimmun *13*, 290-295.

Wu, Y., Singh, S., Georgescu, M. M., and Birge, R. B. (2005). A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. J Cell Sci *118*, 539-553.

Appendix I:

IMMUNOREGULATION OF DENDRITIC CELLS

(published manuscript included with permission)

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Abstract

The paradigm of tolerogenic/immature versus inflammatory/mature dendritic cells (DC) has dominated the recent literature regarding the role of these antigen presenting cells (APC) in mediating immune homeostasis or self-tolerance and response to pathogens, respectively. This issue is further complicated by identification of distinct subtypes of DC which exhibit different APC effector functions. The discovery of pathogen-associated molecular patterns (PAMP) and Toll-like receptors (TLR) provide the mechanistic basis for DC recognition of specific pathogens and induction of appropriate innate and adaptive immune responses. Only recently has insight been gained into how DC contribute to establishing and/or maintaining immunological tolerance to self. Soluble and cellular mediators have been reported to effectively regulate the function of DC by inducing several outcomes ranging from non-inflammatory DC which lack the ability to induce T lymphocyte activation, to DC which actively suppress T lymphocyte responses. A thorough discussion of these stimuli and their

outcomes is essential to understanding the potential for modulating DC function in the treatment of inflammatory disease conditions.

Introduction

DC have long been recognized as highly potent APC. Upon activation, DC process and present antigen and express high levels of co-stimulatory and major histocompatibility complex (MHC) molecules, in addition to secreting various cytokines and chemokines which initiate and/or enhance many T and B lymphocyte responses (Alaniz et al., 2004). These responses include: 1) induction of CD4+ T lymphocyte type 1 and type 2 subset differentiation (Alaniz et al., 2004; Eisenbarth et al., 2003; Maldonado-Lopez and Moser, 2001), 2) CD8+ T lymphocyte activation and enhancement of cytotoxic T lymphocyte (CTL) activity (Smith et al., 2004), and 3) B lymphocyte maturation, Ig class-switching and antibody production (Gerloni et al., 1998; MacPherson et al., 1999). Only recently, however, has the tremendous versatility of DC become apparent. Beyond simple antigen capture, presentation and co-stimulation, DC play a unique role in tailoring immune responses to individual pathogens (de Jong et al., 2004; Proietto et al., 2004). Additionally, DC are important mediators of peripheral immune tolerance and maintenance of immune homeostasis (Gad et al., 2003). In the past, these contrasting roles of DC have been described primarily as a function of maturation. Immature DC largely being non-inflammatory or tolerogenic, whereas mature DC able to elicit a pro-inflammatory response. Although generally correct, this paradigm is now proving to be too simple.

DC arising from myeloid- or lymphoid-derived precursors exhibit an immature phenotype, characterized by a high phagocytic capacity and low expression levels of co-stimulatory

molecules such as CD40, CD80 and CD86 (Mahnke et al., 2002). The many phagocytic receptors of DC allow ingestion of a wide variety of antigens including microbial pathogens or necrotic tissue laden with microbial antigens. Adhesion to, and phagocytosis of microbes by DC is dependent on several receptors including CD14, scavenger receptor-A (SR-A) (van Oosten et al., 1998), and the Fc receptors (FcR) FcγR1, FcγR2b and FcγRIII (Regnault et al., 1999; Schuurhuis et al., 2002; Tobar et al., 2004). These pathways of phagocytosis lead to antigen processing mechanisms that load microbial peptides typically onto MHC class II for presentation to CD4+ T lymphocytes. Notably, DC defined by expression of CD8α are also efficient in shuttling phagocytosed antigens into the MHC class I pathway and subsequent presentation to CD8+ T lymphocytes (Albert et al., 1998a; Albert et al., 1998b). Upon phagocytosis and subsequent activation, DC found residing in peripheral tissues traffick to the draining lymph nodes, undergo further maturation, and present to and stimulate T cells specific for the cognate peptide.

Several factors can enhance DC maturation and promote a pro-inflammatory phenotype. PAMP-containing molecules of bacteria, viruses and parasites, such as lipopolysacchride (LPS), peptidoglycan (PGN), CpG motifs, flagellin, and viral nucleic acids, induce TLR signaling (Werling and Jungi, 2003). TLR signaling in DC serves two purposes. First, bodies that are phagocytosed in the presence of TLR signaling enter a specific phagosome maturation pathway. This pathway results in enhanced degradation of the engulfed material and efficient presentation of antigens (Blander and Medzhitov, 2004). Secondly, DC subsets express unique repertoires of TLRs, allowing for specialized responses to each class of pathogen(Proietto et al., 2004). For example, plasmacytoid DC (pDC) uniquely express TLR-9 and therefore can respond to CpG DNA (Coccia et al., 2004). However, pDC do not

express TLR-4 and therefore respond to LPS weakly. In pDC, TLR-9 ligation results in high production of type-I interferons (IFN-α and IFN-β) enhancing pDC survival and promoting increased MHC expression by neighboring APC. Throughout the DC family, engagement of the various TLRs results in production of several pro-inflammatory cytokines including type-I interferons, TNF-α, IFN-γ, IL-12 and IL-1 (Proietto et al., 2004). These unique responses, tailored by each DC subset, serve to enhance innate immune responses at the site of inflammation, and guide adaptive immunity. The characteristics of murine DC subsets have been thoroughly reviewed by Wilson *et al.* (Wilson and O'Neill, 2003) and are summarized in Table 1.

The above pattern response mechanisms driving inflammation and adaptive immunity to pathogens have established a paradigm for recognition of non-self molecules. Overlooked until recently, however, is the role and in turn how DC prevent autoreactivity and/or tolerize existing autoreactive T cells. For the purpose of this review, we will focus on key soluble and cellular immunoregulators that effectively promote a "tolergenic" phenotype in DC.

Immunoregulation of DC by IL-10

The local cytokine environment is a key contributor to the establishment of DC phenotype. IL-10, a major anti-inflammatory cytokine is produced predominantly by Th2 lymphocytes, Tr1 regulatory lymphocytes, and monocytes and macrophages (Moore et al., 2001). Treatment of immature DC with IL-10 *in vitro* induces an immunoregulatory phenotype that results in inhibition of CD4+ and CD8+ T lymphocyte reactivity in an antigen-specific manner (Steinbrink et al., 1999; Steinbrink et al., 1997; Yang and Lattime, 2003). Among

CD4+ T lymphocytes, both Th1 and Th2 responses can be inhibited by IL-10 treated DC (^{IL10}DC) (Haase et al., 2002). Similarly, ^{IL10}DC mediate tolerance within CD8+ CTL.

IL-10 pretreatment effectively suppresses maturation of immature DC. Upon stimulation, ^{IL10}DC fail to: i) secrete pro-inflammatory cytokines including IL-6, IL-1β, TNF-α and IL-12p70 (Driessler et al., 2004), and ii) up-regulate expression of co-stimulatory molecules such as CD40 and CD86 (McBride et al., 2002). Studies have demonstrated, however, that the tolergenic effect of ^{IL10}DC is not simply due to insufficient co-stimulation. Manavalan *et al.* reported that IL-10 treatment induced up-regulation of the inhibitory immunoglobulin-like transcript 3 (ILT3) and ILT4 (LIR-2) molecules on the surface of DC (Manavalan et al., 2003). Importantly, ^{IL10}DC no longer induced T lymphocyte anergy upon treatment with blocking antibodies specific for ILT3 and ILT4. Furthermore, murine fibroblast L transfectants expressing ILT4 stimulated T lymphocyte proliferation, suggesting that the inhibitory effect of ILT4 is in fact DC-specific (Beinhauer et al., 2004). Evidence indicates that engagement of ILT4 by T lymphocytes promotes signaling within DC necessary for establishing an immunoregulatory phenotype. Interestingly, LPS stimulation of DC has also been found to elicit production of a soluble ILT4 molecule (sILT4) that enhances T lymphocyte activation. sILT4 may competitively block T lymphocyte binding of membrane bound ILT4 (mILT4) to favor a stimulatory DC phenotype. On the other hand, ^{IL10}DC upon LPS treatment fail to express sILT4 which would be expected to further promote the tolergenic capacity of ^{IL10}DC (Beinhauer et al., 2004). Interestingly, donor DC expressing ILT3 and ILT4 are associated with reduced rejection of murine heart transplants (Chang et al., 2002). The immunoregulatory properties of IL-10 upon DC are summarized in Figure 1.

Although ineffective at mediating T lymphocyte activation, Nolan *et al.* demonstrated that ^{IL10}DC maintain the capacity to elicit innate immune responses to microbial pathogens (Nolan et al., 2004). Employing real-time PCR and SAGE, production of TLR-induced pro-inflammatory molecules such as DCIP-1, MIP-2, CXCL-4, CXCL-5 and IL-1, in addition to proteins associated with phagocytosis were either unaffected or up-regulated in ^{IL10}DC treated with LPS. These factors typically enhance: i) recruitment and activation of neutrophils, and ii) phagocytosis of microbial debris, both of which are essential components of innate immunity. Consequently, ^{IL10}DC continue to mediate a pro-inflammatory response and clearance of pathogens. This work has helped to establish a paradigm whereby DC tolerance may not simply be a question of "on versus off" but rather "adaptive versus innate". Future analysis of transcriptional regulation induced by IL-10 may shed light upon molecular switches that induce global changes in the ability of DC to mediate specific responses.

The signaling mechanisms responsible for phenotypic changes in DC following IL-10 treatment remain largely unknown, but recent findings implicate phosphoinositol-3 kinase (PI3K)-and STAT3-dependent inhibition of NF-κB function (Bhattacharyya et al., 2004; Hoentjen et al., 2004). NF-κB is a key transcription factor that regulates various aspects of DC development, maturation and effector function. The IL-10 induced PI3K signaling pathway results in downstream blockade of NF-κB activation (Bhattacharyya et al., 2004). Additionally STAT3 activation, in response to IL-10, results in direct inhibition of NF-κB binding to the IL-12p40 promoter sequence (Hoentjen et al., 2004). Noteworthy is that the effects of IL-10 on signaling are distinct between macrophages and DC, and possibly between subsets of DC. Further research into the signaling mechanisms that control immunoregulatory DC differentiation are warranted.

The many observations that IL-10 modulates DC effector function in a tolerogenic manner have spurred studies to assess the efficacy of using ^{IL10}DC to treat inflammatory disorders. Initial results have been promising and suggest that so-called DC "vaccines" may be useful in the treatment of various autoimmune diseases and rejection of allogeneic organ grafts. In addition, groups have engineered DC to express high levels of IL-10. In this way, the preferential trafficking of DC to sites of inflammation upon adoptive transfer, and the anti-inflammatory properties of IL-10 can be exploited. For example, Coates *et al.* transduced human myeloid DC with an adenoviral recombinant encoding IL-10 gene (AdvIL-10) which upon transfer into a humanized NOD.*scid* mouse model, effectively protected human skin grafts (Coates et al., 2001).

The role of TGF-β1 in regulating DC.

TGF-β1 belongs to a well defined multipotent cytokine family known to regulate several patho-physiological events. TGF-β, produced by T cells, monocytes/macrophages, and granulocytes is typically detected at sites of inflammation (Rastellini et al., 1995). Among the three isoforms of TGF-β, TGF-β1 exhibits the broadest spectrum of biological activities. Notably, TGF-β1 promotes early DC development *in vitro*, and suppression of immature DC activation and maturation (Ellingsworth et al., 1986; Yamaguchi et al., 1997). For example, TGF-β1 inhibits up-regulation of the costimulatory molecules CD83 and CD86 in humans (Strobl and Knapp, 1999) and CD80 and CD86 in mice (Zhang et al., 1999), thereby reducing the efficacy of DC to stimulate T lymphocytes.

One mechanism that contributes to the impaired stimulatory capacity of TGF-β1-treated DC may be the down-regulation of key immunomodulatory receptors. Mou *et al.* observed

that treatment of murine bone marrow-derived DC with TGF-β1 *in vitro* results in decreased expression of TLR-4. This finding correlated with reduced sensitivity to LPS-induced maturation of the DC(Mou et al., 2004). The inability to recognize pro-inflammatory microbial products as well as inhibitory signaling mechanisms likely combine to render TGF-β1 DC ineffective in driving T cell immunity.

Additionally, TGF-β1 affects the trafficking of mature DC (Figure 2). For example, Ogata *et al.* have reported that TGF-β1 inhibits DC expression of the chemokine receptor CCR7. Consequently, TGF-β1 conditioned DC are unable to efficiently traffic to the draining lymph nodes in response to MIP-3β (Ogata et al., 1999). The molecular targets of TGF-β1 mediated suppression in DC remain ill defined. One such target, however, appears to be the transcription factor RunX3. For example, DC lacking expression of RunX3 are resistant to the immunoregulatory effects of TGF-β1(Fainaru et al., 2004).

Several strategies of immunotherapy have focused on the immunoregulatory properties of TGF-β1 upon DC maturation and effector function. Yarlin *et al.* employing TGF-β1-treated DC successfully suppressed experimental autoimmune myasthenia gravis (EAMG), an animal model of myasthenia gravis (MG) (Yarilin et al., 2002). Both EAMG and MG pathologies are the result of T cell-dependent antibody responses to the acetylcholine receptor (AChR). Adoptive transfer of splenic DC pretreated with TGF-β1 after Lewis rats were immunized with AChR protein, effectively suppressed ongoing EAMG characterized by reduced mortality and a diminished frequency of anti-AChR antibody producing cells.

Similarly, many groups have utilized TGF-β1-expressing adenoviral vectors (Adv-TGF) to modify DC function. These studies have consistently reported the ability of Adv-TGF-transduced DC to inhibit syngeneic and allogeneic T cell responses. This inhibition includes

dominant suppression of allogeneic CD4+ and CD8+ effectors that have been primed by untreated DC (Asiedu et al., 2002).

DC and immune homeostasis; the role of apoptotic cells.

DC activation is typically referred to in the context of a response to pathogens. Unperturbed environments foster maintenance of immature DC, while microbial pathogens induce differentiation to a specific mature/activated phenotype. This representation of DC life cycle accurately represents a portion of immune regulation, but the model fails to address the activity of DC under homeostatic conditions. Multicellular organisms constantly harbor a mixed balance of viable, apoptotic and necrotic tissues. The continual exposure to self-antigen necessitates that DC maturation and effector function to be regulated appropriately to maintain immunological tolerance.

The clearance of apoptotic cells *in vivo* is carried out by multiple phagocytes including macrophages, neutrophils and DC. In the case of DC, it is only immature cells that are capable of phagocytosing apoptotic cells. Binding and ingestion of apoptotic cells by immature DC is mediated by a group of receptors including phosphatidylserine receptor (PSR) (Li et al., 2003), CD36, $\alpha_V\beta_5$ integrin (Albert et al., 1998a), complement receptor C1qR and the receptor tyrosine kinase Mertk (Cohen et al., 2002).

In general, when DC encounter apoptotic cells, a non-inflammatory and tolerogenic phenotype typical of immature DC is observed (Steinman et al., 2000). For example, Stuart *et. al.* showed that DC pretreated with apoptotic cells exhibit a reduced capacity to produce IL-12p70 upon stimulation (Stuart et al., 2002). Interestingly, the effect was selective in that secretion of TNF-α was unaltered. Immature DC treated with apoptotic cells also exhibit an

impaired capacity to stimulate T lymphocytes. Notably, LPS treatment is unable overcome the "tolergenic" phenotype, indicating the effect of apoptotic cells is long-lasting and relatively stable (Takahashi and Kobayashi, 2003). As a result, activation of DC carrying the remnants of apoptotic cells is effectively prevented. In addition to maintaining an immature phenotype, apoptotic cells have been reported by Takahashi *et al.* to induce the expression of IL-6 and IL-12p40, and therefore further enhance the tolergenic function of the DC (Takahashi and Kobayashi, 2003). Apoptotic cell-induced IL-6 secretion by DC functions in an autocrine manner to block further maturation of DC. For example, activation of STAT3 following IL-6 pretreatment blocks LPS-induced up-regulation of CD80, CD86, CD40 and MHCII, thereby reducing the T lymphocyte stimulatory capacity of the DC. Furthermore, IL-6 has been reported to suppress TNFα- and PGN-mediated activation and maturation of DC. On the other hand, secretion of significant levels of IL-12p40 results in formation of IL-12p40 homodimers, which competitively inhibit IL-12p70 binding to the IL-12 receptor on T lymphocytes (Trinchieri, 2003). Consequently, differentiation of naïve T lymphocytes into type 1 effectors is suppressed (Figure 3).

Sauter *et al*. have described how phagocytosis of apoptotic cells by DC affects cross-presentation of self-antigens to CD8+ T lymphocytes (Sauter et al., 2000). Employing human DC and apoptotic versus necrotic 293 tumor cells, the importance of the mode of death of the phagocytosed cells was initially defined. Necrotic but not apoptotic 293 cells induced DC maturation based on up-regulation of CD83 and DC-LAMP. Furthermore, pulsing DC with necrotic allogeneic splenocytes resulted in a robust mixed lymphocyte reaction (MLR). In contrast, apoptotic allogeneic splenocytes failed to induce a significant MLR. Furthermore, Furgeson *et al.* demonstrated the potent effects of apoptotic cells on DC effector function *in*

vivo (Ferguson et al., 2002). Injection of apoptotic splenocytes coupled with trinitrophenol blocked *in vitro* recall responses to the hapten. Importantly, CD11c+CD8α+ DC were identified as the critical effectors for inducing the immune tolerance to apoptotic cell antigens.

These data and work by others support a role for DC in maintaining immunological homeostasis s(Basu et al., 2000; Stuart et al., 2002; Xu et al., 2004). In contrast, necrotic cells typical of microbial infections induce DC activation and maturation, and subsequent immunity. This balance in DC reactivity allows the peripheral immune system to continually monitor tissues for signs of death and mount the appropriate responses. The importance of DC in clearance of apoptotic cells and the maintenance of immune homeostasis is highlighted in autoimmune diseases such as systemic lupus erythemetosous (SLE). While not wholly causal for SLE, failure to effectively clear apoptotic cells contributes to the severity of disease (Gaipl et al., 2004).

We have recently undertaken studies to describe the signaling mechanisms responsible for apoptotic cell-induced DC tolerance. Initial results suggest that apoptotic cells block pro-inflammatory stimuli, such as TLR and CD40 signaling through inhibition of NF-κB activation and nuclear translocation. Further analysis of the processes that mediate immune homeostasis by apoptotic cells will help define defects which contribute to autoimmunity, in addition to the development of rational modalities of immunotherapy for prevention and/or treatment of autoimmunity, and other pro-inflammatory diseases.

Signaling via CD80 and CD86 regulates DC activation/maturation.

T lymphocytes are frequently viewed as dependents of DC and other APC. Upon a cognate interaction, however, engagement by T lymphocytes of various cell surface molecules also induce signals which affect DC activation and/or maturation. For example, ligation of CD40 by CD40L expressed on the surface of T lymphocytes delivers a potent activational signal for DC (Bennett et al., 1998; Hermans et al., 1999; Ridge et al., 1998; Schoenberger et al., 1998). More recently, signals delivered by the co-stimulatory molecules CD80 and CD86 have been shown to modulate DC activation and effector function (Zheng et al., 2004). Regulatory T lymphocytes (T_{Reg}) express the surface molecule CTLA-4 which binds CD80 (and CD86) with high affinity(Munn et al., 2004). Following a cognate interaction between T_{Reg} and DC, CD80 signaling is induced which includes STAT-1 activation and subsequent secretion of IFN-γ (Logue and Sha, 2004). IFN-γ has an immediate autocrine effect upon DC resulting in the secretion of the enzyme indoleamine 2,3-dioxygenase (IDO) (Grohmann et al., 2002). IDO is an enzyme that catalyzes the breakdown of the amino acid tryptophan into N-formyl-kynurenine and various downstream metabolites. Importantly, depletion of tryptophan in the surrounding milieu has a profound effect upon T lymphocytes which can be reversed experimentally by substituting excess tryptophan (Munn et al., 1999; Munn et al., 2002). T lymphocytes stimulated by peptide/MHC presented by IDO-producing DC undergo activation and then rapid cell death via apoptosis (Grohmann et al., 2001) (Figure 4).

As is the case for most mechanisms of immune deviation, CTLA-4 induced tolerance has an opposing force to deal with. In a fashion quite similar to the CTLA-4/CD80 signaling axis, activated T lymphocytes provide DC with a pro-inflammatory feedback stimulus. Expression of CD28 by activated T lymphocytes serves to ligate CD86 on DC(Orabona et al.,

2004). This interaction induces a signaling cascade that results in p38 MAPK and NF-κB activation (Logue and Sha, 2004). Subsequently IFN-γ and IL-6 are produced. In this scenario, however, IFN-γ mediated induction of IDO is hampered by IL-6. IL-6 has been shown to dominantly block IFN-γ induced IDO expression and block the regulatory capacity of these DC (Grohmann et al., 2001). Indeed, expression of IL-6 by DC in this case drives a dominant pro-inflammatory response, as opposed to serving a suppressive role described above. This phenomenon further highlights the complexity of DC regulation and serves to warn against assignment of broad descriptive labels (i.e. regulatory) to cytokines.

CD200R, a novel molecule regulating DC effector function.

A second model for IDO induction involves the widely expressed molecule CD200 (OX-2) and its receptor CD200R (Gorczynski et al., 2004). Ligation of CD200R on splenic pDC results in IDO secretion and antigen-specific immunoregulation, which in turn is mediated via induction of T_{Reg} (Fallarino et al., 2004). The mechanism by which IDO secreting pDC induce T_{Reg} has yet to be defined. The ubiquitous nature of CD200 expression by non-immune cells suggests a role for CD200R-mediated tolerance in immune homeostasis. Furthermore, mice lacking CD200 expression demonstrate increased susceptibility to autoimmunity (Barclay et al., 2002; Nathan and Muller, 2001). Manipulation of CD200/CD200R signaling may prove to be a novel mechanism for induction of antigen specific immune tolerance. Such a system could be beneficial for clinical treatment of many inflammatory diseases such as type 1 diabetes, rheumatoid arthritis and multiple sclerosis.

Summary

Scientists have spent the better part of the past century attempting to manipulate the immune system. These efforts have primarily focused upon induction of immunity to pathogens. Currently, a major emphasis is the development of effective immunotherapies for the prevention and/or suppression of pro-inflammatory responses associated with autoimmunity and transplantation. In view of their central role in eliciting and regulating immunity, the application of DC for the purpose of immunotherapy is highly appealing.

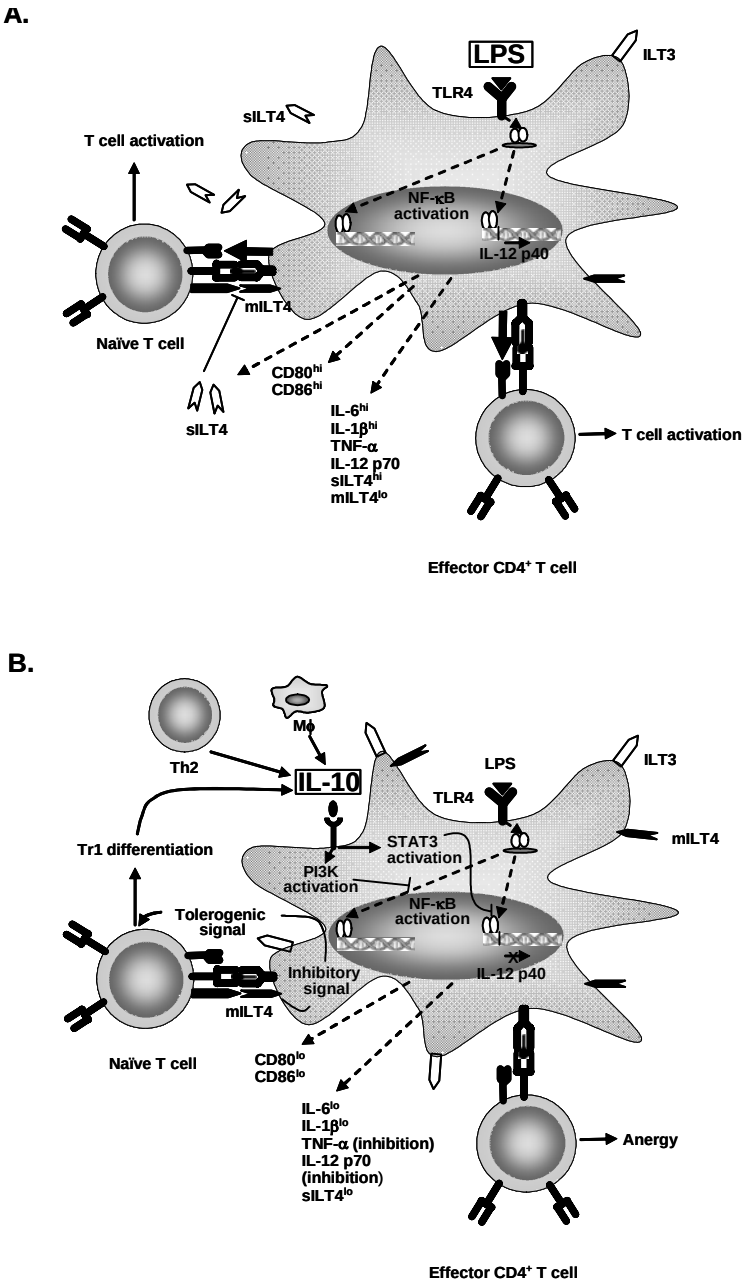
Initial studies using IL-10- or TGF-β1-treated DC to induce antigen-specific immune tolerance have shown promise. As we gain further mechanistic insight into events regulating DC maturation and effector function, the therapeutic efficacy of DC-based "vaccines" will be enhanced. Techniques that allow for harvest, *ex vivo* culture and re-infusion of autologous DC have already been developed. Together with advances in gene therapy, the use of DC-based vaccines will allow clinicians to manipulate the immune system in a safe and effective manner.

Table 1

Multiple subsets of murine splenic dendritic cells perform specialized functions.

DC subset	Markers	Function
Myeloid	$CD11c^{+}CD205^{-}CD11b^{+}$ $CD8\alpha^{-}CD4^{+/-}$	Located in splenic marginal zones. Efficient stimulation of $CD4^{+}$ and $CD8^{+}$ T cells. Favor Th2 differentiation during inflammatory conditions
Lymphoid	$CD11c^{+}CD205^{+}CD11b^{-}$ $CD8\alpha^{+}CD4^{-}$	Efficiently cross-present exogneous antigens to $CD8^{+}$ CTL. Favor Th1 differentiation during inflammatory conditions. Maintain cross-tolerance to self antigens.
Plasmacytoid	$CD11c^{+}CD11b^{-}B220^{+}$ $GR1^{+/-}$	Possibly contribute to peripheral self-tolerance. Mediate anti-viral responses through production of IFN-α.

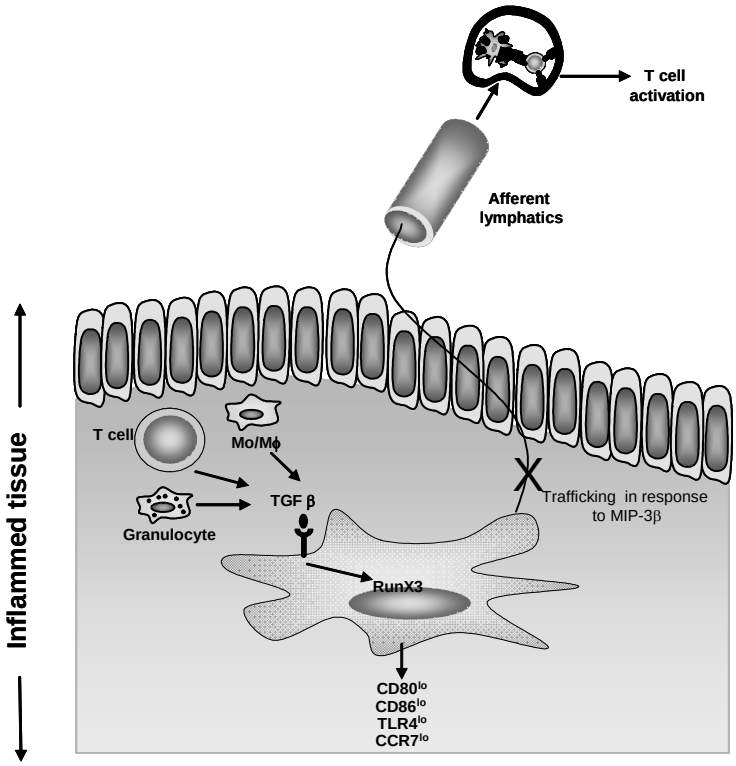
Figure 1



IL-10 induces immunoregulation of DC maturation. (A) LPS promotes T cell activation. When immature DC encounter inflammatory stimuli such as LPS, maturation ensues. NF-κB is activated, translocated to the nucleus and transcription of multiple genes is induced. Co-stimulatory molecules CD80 and CD86, inflammatory cytokines (IL-6, IL-1β, TNF-α and IL-12p70) and soluble ILT4 (sILT4, sLIR2) are produced, which supports activation of T cells. (B) IL-10 blocks the effects of LPS upon DC. IL-10 produced by monocytes, TH2 cells and Tr1 cells induces PI3K and STAT3 activation by DC. PI3K activation results in blockade of NF-κB activation and STAT3 blocks binding of NF-κB to the IL-12p40 promoter. Consequently, differentiation of Th1 T lymphocytes is inhibited. Additionally, IL-10 inhibits

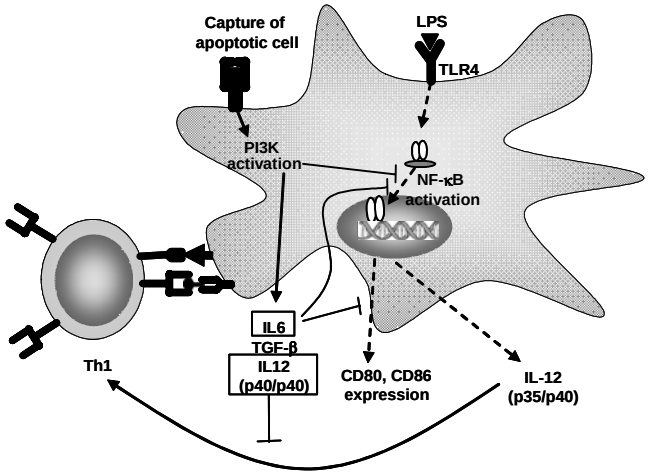
production of sILT4, favoring instead expression of mILT4. Ligation of DC mILT4 by T lymphocytes induces a regulatory DC phenotype that induces Tr1 differentiation.

Figure 2



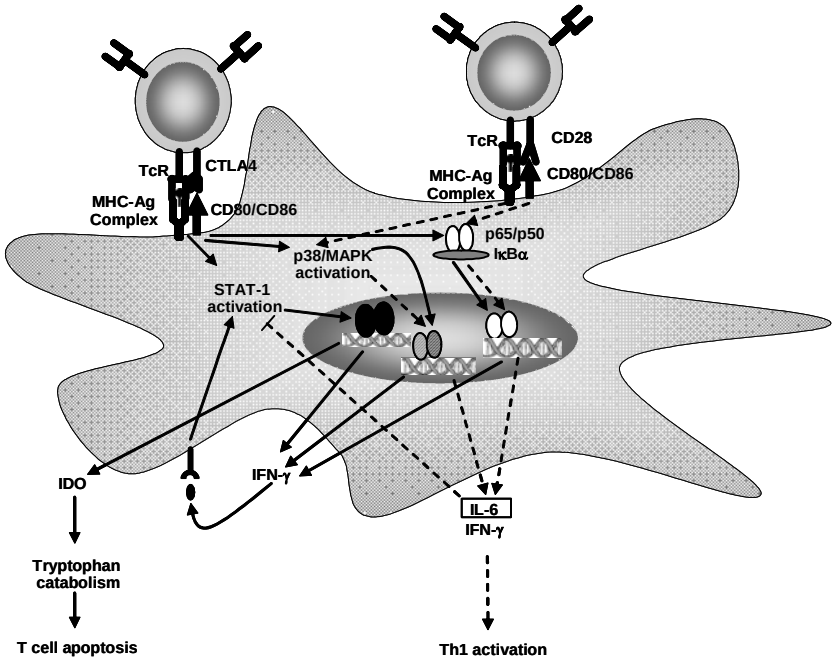
TGF-β1 modulated DC phenotype and T lymphocyte activation. TGF-β1 is produced by monocyte/macrophages, T lymphocytes or granulocytes or provided exogenously as a means of immunotherapy. DC which encounter TGF-β1 down regulate expression of CD80, CD86, TLR4 and importantly CCR7. Low expression of CCR7 inhibits MIP-3β-induced migration to lymph nodes. Therefore, T lymphocyte stimulation is inhibited.

Figure 3



Apoptotic cells induce immunoregulation and tolerance induction through DC. DC scavenge tissues for microbes and dying tissue. Apoptotic cells induce PI3K dependent signaling resulting in inhibition of NF-κB translocation and co-stimulatory molecule (CD80/86) expression. Production of IL-6, TGF-β and IL-12p40 ensue. IL-12p40 homdimers inhibit the effects of IL-12p70 upon T lymphocytes, effectively inhibiting Th1 induction. Apoptotic cells induce a lasting inhibitory effect which renders DC refractory to LPS induced stimulation of Th1 lymphocytes.

Figure 4



CTLA-4 and CD28 induce divergent outcomes in DC maturation. T_{Reg} cells expressing CTLA-4 ligate CD80 on DC inducing STAT-1 activation, IFN-γ production and IDO production. IDO catalyzes the breakdown of tryptophan which results in T lymphocyte activation followed by rapid induction of apoptosis. In contrast, activated T lymphocytes expressing CD28 induce activational signals through CD80/CD86. Receptor ligation induces p38 MAPK and NF-κB activation. IL-6 and IFN-γ are then produced. IL-6 induces a dominant antagonistic effect upon IDO production. DC are now capable of inducing T lymphocyte activation and proliferation.

References

Alaniz, R. C., Sandall, S., Thomas, E. K., and Wilson, C. B. (2004). Increased dendritic cell numbers impair protective immunity to intracellular bacteria despite augmenting antigen-specific CD8+ T lymphocyte responses. J Immunol *172*, 3725-3735.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med *188*, 1359-1368.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature *392*, 86-89.

Asiedu, C., Dong, S. S., Pereboev, A., Wang, W., Navarro, J., Curiel, D. T., and Thomas, J. M. (2002). Rhesus monocyte-derived dendritic cells modified to over-express TGF-beta1 exhibit potent veto activity. Transplantation *74*, 629-637.

Barclay, A. N., Wright, G. J., Brooke, G., and Brown, M. H. (2002). CD200 and membrane protein interactions in the control of myeloid cells. Trends Immunol *23*, 285-290.

Basu, S., Binder, R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. (2000). Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol *12*, 1539-1546.

Beinhauer, B. G., McBride, J. M., Graf, P., Pursch, E., Bongers, M., Rogy, M., Korthauer, U., de Vries, J. E., Aversa, G., and Jung, T. (2004). Interleukin 10 regulates cell surface and soluble LIR-2 (CD85d) expression on dendritic cells resulting in T cell hyporesponsiveness in vitro. Eur J Immunol *34*, 74-80.

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478-480.

Bhattacharyya, S., Sen, P., Wallet, M., Long, B., Baldwin, A. S., Jr., and Tisch, R. (2004). Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of I{kappa}B kinase activity. Blood *104*, 1100-1109.

Blander, J. M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. Science *304*, 1014-1018.

Chang, C. C., Ciubotariu, R., Manavalan, J. S., Yuan, J., Colovai, A. I., Piazza, F., Lederman, S., Colonna, M., Cortesini, R., Dalla-Favera, R., and Suciu-Foca, N. (2002). Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nat Immunol *3*, 237-243.

Coates, P. T., Krishnan, R., Kireta, S., Johnston, J., and Russ, G. R. (2001). Human myeloid dendritic cells transduced with an adenoviral interleukin-10 gene construct inhibit human skin graft rejection in humanized NOD-scid chimeric mice. Gene Ther *8*, 1224-1233.

Coccia, E. M., Severa, M., Giacomini, E., Monneron, D., Remoli, M. E., Julkunen, I., Cella, M., Lande, R., and Uze, G. (2004). Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. Eur J Immunol *34*, 796-805.

Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A., Earp, H. S., Matsushima, G., and Reap, E. A. (2002). Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med *196*, 135-140.

de Jong, E. C., Smits, H. H., and Kapsenberg, M. L. (2004). Dendritic cell-mediated T cell polarization. Springer Semin Immunopathol.

Driessler, F., Venstrom, K., Sabat, R., Asadullah, K., and Schottelius, A. J. (2004). Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. Clin Exp Immunol *135*, 64-73.

Eisenbarth, S. C., Piggott, D. A., and Bottomly, K. (2003). The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. Curr Opin Immunol *15*, 620-626.

Ellingsworth, L. R., Brennan, J. E., Fok, K., Rosen, D. M., Bentz, H., Piez, K. A., and Seyedin, S. M. (1986). Antibodies to the N-terminal portion of cartilage-inducing factor A and transforming growth factor beta. Immunohistochemical localization and association with differentiating cells. J Biol Chem *261*, 12362-12367.

Fainaru, O., Woolf, E., Lotem, J., Yarmus, M., Brenner, O., Goldenberg, D., Negreanu, V., Bernstein, Y., Levanon, D., Jung, S., and Groner, Y. (2004). Runx3 regulates mouse TGF-beta-mediated dendritic cell function and its absence results in airway inflammation. Embo J *23*, 969-979.

Fallarino, F., Asselin-Paturel, C., Vacca, C., Bianchi, R., Gizzi, S., Fioretti, M. C., Trinchieri, G., Grohmann, U., and Puccetti, P. (2004). Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. J Immunol *173*, 3748-3754.

Ferguson, T. A., Herndon, J., Elzey, B., Griffith, T. S., Schoenberger, S., and Green, D. R. (2002). Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. J Immunol *168*, 5589-5595.

Gad, M., Claesson, M. H., and Pedersen, A. E. (2003). Dendritic cells in peripheral tolerance and immunity. Apmis *111*, 766-775.

Gaipl, U. S., Franz, S., Voll, R. E., Sheriff, A., Kalden, J. R., and Herrmann, M. (2004). Defects in the disposal of dying cells lead to autoimmunity. Curr Rheumatol Rep *6*, 401-407.

Gerloni, M., Lo, D., and Zanetti, M. (1998). DNA immunization in relB-deficient mice discloses a role for dendritic cells in IgM-->IgG1 switch in vivo. Eur J Immunol *28*, 516-524.

Gorczynski, R. M., Chen, Z., Kai, Y., Wong, S., and Lee, L. (2004). Induction of tolerance-inducing antigen-presenting cells in bone marrow cultures in vitro using monoclonal antibodies to CD200R. Transplantation *77*, 1138-1144.

Grohmann, U., Fallarino, F., Bianchi, R., Belladonna, M. L., Vacca, C., Orabona, C., Uyttenhove, C., Fioretti, M. C., and Puccetti, P. (2001). IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase. J Immunol *167*, 708-714.

Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candeloro, P., Belladonna, M. L., Bianchi, R., Fioretti, M. C., and Puccetti, P. (2002). CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol *3*, 1097-1101.

Haase, C., Jorgensen, T. N., and Michelsen, B. K. (2002). Both exogenous and endogenous interleukin-10 affects the maturation of bone-marrow-derived dendritic cells in vitro and strongly influences T-cell priming in vivo. Immunology *107*, 489-499.

Hermans, I. F., Ritchie, D. S., Daish, A., Yang, J., Kehry, M. R., and Ronchese, F. (1999). Impaired ability of MHC class II-/- dendritic cells to provide tumor protection is rescued by CD40 ligation. J Immunol *163*, 77-81.

Hoentjen, F., Sartor, R. B., Ozaki, M., and Jobin, C. (2004). STAT3 regulates NF-{kappa}B recruitment to the IL-12p40 promoter in dendritic cells. Blood.

Li, M. O., Sarkisian, M. R., Mehal, W. Z., Rakic, P., and Flavell, R. A. (2003). Phosphatidylserine receptor is required for clearance of apoptotic cells. Science *302*, 1560-1563.

Logue, E. C., and Sha, W. C. (2004). CD28-B7 bidirectional signaling: a two-way street to activation. Nat Immunol *5*, 1103-1105.

MacPherson, G., Kushnir, N., and Wykes, M. (1999). Dendritic cells, B cells and the regulation of antibody synthesis. Immunol Rev *172*, 325-334.

Mahnke, K., Schmitt, E., Bonifaz, L., Enk, A. H., and Jonuleit, H. (2002). Immature, but not inactive: the tolerogenic function of immature dendritic cells. Immunol Cell Biol *80*, 477-483.

Maldonado-Lopez, R., and Moser, M. (2001). Dendritic cell subsets and the regulation of Th1/Th2 responses. Semin Immunol *13*, 275-282.

Manavalan, J. S., Rossi, P. C., Vlad, G., Piazza, F., Yarilina, A., Cortesini, R., Mancini, D., and Suciu-Foca, N. (2003). High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. Transpl Immunol *11*, 245-258.

McBride, J. M., Jung, T., de Vries, J. E., and Aversa, G. (2002). IL-10 alters DC function via modulation of cell surface molecules resulting in impaired T-cell responses. Cell Immunol *215*, 162-172.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol *19*, 683-765.

Mou, H. B., Lin, M. F., Cen, H., Yu, J., and Meng, X. J. (2004). TGF-beta1 treated murine dendritic cells are maturation resistant and down-regulate Toll-like receptor 4 expression. J Zhejiang Univ Sci *5*, 1239-1244.

Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., and Mellor, A. L. (1999). Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med *189*, 1363-1372.

Munn, D. H., Sharma, M. D., Lee, J. R., Jhaver, K. G., Johnson, T. S., Keskin, D. B., Marshall, B., Chandler, P., Antonia, S. J., Burgess, R., *et al.* (2002). Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science *297*, 1867-1870.

Munn, D. H., Sharma, M. D., and Mellor, A. L. (2004). Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. J Immunol *172*, 4100-4110.

Nathan, C., and Muller, W. A. (2001). Putting the brakes on innate immunity: a regulatory role for CD200? Nat Immunol *2*, 17-19.

Nolan, K. F., Strong, V., Soler, D., Fairchild, P. J., Cobbold, S. P., Croxton, R., Gonzalo, J. A., Rubio, A., Wells, M., and Waldmann, H. (2004). IL-10-conditioned dendritic cells, decommissioned for recruitment of adaptive immunity, elicit innate inflammatory gene products in response to danger signals. J Immunol *172*, 2201-2209.

Ogata, M., Zhang, Y., Wang, Y., Itakura, M., Zhang, Y. Y., Harada, A., Hashimoto, S., and Matsushima, K. (1999). Chemotactic response toward chemokines and its regulation by transforming growth factor-beta1 of murine bone marrow hematopoietic progenitor cell-derived different subset of dendritic cells. Blood *93*, 3225-3232.

Orabona, C., Grohmann, U., Belladonna, M. L., Fallarino, F., Vacca, C., Bianchi, R., Bozza, S., Volpi, C., Salomon, B. L., Fioretti, M. C., *et al.* (2004). CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. Nat Immunol *5*, 1134-1142.

Proietto, A. I., O'Keeffe, M., Gartlan, K., Wright, M. D., Shortman, K., Wu, L., and Lahoud, M. H. (2004). Differential production of inflammatory chemokines by murine dendritic cell subsets. Immunobiology *209*, 163-172.

Rastellini, C., Lu, L., Ricordi, C., Starzl, T. E., Rao, A. S., and Thomson, A. W. (1995). Granulocyte/macrophage colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival. Transplantation *60*, 1366-1370.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med *189*, 371-380.

Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature *393*, 474-478.

Sauter, B., Albert, M. L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med *191*, 423-434.

Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schuurhuis, D. H., Ioan-Facsinay, A., Nagelkerken, B., van Schip, J. J., Sedlik, C., Melief, C. J., Verbeek, J. S., and Ossendorp, F. (2002). Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. J Immunol *168*, 2240-2246.

Smith, C. M., Wilson, N. S., Waithman, J., Villadangos, J. A., Carbone, F. R., Heath, W. R., and Belz, G. T. (2004). Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. Nat Immunol *5*, 1143-1148.

Steinbrink, K., Jonuleit, H., Muller, G., Schuler, G., Knop, J., and Enk, A. H. (1999). Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood *93*, 1634-1642.

Steinbrink, K., Wolfl, M., Jonuleit, H., Knop, J., and Enk, A. H. (1997). Induction of tolerance by IL-10-treated dendritic cells. J Immunol *159*, 4772-4780.

Steinman, R. M., Turley, S., Mellman, I., and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med *191*, 411-416.

Strobl, H., and Knapp, W. (1999). TGF-beta1 regulation of dendritic cells. Microbes Infect *1*, 1283-1290.

Stuart, L. M., Lucas, M., Simpson, C., Lamb, J., Savill, J., and Lacy-Hulbert, A. (2002). Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. J Immunol *168*, 1627-1635.

Takahashi, M., and Kobayashi, Y. (2003). Cytokine production in association with phagocytosis of apoptotic cells by immature dendritic cells. Cell Immunol *226*, 105-115.

Tobar, J. A., Gonzalez, P. A., and Kalergis, A. M. (2004). Salmonella escape from antigen presentation can be overcome by targeting bacteria to Fc gamma receptors on dendritic cells. J Immunol *173*, 4058-4065.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol *3*, 133-146.

van Oosten, M., van de Bilt, E., van Berkel, T. J., and Kuiper, J. (1998). New scavenger receptor-like receptors for the binding of lipopolysaccharide to liver endothelial and Kupffer cells. Infect Immun *66*, 5107-5112.

Werling, D., and Jungi, T. W. (2003). TOLL-like receptors linking innate and adaptive immune response. Vet Immunol Immunopathol *91*, 1-12.

Wilson, H. L., and O'Neill, H. C. (2003). Murine dendritic cell development: difficulties associated with subset analysis. Immunol Cell Biol *81*, 239-246.

Xu, D. L., Liu, Y., Tan, J. M., Li, B., Zhong, C. P., Zhang, X. H., Wu, C. Q., and Tang, X. D. (2004). Marked prolongation of murine cardiac allograft survival using recipient immature dendritic cells loaded with donor-derived apoptotic cells. Scand J Immunol *59*, 536-544.

Yamaguchi, Y., Tsumura, H., Miwa, M., and Inaba, K. (1997). Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. Stem Cells *15*, 144-153.

Yang, A. S., and Lattime, E. C. (2003). Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses. Cancer Res *63*, 2150-2157.

Yarilin, D., Duan, R., Huang, Y. M., and Xiao, B. G. (2002). Dendritic cells exposed in vitro to TGF-beta1 ameliorate experimental autoimmune myasthenia gravis. Clin Exp Immunol *127*, 214-219.

Zhang, Y., Zhang, Y. Y., Ogata, M., Chen, P., Harada, A., Hashimoto, S., and Matsushima, K. (1999). Transforming growth factor-beta1 polarizes murine hematopoietic progenitor cells to generate Langerhans cell-like dendritic cells through a monocyte/macrophage differentiation pathway. Blood *93*, 1208-1220.

Zheng, Y., Manzotti, C. N., Liu, M., Burke, F., Mead, K. I., and Sansom, D. M. (2004). CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. J Immunol *172*, 2778-2784.

APPENDIX II:

APOPTOTIC CELLS INDUCE MERTK-DEPENDENT BLOCKADE OF NF-κB IN DENDRITIC CELLS

(Manuscript submitted for publication but not yet accepted)

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Abstract

Dendritic cells (DC) play a key role in immune homeostasis and maintenance of self-tolerance. Tolerogenic DC can be established by an encounter with apoptotic cells (AC) and subsequent inhibition of maturation and effector functions. The receptor(s) and signaling pathway(s) involved in AC-induced inhibition of DC have yet to be defined. We demonstrate that pretreatment with apoptotic but not necrotic cells, inhibits activation of IκB kinase (IKK) and downstream NF-κB. Notably, receptor tyrosine kinase Mer (MerTK) binding of AC is required for mediating this effect. DC lacking MerTK expression ($MerTK^{KD}$) or treated with blocking MerTK-specific Ab are resistant to AC-induced inhibition and continue to activate

NF-κB, and secrete proinflammatory cytokines. Blocking MerTK activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway prevents AC-induced inhibition. These results demonstrate an essential role for MerTK-mediated regulation of the PI3K/AKT and NF-κB pathways in AC-induced inhibition of DC.

Introduction

Dendritic cells (DC) are potent mediators of T cell activation and proinflammatory immune responses to foreign antigens and pathogens (Banchereau et al., 2000; Pulendran et al., 2001). However, DC also have an important role in maintaining immune homeostasis and tolerance to self-proteins (Hawiger et al., 2001; Scheinecker et al., 2002; Liu et al., 2002; Belz et al., 2002; Steinman et al., 2003). These two opposing functions are believed in part to reflect differences in DC activation, maturation, and/or subset. Tolerogenic DC typically exhibit an immature phenotype characterized by low cell surface expression of MHC and co-stimulatory molecules, and do not secrete proinflammatory cytokines. Furthermore, soluble and cellular mediators that inhibit DC activation and maturation can establish a tolerogenic phenotype. For example, binding to and phagocytosis of apoptotic cells (AC) by immature DC inhibits activation and maturation induced by various stimuli (Sauter et al., 2000; Stuart et al., 2002). This inhibitory effect serves an important role since AC are present in tissues under both homeostatic and inflamed conditions, and provide a potential source of self-proteins to mediate autoimmunity. Defective clearance of AC has been linked to different types of autoimmunity (Botto et al., 1998; O'Brien et al., 2002). A number of receptors expressed by immature DC such as the phosphatidylserine (PS) receptor, CD36, $\alpha_v\beta_5$

integrin and complement receptor C1qR are involved in AC binding and/or ingestion (Chen et al., 2004; Fadok et al., 2001; Savill et al., 2002; Roos et al., 2004). However, the relative contribution of these receptors in mediating the immunoregulatory effect(s) of AC on immature DC is unclear, and the molecular basis for this inhibition has not been defined in DC.

Recently, the Axl/Mer/Tyro3 receptor tyrosine kinase (RTK) family has been implicated in homeostatic regulation of antigen presenting cell (APC) activation (Lu and Lemke, 2001; Lemke and Lu, 2003). This family consisting of Axl, Tyro3 and MerTK is expressed by a variety of cell types including macrophages (Mϕ) and DC. Mice lacking expression of all three RTKs exhibit hyperactivated Mϕ and DC, which in turn drive lymphoproliferation and systemic autoimmunity (Lu and Lemke, 2001). Similarly, our group has shown that mice lacking MerTK expression ($MerTK^{KD}$) develop lupus-like autoimmunity and are more prone to lipopolysaccharide (LPS)-induced endotoxic shock (Camenisch et al., 1999; Scott et al., 2001; Cohen et al., 2002). Autoimmunity in $MerTK^{KD}$ mice correlates with a reduced rate of *in vivo* clearance of AC, which is consistent with findings that MerTK mediates AC phagocytosis by Mϕ (Scott et al., 2001; Cohen et al., 2002). A ligand for MerTK is growth arrest specific gene (GAS) 6, which binds to PS expressed on the inverted plasma membrane of AC (Chen et al., 1997). Recognition of a GAS6-PS complex facilitates binding of AC and subsequent phagocytosis by Mϕ. Accordingly, MerTK has been proposed to facilitate phagocytosis of AC and down-regulate activation in Mϕ. Whether MerTK functions similarly in DC has yet to be determined.

We and others (Burkly et al., 1995; Rescigno et al., 1998; Wu et al., 1998; Weaver et al., 2001; Ouaaz et al., 2002; Poligone et al., 2002) have demonstrated a key role for the

transcription factor NF-κB in regulating gene expression associated with the development, activation, maturation and APC function of DC. The NF-κB complex consists of homo- and heterodimers of the structurally related proteins p50, p52, p65 (RelA), c-Rel and RelB. NF-κB is typically sequestered in the cytoplasm bound by the inhibitory molecules IκBα, IκBβ and IκBε (Baldwin, 1996; Ghosh et al., 1998; Ghosh and Karin, 2002). In response to a broad range of stimuli, including LPS and CD40 engagement, the multi-subunit complex IκB kinase (IKK) consisting of IKK1/IKKα, IKK2/IKKβ, and IKKγ/NEMO is activated upon phosphorylation (Delhase et al., 1999; Karin, 1999; Li et al., 1999; Tanaka et al., 1999). Activated IKK phosphorylates the IκB proteins, which in turn undergo polyubiquitination and subsequent degradation via the 26S proteasome (Ghosh et al., 1998; Ghosh and Karin 2002). The latter permits nuclear translocation of NF-κB that binds to consensus sequences and induces gene transcription. We recently demonstrated that the immunosuppressive effect of IL-10 on DC maturation and APC function is mediated by inhibition of IKK activity and downstream NF-κB activation (Bhattacharyya et al., 2004) further arguing that the NF-κB pathway is a key target for immunoregulation of DC. In addition, IL-10-induced inhibition of DC was dependent on suppression of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway. Studies have shown that NF-κB activation can be regulated by the PI3K/AKT pathway via different mechanisms (Madrid et al., 2000; Guha and Mackman, 2002; Martin et al., 2003; Gustin et al., 2004).

The current study was initiated to define the molecular basis of AC-induced inhibition of DC activation and effector function. Evidence is provided that AC inhibit activation of the NF-κB signaling pathway in DC, and that MerTK via PI3K/AKT signaling serves a major role in mediating this immunoregulatory effect.

Materials and methods

Mice

NOD/LtJ. BALB/c and C57BL/6 (B6) mice were maintained and bred under specific-pathogen free conditions. Establishment of MerTK^{KD} mice has been described (Camenisch et al., 1999). Briefly, the tyrosine kinase domain of *Mertk* was replaced with a neomycin resistance gene, and B6.MerTK^{KD} mice established. NOD MerTK^{KD} mice were generated by breeding B6.MerTK^{KD} and NOD mice, and then backcrossing the *Mertk^{KD}* gene onto the NOD genome for an additional 11 generations. At N11, Mouse MapPairs™ distinguishing B6, 129/Ola, and NOD/LtJ [Chr. 2 (D2Mit378, D2Mit94, D2Mit14, D2Mit393, D2Mit395, D2Mit190, D2Mit164, D2Mit256, D2Mit304, D2Mit224, D2Mit338, D2Mit307, D2Mit260, D2Mit309, D2Mit493, D2Mit451, D2Mit496, D2Mit287, D2Mit456, D2Mit265) (Invitrogen, Carlsbad, CA) were used in PCR according to the supplier's directions to define a 17 cM segment derived from 129/Ola and containing *Mertk^{KD}*. Use of mice was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Preparation of DC

Bone marrow-derived DC (BMDC) and splenic DC (sDC) were prepared from male or female mice between 8-12 weeks as described (Bhatacharyya et al., 2004).

Flow cytometry

The following monoclonal Abs used for fluorescence staining were purchased from BD PharMingen (San Diego, CA): FITC-αCD40, FITC-αCD86, FITC-αCD80, PE-αCD11c, PE-

αH2K^d and PE-αCD11b. PE-αmouse IgG, FITC-αmouse IgG and streptavidin-PE were also purchased from BD PharMingen. Polyclonal goat-αMerTK, and normal goat IgG were purchased from R&D Systems and biotin-αgoat IgG was purchased from Vector. Stained cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA) using Summit Software (Cytomation, Ft. Collins, CO).

DC pretreatment with AC, necrotic cells, or PI3K inhibitors.

Thymocytes prepared from 4-6 week old mice, were adhered to plastic for 2 hr to remove APC, irradiated at 600R and then cultured in base medium for 12 hr. Flow cytometry demonstrated >95% apoptotic and <1% necrotic thymocytes based on Annexin-V and propidium iodide staining. Apoptosis was confirmed via DNA fragmentation analysis. DC were co-cultured with AC at a ratio of 1:5 (DC:AC) for indicated times. For necrotic cell preparations, thymocytes were frozen at -80^{o}C and thawed for 4 cycles, and then co-cultured with DC at a 5:1 ratio (necrotic cell:DC equivalence) for 3 hr. Following pretreatment with AC or necrotic cells, DC were resuspended accordingly, and stimulated with LPS.

In some experiments, DC were treated with αMerTK Ab prior to AC incubation. Briefly, DC (5x10^6/well) were incubated with αmouse FcγIII/II (BD PharMingen) in 6-well ultra-low cluster plates for 0.5 hr at 37^{o}C to block Fc receptor binding. DC were then treated for 1 hr at 37^{o}C with 20 μg/ml of either goat αMerTK Ab (AF591, R&D Systems, Inc., Minneapolis, MN) or goat IgG (R&D Systems), an isotype control.

Alternatively, DC (5x10^6 cells/well) were treated with wortmannin (200 nM) or Ly294002 (50 μM) (Cell Signaling Technology^{TM}, Beverly, MA) for 1 hr prior to AC treatment or LPS stimulation as described (Bhatacharyya et al., 2004).

EMSA and Western blotting

Nuclear and cytoplasmic extracts were prepared from DC as described (Beg et al., 1993). EMSA was performed using ^{32}P-labeled DNA probes containing NF-κB binding sites derived from MHC class I H2K promoter: 5'-CAGGCTGGGGATTCCCATCTCCACAGTTTCACTTC-3' (Weaver et al., 2001). A double stranded OCT-1 DNA probe: 5'-TGTCGAATGCAAATCACTAGAA-3' was used as control. Bands were visualized using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). For each treatment at least three EMSAs were run.

Western blotting was carried out as described (Bhatacharyya et al., 2004). Membranes were probed with Abs specific for: IκBα, IκBβ, IκBε, IKK1, ERK1, and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA); IKK2, pIκBα, pIKK1 (Ser180)/pIKK2 (Ser181), pAKT (Thr308), AKT, pSAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-p38MAPK (Thr180/Tyr182), p38MAPK, pERK1/pERK2 (Thr202/Tyr204), and phospho-tyrosine (Cell Signaling Technology™); MerTK (R&D Systems); β-actin (Sigma, St. Louis, MO). Binding of secondary HRP-labeled goat-αrabbit, donkey-αgoat or goat-αmouse Abs (Santa Cruz Biotechnology) was analyzed using SuperSignal® West Pico or West Dura Chemiluminescent Substrate (Pierce, Rockland, IL).

IKK Assay

IKK signalosome was immunoprecipitated from 700 μg of a whole DC lysate using Protein G agarose beads (Upstate, Lake Placid, NY) and rabbit polyclonal αIKK1 as per the manufacturer's instruction. *In vitro* kinase reaction was performed by incubating the IKK signalosome-bead complex with IκBα-GST and 20 μl of a magnesium/ATP cocktail

(Upstate) for 60 min at 30°C in kinase buffer. Supernatants were collected and kinase activity determined by measuring phosphorylation of the IκBα-GST substrate via immunoblot probed with αpIκBα Ab.

AKT kinase assay

DC ($5x10^6$) were stimulated as described above and whole cell lysates prepared. AKT kinase activity was determined using an AKT kinase assay kit (Cell Signaling Technology). Briefly, AKT was immunoprecipitated according to the manufacturer's instructions, and kinase activity assessed by measuring phosphorylation of a GSK3 substrate via immunoblot using αpGSK3 Ab.

Immunoprecipitation of MerTK

Whole cell lysates prepared from DC ($5x10^6$) were pre-cleared with protein G sepharose beads, and MerTK immunopreciptated using Protein G sepharose beads precoated with αMerTK Ab. For "pull-down" experiments, DC (10^7) were treated with AC (1:5) for varying times, chilled on ice, resuspended in 1 ml of the cell-permeable protein cross-linker dimethyl 3,3'-dithiopropionimidate dihydrochloride (Sigma) in PBS (2 mg/ml), and incubated at room tempertature for 20 min. A whole cell lysate was prepared and MerTK immunoprecipitated as above. In some experiments, Western blots were probed with Ab specific for the PI3K subunits p85α, p110α (Cell Signaling Technology™), p110β, p110δ (Santa Cruz Biotechnology) or Axl (R&D Systems) and Tyro3 (BD PharMingen) specific Ab.

Measurement of TNFα production from DC

DC ($9x10^5$/well) were pretreated with or without AC for 3 hr, washed and then stimulated with LPS for 48 hr. Supernatants were collected and assayed for TNFα in triplicate using an ELISA kit (BD PharMingen) following the manufacturer's instructions.

Results

NF-κB activation is inhibited in DC by AC pretreatment.

Binding of AC by immature DC impairs subsequent activation, maturation, and APC function (Sauter et al., 2000; Stuart et al., 2002). Since these events are largely regulated by NF-κB, the effect of AC on the NF-κB signaling pathway was investigated. For this purpose, NOD BMDC which are $CD11b^+CD8\alpha^-$ and exhibit an immature phenotype ($CD40^{lo}$, $CD80^{lo}$, $CD86^{lo}$) (Weaver et al., 2001) were examined. BMDC were pretreated at a ratio of 1:5 with AC for varying times, stimulated with LPS (50 ng/ml) for 0.5 hr, and nuclear NF-κB DNA binding activity assessed via EMSA. BMDC treated with LPS-only exhibited a 5.7-fold increase in NF-κB DNA binding relative to untreated BMDC (Figure 1A). In contrast, AC pretreatment resulted in a temporal loss of LPS-stimulated NF-κB DNA binding, which was completely inhibited after a 3 hr incubation with AC (Figure 1A). No effect on DNA binding of OCT-1 was detected, indicating that the inhibitory effect of AC was NF-κB-specific (Figure 1A). Titrating AC showed that 1:5 BMDC to AC was the lowest ratio at which maximum suppression of NF-κB activity induced by LPS (50 and 500 ng/ml) was observed (data not shown). This ratio was used for all subsequent experiments. Analogous to BMDC,

a reduction (~5-fold) in LPS-stimulated nuclear NF-κB activity was detected in NOD DC prepared from the spleen (sDC), and pretreated with AC (Figure 1B). Inhibition of NF-κB activity was specific for AC. For example, LPS-stimulated NF-κB DNA binding was unaffected by pretreatment of BMDC with either necrotic cells or polystyrene latex beads (Figure 1C). Furthermore, *de novo* protein synthesis was required for AC-induced inhibition. Despite AC pretreatment, LPS-stimulated NF-κB activation was readily detected in BMDC incubated with 10 μg/ml cyclohexamide (Figure 1D).

In agreement with the above EMSA data, LPS-stimulated degradation of IκBα, IκBβ, and IκBε in BMDC was progressively inhibited with increasing times of AC pretreatment (Figure 2A). Furthermore, inhibition of LPS-induced IκB degradation by AC pretreatment coincided with a reduction in IκBα phosphorylation (Figure 2B). IκBα degradation stimulated by LPS was also inhibited in sDC pretreated with AC for 3 hr (Figure 2C).

The lack of LPS-induced IκB degradation suggested that upstream IKK activity was inhibited by AC pretreatment. To test this possibility, BMDC were pretreated with AC for 0.5 or 3 hr and stimulated with LPS for 15 min. Temporal analyses demonstrated that maximum IKK activity in BMDC was detected after a 15 min incubation with LPS (data not shown). Cytoplasmic extracts were prepared, the IKK signalosome immunoprecipitated and kinase activity of the complex determined by measuring phosphorylation of an IκBα-GST substrate *in vitro*. Phosphorylation of the IκBα-GST substrate was readily detected in LPS-stimulated versus untreated BMDC (Figure 2D). In comparison, when LPS-stimulated BMDC where pretreated with AC for 0.5 and 3 hr, phosphorylation of the IκBα-GST substrate was reduced 6.8- and 54-fold, respectively (Figure 2D). No significant difference was detected in the amount of IKK1 and IKK2 protein in the respective BMDC lysates

(Figure 2D), indicating that the lack of *in vitro* phosphorylation of the IκBα-GST substrate was due to reduced IKK activity and not inefficient immunoprecipitation of the IKK complex.

AC pretreatment of DC had no significant effect on LPS-stimulated activation of the mitogen-activated protein kinase (MAPK) pathway. LPS-stimulated phosphorylation of JNK, ERK1/2 and p38 MAPK was unaffected by AC pretreatment (Figure 2E-G). These data demonstrate that AC pretreatment of either BMDC or sDC results in inhibition of IKK signalosome activation, downstream phosphorylation and degradation of the IκB proteins, and NF-κB DNA binding activity induced by LPS stimulation. Furthermore, AC pretreatment selectively inhibits LPS-induced NF-κB signaling.

MerTK is required to mediate AC-induced inhibition of NF-κB activation in DC.

MerTK is necessary for efficient phagocytosis of AC by Mϕ, and is associated with down-regulation of Mϕ activation (Lu and Lemke, 2001; Camenisch et al., 1999; Scott et al., 2001; Hu et al., 2004; Todt et al., 2004). Whether MerTK mediates AC-induced inhibition of NF-κB activity in DC was therefore investigated. DC which lack MerTK expression were prepared from NOD mice homozygous for the *Mertk*^{KD} null mutation (NOD.MerTK^{KD}), and the effect of AC pretreatment on LPS-stimulated NF-κB activation determined. Whereas NF-κB DNA binding was inhibited in NOD BMDC, pretreatment with AC had no significant effect on the induction of NF-κB activity in NOD.MerTK^{KD} BMDC stimulated with LPS (Figure 3A). LPS-induced NF-κB DNA binding was also unaffected by AC pretreatment in NOD.MerTK^{KD} sDC (Figure 3D).

Analyses of IκB protein degradation and IKK activity confirmed that AC failed to inhibit NF-κB activity in NOD.MerTK^{KD} DC. Degradation of the IκB proteins was observed in NOD.MerTK^{KD} but not NOD BMDC or sDC pretreated with AC and stimulated with LPS (Figures 3B,E). Furthermore, pretreatment with AC failed to inhibit *in vitro* IKK activity in lysates prepared from NOD.MerTK^{KD} but not NOD BMDC (Figure 3C). Similarly, LPS-stimulated IKK activation as measured by phosphorylation of IKK1 and IKK2 was unaffected by AC pretreatment in NOD.MerTK^{KD} sDC, but was significantly reduced in NOD sDC (Figure 3F). Moreover, AC failed to inhibit NF-κB DNA binding and phosphorylation of IKK in LPS-stimulated BMDC prepared from B6 mice lacking MerTK (B6.MerTK^{KD}) (Figure 8), demonstrating that the inability of AC to block NF-κB activation in NOD.MerTK^{KD} DC was not intrinsic to the NOD genotype.

To confirm the role of MerTK in AC-induced inhibition of NF-κB activity, the effect of treating wild-type DC with a blocking αMerTK Ab was investigated. BMDC were treated with either αMerTK or isotype control Abs, incubated with AC for 3 hr, and then stimulated with LPS. LPS-induced NF-κB DNA binding was efficiently inhibited in BMDC treated with the isotype control Ab and AC (Figure 4A). In contrast, LPS-induced NF-κB DNA binding was readily detected in BMDC treated with the αMerTK Ab despite AC pretreatment (Figure 4A). Furthermore, AC failed to block LPS-induced degradation of the IκB proteins in DC treated with αMerTK but not isotype control Abs (Figure 4B). The effect of MerTK Ab blocking on AC-induced inhibition of NF-κB signaling was also detected in BMDC cultured from BALB/c. Whereas AC inhibited LPS-induced NF-κB DNA binding and IκB protein degradation in BALB/c BMDC treated with the isotype control Ab, αMerTK Ab blocked this effect (Figure 4C,D). Collectively, these results demonstrate that MerTK signaling is

necessary to mediate AC-induced inhibition of the NF-κB pathway, independent of the genotype of the DC.

AC-induced inhibition of NF-κB activation is mediated through the PI3K/AKT pathway in DC.

Signaling via a MerTK chimeric molecule or MerTK activates the PI3K/AKT pathway in 32D transfectant cells and retinal pigment epithelial cells, respectively (Guttridge et al., 2002; Hall et al., 2003). In addition, the PI3K/AKT pathway negatively regulates NF-κB activation in human monocytes (Guha and Mackman, 2002). Accordingly, a role for the PI3K/AKT pathway in mediating AC-induced inhibition of NF-κB activation was assessed. Initially, AKT kinase activity was measured in lysates prepared from NOD BMDC treated with AC. AC treatment induced a 3-fold increase in AKT kinase activity relative to untreated NOD BMDC (Figure 5A). Furthermore, pretreatment of DC with the PI3K inhibitors wortmannin (Wort) or LY294002 (LY) for 1 hr effectively blocked AC-induced AKT kinase activity (Figure 5A). Importantly, AC treatment failed to induce AKT kinase activity in NOD.MerTK^{KD} BMDC (Figure 5A). In addition, phosphorylation of AKT induced by AC (Figure 5B) was blocked by αMerTK but not isotype Ab treatment.

Next, the effect of Wort or LY pretreatment on AC-induced inhibition of NF-κB activation was determined. Whereas AC blocked LPS-stimulated nuclear NF-κB DNA binding, NF-κB activation was readily detected in NOD BMDC pretreated with Wort or LY and then incubated with AC (Figure 5C). In addition, AC failed to inhibit LPS-stimulated IκB protein degradation (Figure 5E) and IKK activity (Figure 5G) in NOD BMDC treated with Wort or LY. The two PI3K inhibitors similarly prevented AC-induced inhibition of nuclear NF-κB

DNA binding activity and degradation of the IκBs in NOD sDC (Figure 5D,F) or BALB/c BMDC (Figure 9) stimulated with LPS.

The intra-cytoplasmic domain of MerTK contains a PI3K binding motif (YDIM). To determine whether PI3K is directly associated with MerTK, NOD BMDC were treated with AC and proteins chemically cross-linked. MerTK was then immunoprecipitated and the resulting complexes analyzed via Western blot. MerTK but not p85α/PI3K was detected in untreated NOD BMDC (Figure 6A). In contrast, a temporal increase in p85α/PI3K was seen in AC-treated NOD BMDC, with no significant change in the level of MerTK (Figure 6A). Furthermore, the PI3K catalytic subunit p110δ, but not p110α or p110β was found complexed with immunoprecipitated MerTK (Figure 6B). These results demonstrate that AC-induced inhibition of NF-κB signaling is dependent on MerTK activation of the PI3K/AKT pathway.

AC-induced inhibition of DC maturation is mediated by MerTK and PI3K/AKT signaling.

The role of MerTK and PI3K/AKT signaling in AC-induced inhibition of DC maturation, as determined by TNFα secretion, was investigated. Initially, NOD and NOD.MerTK^{KD} BMDC were compared. As demonstrated in Figure 7A NOD and NOD.MerTK^{KD} BMDC secreted similar levels of TNFα upon LPS stimulation. However, AC pretreatment significantly inhibited LPS-stimulated TNFα secretion by NOD BMDC ($p<10^{-3}$), whereas NOD.MerTK^{KD} BMDC continued to produce TNFα (Figure 7A). Secretion of TGFβ and IL-10 was not detected in either NOD or NOD.MerTK^{KD} BMDC following pretreatment with AC (data not shown). Furthermore, LPS-stimulated TNFα secretion was significantly increased in cultures of NOD BMDC pretreated with Wort or LY plus AC relative to cultures

treated with AC alone ($P<10^{-3}$) (Figure 7B). These data demonstrate that AC inhibition of DC maturation is mediated by MerTK and PI3K/AKT signaling.

Discussion

DC are key contributors to the establishment and maintenance of peripheral tolerance to self. Furthermore, AC are believed to have an important role in establishing "tolerogenic" DC *in vivo* (Scheinecker et al., 2002; Liu et al., 2002). The latter is supported by reports demonstrating the potent inhibitory properties of AC on DC *in vitro* (Sauter et al., 2000; Stuart et al., 2002; Ip and Lau, 2004). However, the molecular basis for AC-induced inhibition of DC is ill-defined. Here we have identified critical signaling pathways targeted in DC upon binding of AC, and the key receptor mediating this inhibitory effect is MerTK.

Pretreatment with AC blocked activation of the NF-κB signaling pathway in both BMDC and sDC. This effect was marked by inhibition of LPS-induced IKK activation as measured by *in vitro* kinase activity or phosphorylation of IKK1 and IKK2 (Figures 2,3). Consistent with inhibition of the IKK complex was the lack of downstream IκB protein phosphorylation and degradation, and NF-κB nuclear translocation and DNA binding following LPS stimulation of DC pretreated with AC (Figures 1-3). In addition, NF-κB activation induced by CD40 crosslinking was inhibited (Figure 10), indicating that AC block multiple pathways that engage NF-κB. Blockade of NF-κB signaling was seen only by AC pretreatment. Incubation with necrotic cells or phagocytosis of latex beads had no significant effect on LPS-induced NF-κB activation (Figure 1C). Furthermore, the inhibitory effect of AC showed selectivity among LPS-induced pathways; in this case the activation of NF-κB. For example,

LPS-stimulated phosphorylation of the MAPK molecules JNK, ERK1/2 and p38 MAPK was unaltered by AC pretreatment (Figure 2E-G). This observation further supports the hypothesis that blockade of NF-κB activation is essential for promoting AC-mediated DC suppression. Indeed, the effects of AC can be mimicked by gene transfer of a modified IκBα recombinant that specifically inhibits NF-κB activation, and alone is sufficient to prevent up-regulation of co-stimulatory molecule expression, proinflammatory cytokine secretion and T cell stimulation by immature DC (Weaver et al., 2001; Ouaaz et al., 2002; Poligone et al., 2002; Hackstein et al., 2001). Interestingly, inhibition of NF-κB signaling by AC appears to be DC-specific. In Mϕ, pro-inflammatory responses are down-regulated by AC pretreatment yet NF-κB activation is still inducible (McDonald et al., 1999; Cvetanovic and Ucker, 2004). This disparity may reflect the relative importance of the NF-κB pathway in regulating activation and the effector functions of DC versus Mϕ.

The second important observation made in this study is that MerTK is required for AC-induced inhibition of DC. This was shown in both genetic and specific Ab blocking experiments. First, AC pretreatment of NOD.MerTK^{KD} BMDC or sDC failed to establish a tolerogenic phenotype seen in MerTK-expressing DC. Despite AC pretreatment, NF-κB signaling was readily induced in NOD.MerTK^{KD} DC, which correlated with continued APC function (Figures 3,7). Second, the inability of AC to inhibit NF-κB activation was also observed when wild-type NOD or BALB/c DC were treated with αMerTK Ab to block MerTK binding of AC (Figure 4). The αMerTK Ab inhibits signaling by preventing AC-induced phosphorylation of MerTK tyrosine residues (Y.H. and R.T., unpublished results). The failure of AC to inhibit NOD.MerTK^{KD} DC therefore was due to the lack of MerTK expression and not a secondary defect. One possibility is that family members Axl and Tyro3

are also involved in AC-induced inhibition. For example, AC may bind a heterodimeric complex consisting of MerTK and Axl and/or Tyro3, and in turn the absence of MerTK expression or blocking MerTK activation with Ab may disrupt such a complex. However, this scenario is unlikely since neither Axl nor Tyro3 were co-immunoprecipitated with MerTK prepared from chemically cross-linked lysates of AC pretreated NOD BMDC (Figure 11).

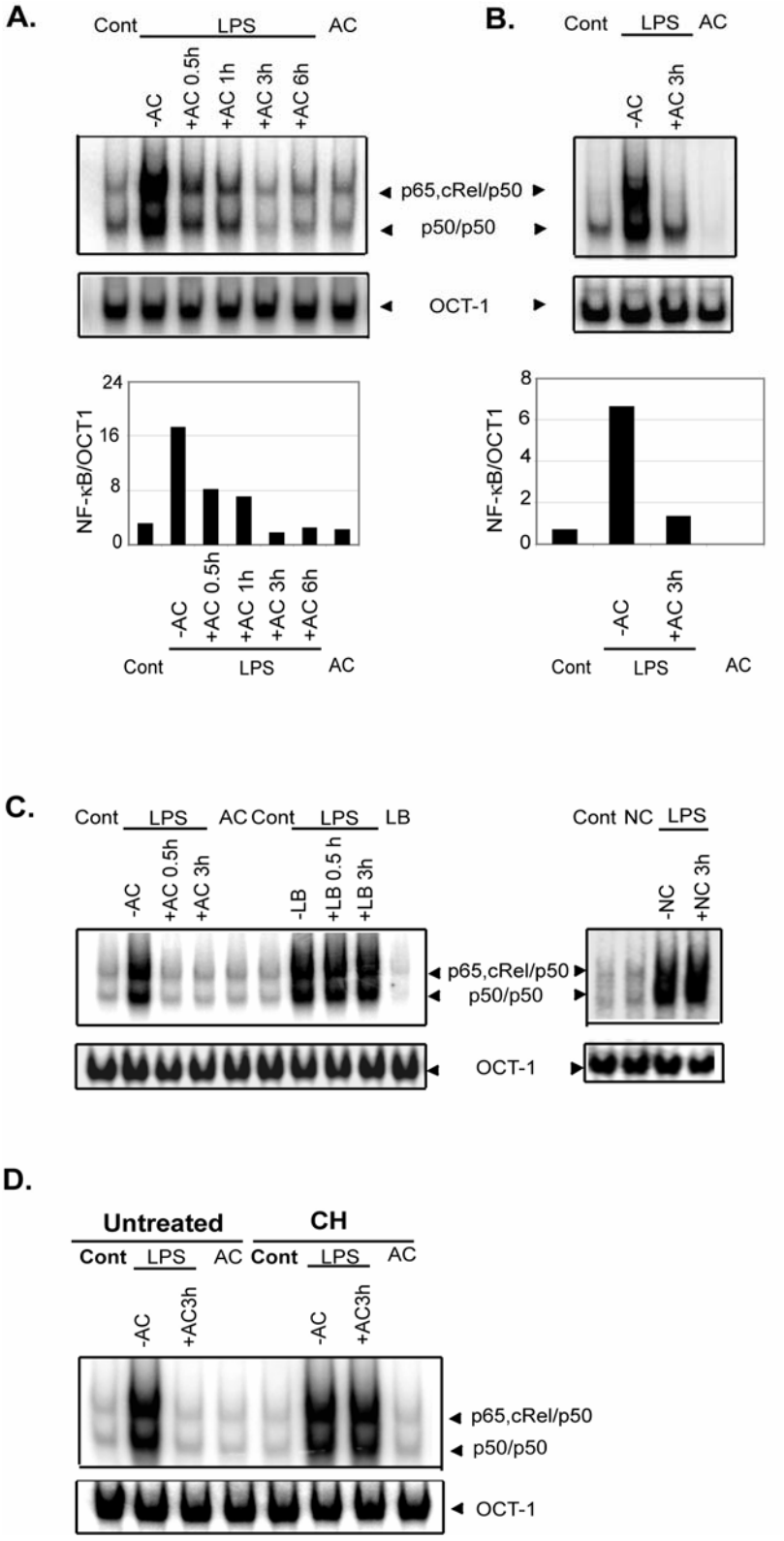
Another key finding is that AC-induced inhibition of DC is dependent on MerTK activation of the PI3K/AKT pathway. AC treatment elicited AKT kinase activity in NOD but not NOD.MerTK^{KD} BMDC (Figure 5A). A role for PI3K/AKT signaling was demonstrated by the use of Wort and LY, which effectively blocked the capacity of AC to inhibit NF-κB signaling and DC maturation (Figures 5,7B). The latter was observed by measuring TNFα secretion, although the capacity of AC to inhibit upregulation of CD40, CD80 and CD86 expression following LPS stimulation was also blocked by the PI3K inhibitors (Figure 12). These results are consistent with a consensus PI3K docking site located in the MerTK intra-cytoplasmic domain, and detection of a p85α/p110δ complex associated with MerTK following AC incubation (Figure 6). How PI3K/AKT signaling blocks downstream activation of the IKK signalsome is currently under investigation. The fact that cyclohexamide pretreatment of BMDC blocked AC-induced inhibition of NF-κB activation (Figure 1D), suggests that PI3K/AKT signaling mediates downstream *de novo* protein synthesis of regulatory molecules. Preliminary findings suggest that LPS-stimulated activation of transforming growth factor β-activated kinase 1 (TAK1) is unaffected by AC pretreatment (Z.Y. and R.T., unpublished results). TAK1 is activated by interacting with tumor necrosis factor receptor-associated factor 6 (Ninomiya et al., 1999), suggesting that

events downstream of this complex are targeted by AC pretreatment. Although other molecules (and pathways) such as phosphatidylinositol-specific phospholipase Cγ2 (Todt et al., 2004) and the guanine exchange factor Vav1 (Mahajan and Earp, 2003) may be involved in MerTK signaling, inhibition of the PI3K/AKT pathway in DC is nevertheless sufficient to block the effects of AC. A chimeric molecule containing the intracellular domain of MerTK has been reported to activate both PI3K/AKT and NF-κB pathways in pro-B cell transfectants (Georgescu et al., 1999). This finding coupled with our own suggests that the nature of MerTK signaling is cell-dependent. It is also noteworthy that induction of the PI3K/AKT pathway in DC can either inhibit (Figure 5) or promote activation of the NF-κB pathway (Bhattacharyya et al., 2004; Yu et al., 2004). Distinct effects of PI3K/AKT signaling may reflect the subunit composition of the PI3K heterodimer and/or the isoform of AKT. Fukao *et al*. reported that the inhibitory effect of PI3K on LPS-stimulated activation of p38MAPK in DC is mediated by a p85α/p110β complex (Fukao et al., 2002). In contrast, AC induction of a p85α/p110δ complex appears to have no effect on LPS-stimulated p38MAPK activation (Figure 2G). Finally, activation of other signaling events engaged by a given receptor may alter the "context" and in turn the downstream effect(s) of the PI3K/AKT pathway.

An essential role for MerTK in efficient phagocytosis of AC by Mϕ has been previously demonstrated (Scott et al., 2001; Cohen et al., 2002). In contrast, Cohen and colleagues demonstrated unaltered AC phagocytosis by B6.MerTK^{KD} BMDC (Behrens et al., 2003) suggesting that the primary function of MerTK is to transduce inhibitory signals upon binding of AC. Importantly, the role of MerTK in regulating DC activation and function is AC-dependent. No significant differences were observed between NOD and NOD.MerTK^{KD} DC in NF-κB signaling or TNFα secretion when AC pretreatment was not included, and DC

were stimulated with LPS-only (Figures 3,7A). A number of DC subsets have been defined, and whether MerTK serves the same function among different DC is of interest. For example, the relative contribution of different receptors for AC may vary depending on the subset of DC, and the nature and concentration of the corresponding ligand. However, the fact that the lack of expression and/or blocking of MerTK efficiently inhibited the effects of AC on NOD, BALB/c and B6 BMDC and/or sDC strongly argues that this RTK plays a major role in regulating DC activation and maturation. Potential defects in MerTK function may in turn contribute to autoimmunity.

Figure 1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NF-κB/OCT1
24
16
8
0
B.
Cont
LPS
AC
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4
2
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C.
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LPS
LB
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+AC 0.5h
+AC 3h
-LB
+LB 0.5 h
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Cont
NC
LPS
-NC
+NC 3h
p65,cRel/p50
p50/p50
OCT-1
D.
Untreated
CH
Cont
LPS
AC
-AC
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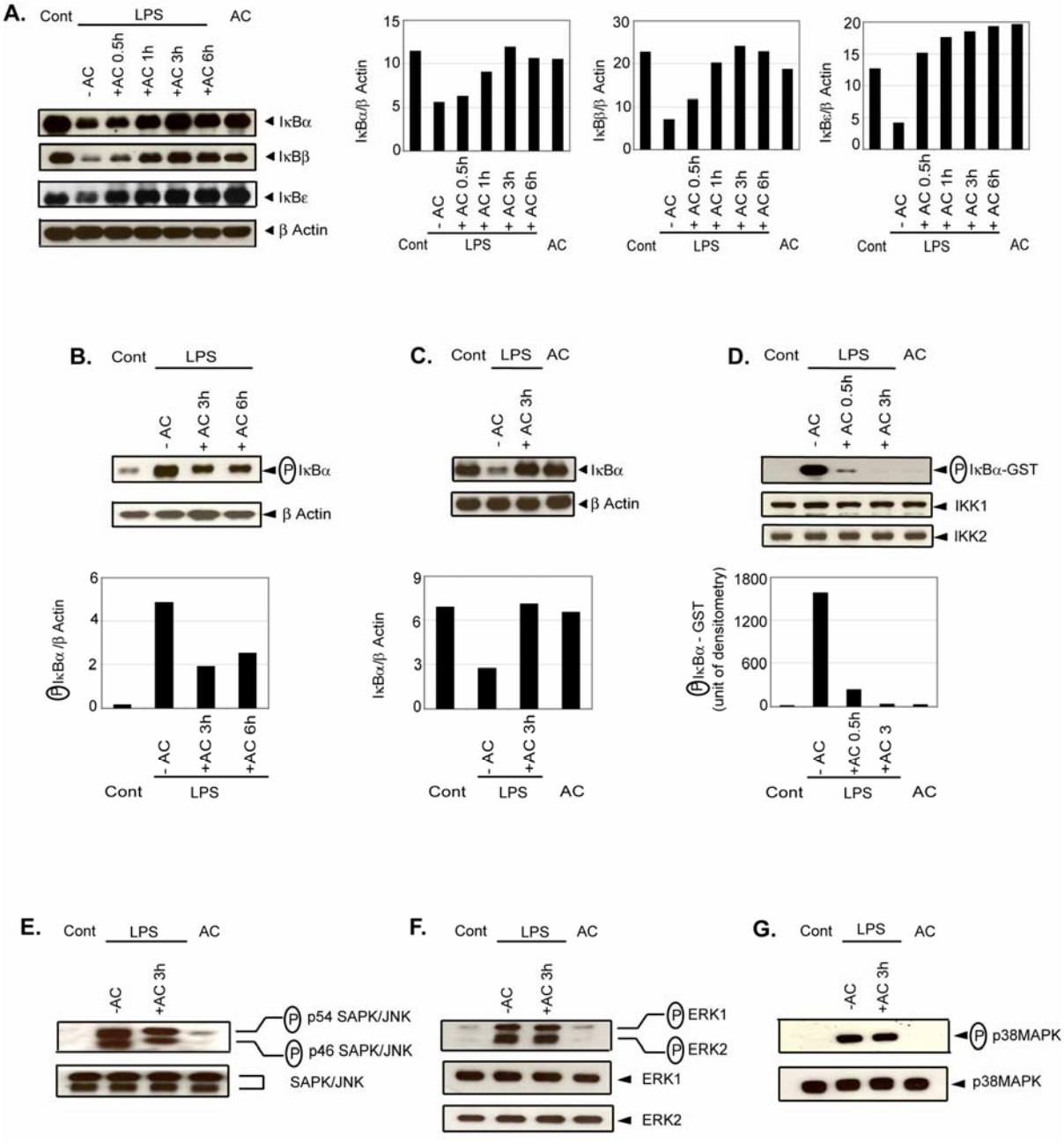
p65,cRel/p50
p50/p50
OCT-1

Pretreatment of DC with AC inhibits DNA binding activity of NF-κB. NOD BMDC (A) or sDC (B) were pretreated with AC at a ratio of 1:5 (DC to AC) for the indicated times or left untreated, and then stimulated with LPS (50 ng/ml) for 0.5 hr. Nuclear NF-κB DNA binding was measured via EMSA. Composition of NF-κB complexes was previously determined via super-shift analysis. A double stranded OCT-1 DNA probe was used as an internal control. Densitometric analyses represent the ratio of intensity of NF-κB to OCT-1 binding per unit area and are represented as arbitrary units for the respective EMSAs. (C) Nuclear NF-κB binding activity was measured in NOD BMDC pretreated with AC, polystyrene latex beads (LB), or necrotic cells (NC) for specified times or left untreated, and then stimulated with LPS for 0.5 hr. (D) NOD BMDC were preincubated with cyclohexamide (10 μg/ml) (CH) for 15 min, treated with AC for 3 hr, stimulated with LPS (50 ng/ml) for 0.5 hr and nuclear NF-κB DNA binding activity determined. Data are representative of three independent experiments.

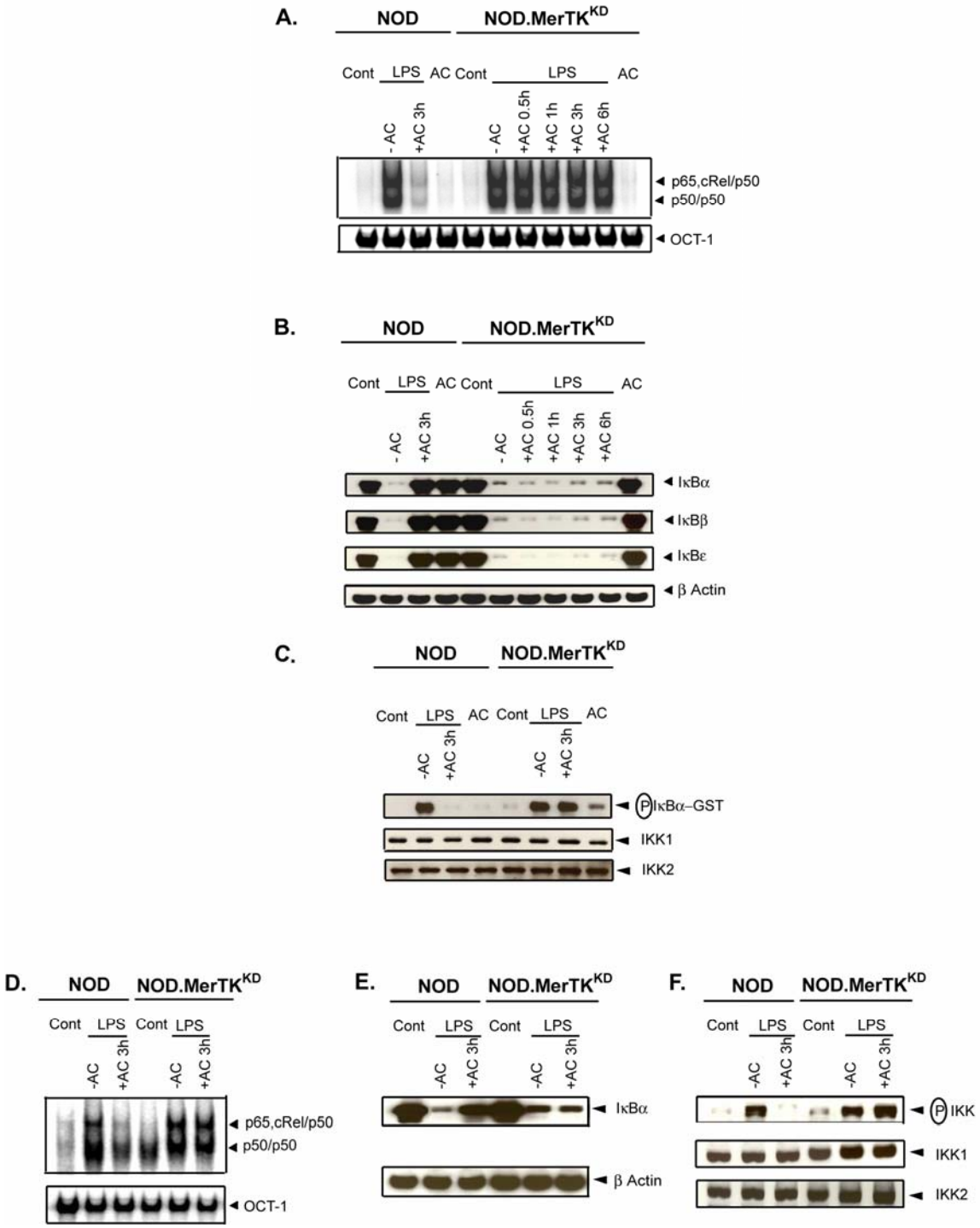
Figure 2



AC pretreatment inhibits IκB protein degradation and IKK activity. NOD BMDC (A, B, D, E-G) or sDC (C) were pretreated with AC and stimulated with LPS as in Figure Legend 1. (A, C) Cytoplasmic IκBα, IκBβ, IκBε, and β-actin protein was detected by Western using the same blot. Densitometric readings represent the ratio of intensity of IκB protein to β-actin expression per unit area, and are represented as an arbitrary unit. (B) Expression of phospho (P)-IκBα in cytoplasmic extract was determined by Western blot. Densitometric analysis represents the ratio of intensity of phospho-IκBα to β-actin expression per unit area. (D) *In vitro* IKK activity was determined by measuring phosphorylation of an IκBα–GST

substrate. Densitometric analysis represents the intensity of phosphorylated (P) IκBα-GST in arbitrary units. The amount of IKK1 and IKK2 immunoprecipitated in the samples was analyzed by Western blot. Western blot was used to detect (E) phospho-JNK versus JNK, (F) phospho-ERK versus ERK and (G) phospho-p38MAPK versus p38MAPK in whole cell lysates.Data are representative of three independent experiments.

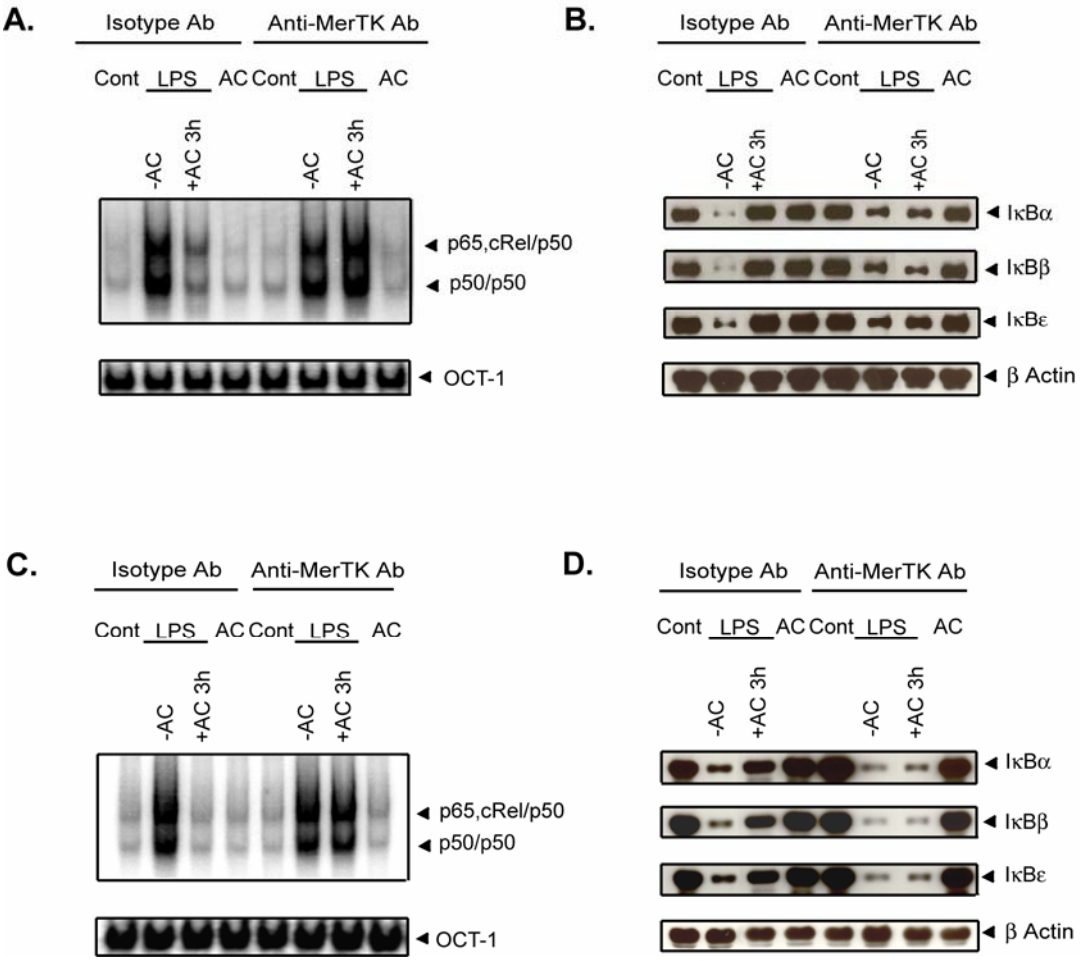
Figure 3



Pretreatment with AC inhibits NF-κB and IKK activation in NOD but not NOD.MerTK^{KD} DC. NOD and NOD.MerTK^{KD} BMDC (A-C) or sDC (D-F) were pretreated with AC and stimulated with LPS as in Figure Legend 1. (A, D) DNA binding activity of nuclear NF-κB or OCT-1 was determined via EMSA. (B, E) Cytoplasmic

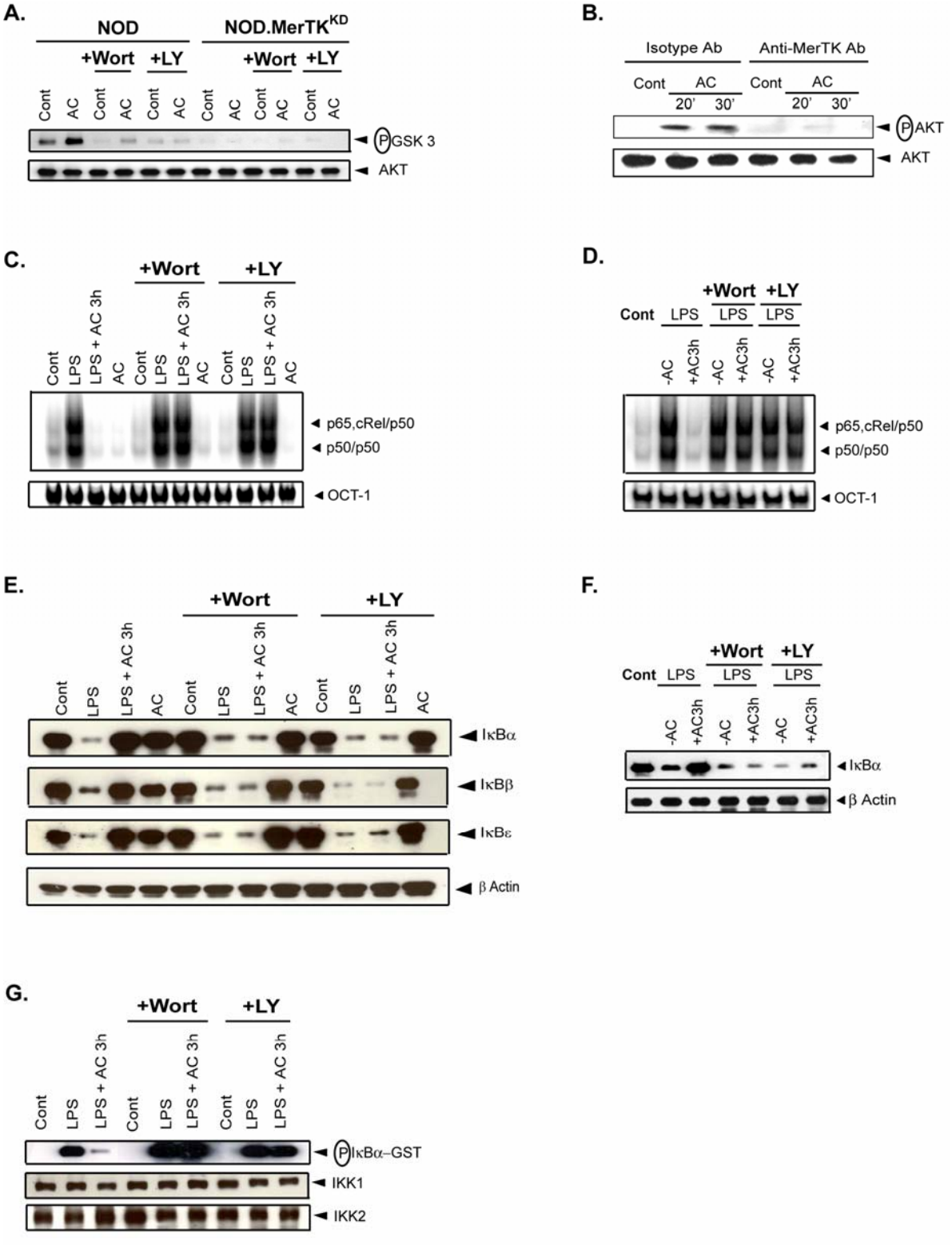
IκBα, IκBβ, IκBε and β-actin protein was detected via Western using the same blot. (C) *In vitro* IKK activity and IKK1 and IKK2 protein expression were determined as in Figure Legend 2. (F) IKK phosphorylation was detected via Western, and the same blot reprobed to for IKK1 and IKK2 protein. Data are representative of three independent experiments.

Figure 4



αMerTK Ab blocks AC-mediated inhibition of NF-κB activation in NOD and BALB/c DC. NOD (A, B) or BALB/c (C, D) BMDC were pretreated with 20 μg/ml of αMerTK or isotype control Ab. BMDC were then treated with AC and stimulated with LPS as in Figure Legend 1. (A, C) EMSA was used to measure DNA binding activity of nuclear NF-κB or OCT-1. (B, D) Cytoplasmic IκBα, IκBβ, IκBε and β-actin was detected via Western with the same blot. Data are representative of three independent experiments.

Figure 5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AC
PGSK 3
AKT
B.
Isotype Ab
Anti-MerTK Ab
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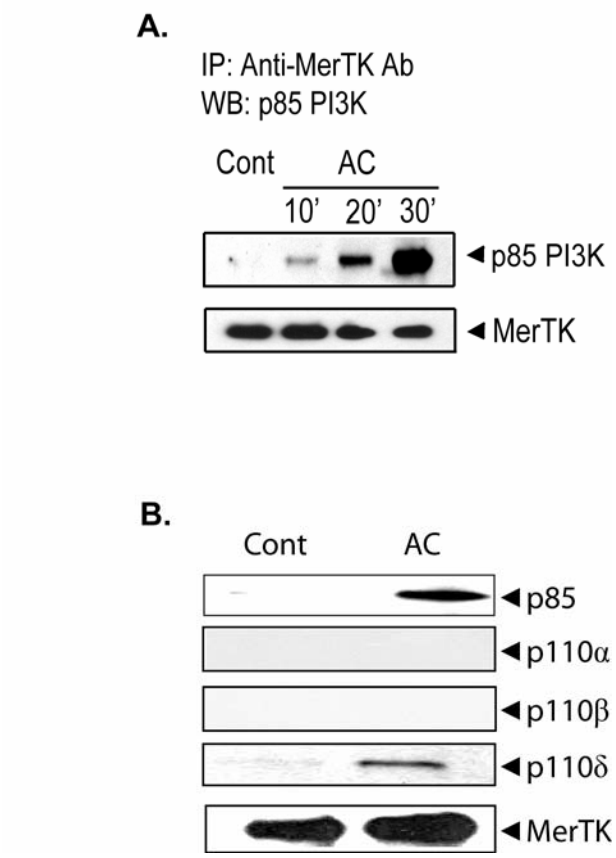
PAKT
AKT
C.
LPS + AC 3h
p65,cRel/p50
p50/p50
OCT-1
D.
-AC
+AC3h
E.
IκBα
IκBβ
IκBε
β Actin
F.
G.
PIκBα–GST
IKK1
IKK2

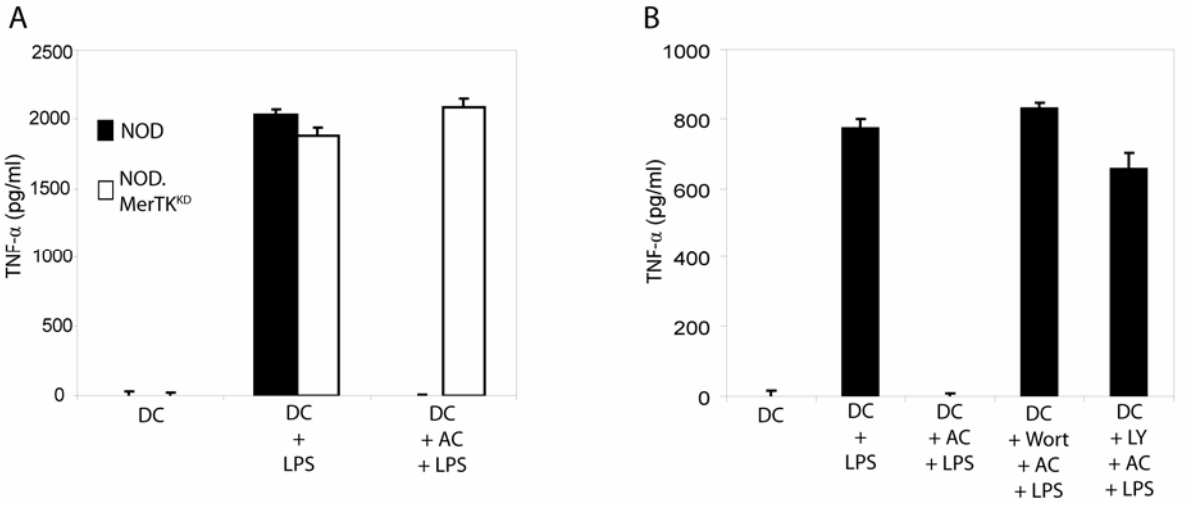
The PI3K/AKT pathway mediates AC-induced inhibition of NF-κB activation in NOD DC. NOD or NOD.MerTK^{KD} BMDC (A-C,E,G) and sDC (D,F) were incubated with 200 nM Wort or 50 μM LY for 1 hr, and then treated with AC for 3 hr or left untreated. In some experiments (C-G), DC were subsequently stimulated with LPS (50 ng/ml) for 0.5 hr. (A) *In vitro* AKT activity was determined by measuring phosphorylation of a GSK3 substrate by Western blot. The same blot was reprobed for AKT protein. (B) NOD BMDC were pretreated either with isotype control or αMerTK Abs for 1 hr and then incubated with AC at specified times. Phosphorylation of AKT in cytoplasmic extracts was determined via Western blot using an αphospho AKT Ab. The same blot was reprobed for AKT protein. (C, D) Nuclear NF-κB or OCT-1 DNA binding activity was determined via EMSA. (E, F) Cytoplasmic IκBs and β-actin protein were detected by Western using the same blot. (G) *In vitro* IKK activity was measured as in Figure Legend 2. The same blot was reprobed for IKK1 and IKK2 protein. Data are representative of three independent experiments.

Figure 6



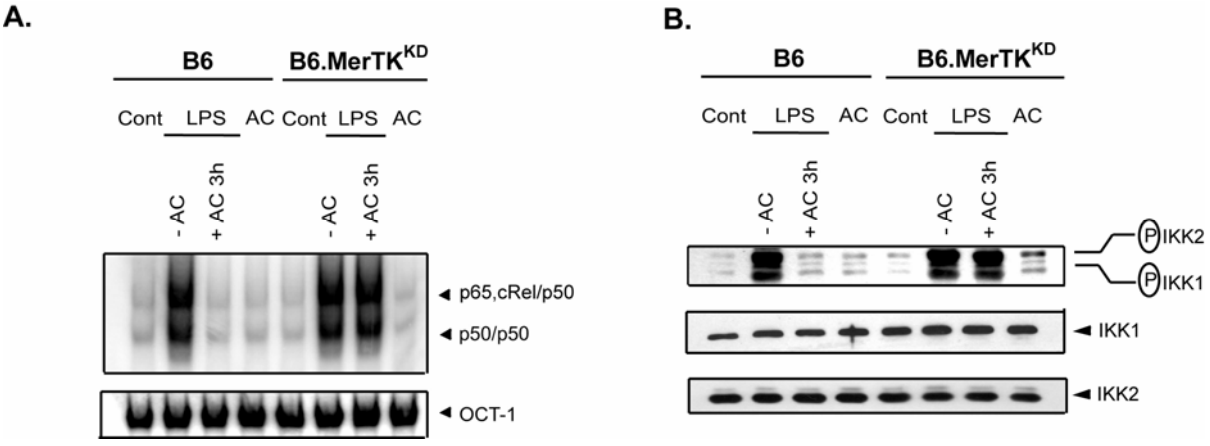
A PI3K p85α/p100δ complex is associated with MerTK upon AC pretreatment of NOD BMDC. MerTK was immunoprecipitated with αMerTK Ab from total cell lysates prepared from NOD BMDC pretreated with AC at specified times (A) or for 30 min (B) or not (Cont). Western blots were probed for p85α/PI3K, MerTK (A, B), and p110α, p110β, and p110δ (B) using the same membranes.

Figure 7



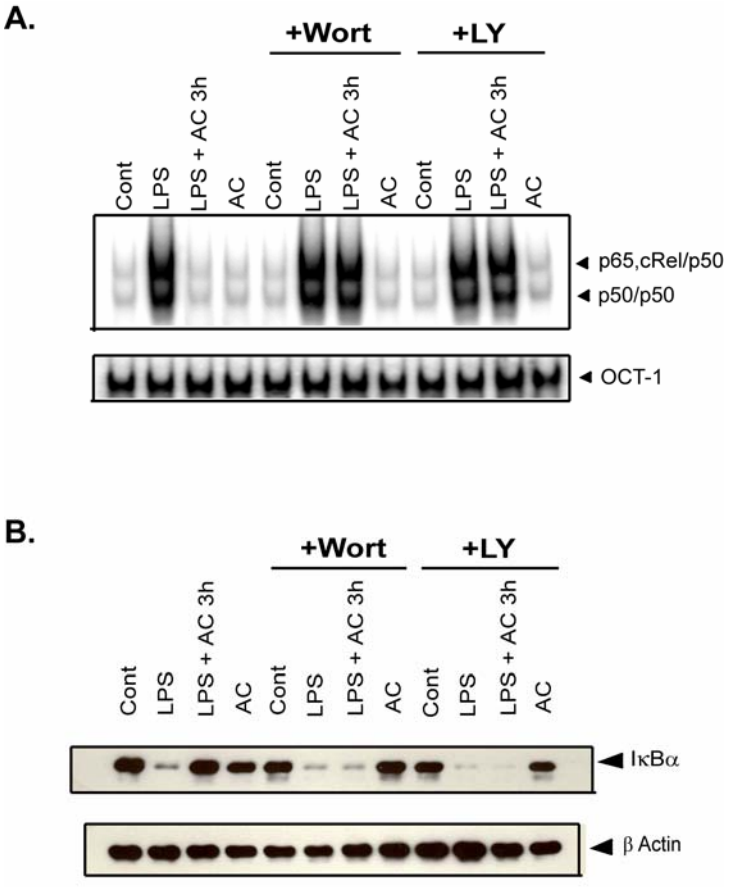
AC-induced inhibition of TNFα secretion by NOD DC is mediated by MerTK-dependent PI3K signaling. (A) NOD and NOD.MerTK^{KD} BMDC were pretreated with AC for 3 hr, stimulated with LPS as in Figure Legend 1, and TNFα secretion measured via ELISA. (B) NOD BMDC were incubated with 200 nM Wort or 50 μM LY for 1 hr, treated with AC and LPS, and TNFα secretion measured as above.

Figure 8



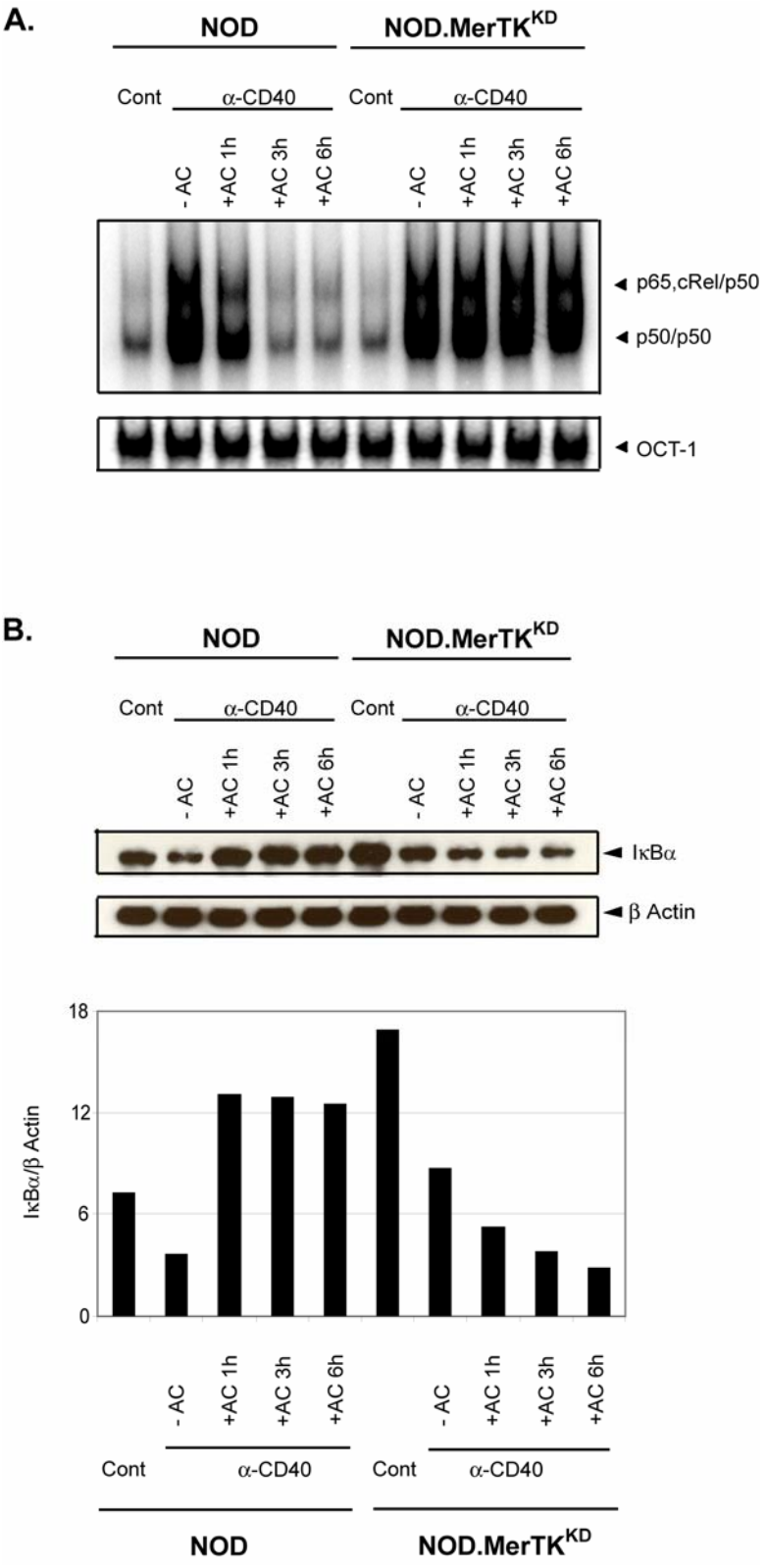
AC pretreatment inhibits NF-κB and IKK activation in B6 but not B6.MerTK^{KD} BMDC. B6 or B6.MerTK^{KD} BMDC were pretreated with AC for 3hr and then stimulated with LPS as in Figure Legend 1. (A) DNA binding activity of nuclear NF-κB or OCT-1 was determined via EMSA. (B) Phospho-IKK1 and -IKK2 were detected by Western and the same blot reprobed for IKK1 and IKK2 protein. Data are representative of three independent experiments.

Figure 9



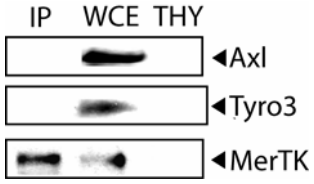
Pretreatment with PI3K inhibitors blocks AC-induced inhibition of NF-κB activation in BALB/c BMDC. BALB/c BMDC were preincubated with Wort (200 nM) or LY (50 μM) for 1 hr, treated with AC for 3 hr or left untreated, and then stimulated with LPS (50 ng/ml) for 0.5 hr. (A) Nuclear NF-κB or OCT-1 DNA binding activity was determined via EMSA. (B) Cytoplasmic IκBα protein was assessed via Western and the same blot reprobed for β-actin protein.

Figure 10


A.
NOD
NOD.MerTK^KD
Cont
α-CD40
- AC
+AC 1h
+AC 3h
+AC 6h
p65,cRel/p50
p50/p50
OCT-1
B.
IκBα
β Actin
IκBα/β Actin
18
12
6
0

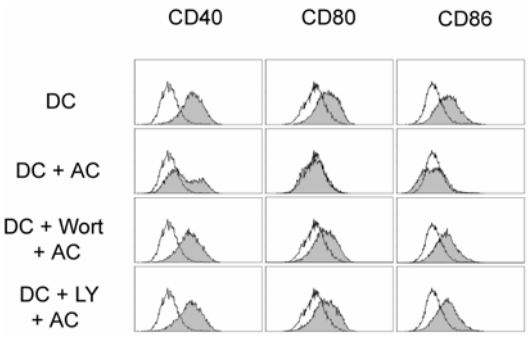
Pretreatment with AC inhibits αCD40 Ab induced NF-κB activation and IκBα degradation in NOD but not NOD.MerTK^{KD} DC. NOD and NOD.MerTK^{KD} BMDC were pretreated with AC for indicated times or left untreated, and then stimulated with 10 μg/ml of hamster-αCD40 (clone HM40-3) and 20 μg/ml αhamster (clone G188-2) Abs for 1 hr. (A) DNA binding activity of nuclear NF-κB or OCT1 was determined via EMSA. (B) Expression of cytoplasmic IκBα was analyzed by Western and the same blot reprobed for β-actin. Corresponding densitometric analysis represents the ratio of intensity of IκBα expression to β-actin expression per unit area.

Figure 11



Axl and Tyro3 are not associated with immunoprecipitated MerTK. NOD BMDC were pretreated with AC and MerTK immunoprecipitated (IP) with αMerTK Ab. Western blot was used to detect MerTK, Axl, and Tyro3 by reprobing the same membrane. Alternatively, whole cell extracts prepared from AC-pretreated NOD BMDC (WCE) or NOD thymocytes (THY; negative control) were similarly analyzed.

Figure 12



AC-induced inhibition of NOD DC activation is mediated by PI3K signaling. NOD BMDC were preincubated with 200 nM Wort or 50 μM LY and treated with AC or left untreated. DC surface phenotype was determined via flow cytometry 24 hr +/- LPS (100 ng/ml) stimulation. Open and shaded histograms represent untreated versus LPS-treated cells NOD BMDC, respectively.

References

Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. 1996;14:649-681.

Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu. Rev. Immunol. 2000; 18:767-811.

Beg AA, Finco TS, Nantermet PV, Baldwin AS Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. 1993;13:3301-3310.

Behrens EM, Gadue P, Gong SY, Garrett S, Stein PL, Cohen PL. The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. Eur. J. Immunol. 2003;33:2160-2167.

Belz GT, Behrens GMN, Smith CM, et al. The $CD8\alpha^+$ dendritic cell is responsible for inducing peripheral self-tolerance to tissue associated antigens. J. Exp. Med. 2002;196:1099-1104.

Bhattacharyya S, Sen P, Wallet M, Long B, Baldwin AS Jr, Tisch R. Immunoregulation of dendritic cells by IL-10 is mediated through suppression of PI3K/Akt pathway and of IκB kinase activity. Blood. 2004;104:1100-1109.

Botto M, Dell'Agnola C, Bygrave AE, et al. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. 1998;19:56-59.

Burkly L, Hession C, Ogata L, et al. Expression of relB is required for the development of thymic medulla and dendritic cells. Nature. 1995;373:531-536.

Camenisch TD, Koller BH, Earp HS, Matsushima GK. A novel receptor tyrosine kinase, Mer, inhibits TNF-α production and lipopolysachcharide-induced endotoxic shock. J. Immunol. 1999;162:3498-3503.

Chen J, Carey K, Godowski PJ. Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. Oncogene. 1997;14:2033-2039.

Chen X, Doffek K, Sugg SL, Shilyansky S. Phosphatidylserine regulates the maturation of human dendritic cells. J. Immunol. 2004;173:2985-2994.

Cohen PL, Caricchio R, Abraham VA, et al. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J. Exp.Med. 2002;196:135-140.

Cvetanovic M, Ucker DS. Innate immune discrimination of apoptotic cells: Repression of proinflammatory macrophage transcription is coupled directly to specific recognition. J. Immunol. 2004;172:880-889.

Delhase M, Hayakawa M, Chen Y, Karin M. Positive and negative regulation of IκB kinase activity through IKKβ subunit phosphorylation. Science. 1999;284:309-313.

Fadok VA, Bratton DL, Henson PT. Phagocyte receptors for apoptotic cells: recognition, uptake and consequences. J. Clin. Invest. 2001;108:957-962.

Fukao T, Tanabe M, Terauchi Y, et al. PI3K-mediated negative feedback regulation of IL-12 production in DCs. Nat. Immunol. 2002;3:875-881.

Georgescu M-M, Kirsch KH, Shishido T, Zong C, Hanafusa H. Biological effects of c-Mer receptor tyrosine kinase in hematopoietic cells depend on Grb2 binding site in the receptor and activation of NF-κB. Mol. Cell. Biol. 1999;19:1171-1181.

Ghosh S, Karin M. Missing pieces in the NF-κB puzzle. Cell. 2002;109:S81-S96.

Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 1998;16:225-260.

Guha M, Mackman N. The phosphatidylinositol 3 kinase-Akt limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J. Biol. Chem. 2002;277:32124-32132.

Gustin JA, Ozes ON, Akca H, et al. Cell type-specific expression of the IκB kinases determines the significance of PI3-kinase/Akt signaling to NF-κB activation. J. Biol. Chem. 2004;279:1615-1620.

Guttridge KL, Luft JC, Dawson TL, et al. Mer receptor tyrosine kinase signaling: Prevention of apoptosis and alteration of cytoskeletal architecture without stimulation or proliferation. J. Biol. Chem. 2002;277:24057-24066.

Hackstein H, Morelli AE, Larregina AT, et al. Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells. J Immunol. 2001;166:7053-7062.

Hall MO, Agnew BJ, Abrams TA, Burgess BL. The phagocytosis of os is mediated by the PI3-kinase linked tyrosine kinase receptor, mer, and is stimulated by GAS6. Adv. Exp. Med. Biol. 2003;533:331-336.

Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. J. Exp. Med. 2001;194:769-779.

Hu B, Jennings JH, Sonstein J, et al. Resident murine alveolar and peritoneal macrophages differ in adhesion of apoptotic thymocytes. Am. J. Respir. Cell Mol. Biol. 2004;30:687-693.

Ip W-K, Lau Y-L. Distinct maturation of, but not migration between, human monocyte-derived dendritic cells upon ingestion of apoptotic cells or early and late phases. J. Immunol. 2004;173:189-196.

Karin M. How NF-κB is activated: the role of the IκB kinase (IKK) complex. Oncogene. 1999;18:6867-6874.

Lemke G, Lu Q. Macrophage regulation by Tyro 3 family receptors. Curr. Opin. Immunol. 2003;15:31-36.

Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the IκB kinase 2 gene. Science 1999;284:321-325.

Liu K, Iyoda T, Saternus M, Kimura Y, Inaba, K, Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells *in situ*. J. Exp. Med. 2002;196:1091-1097.

Lu Q, Lemke G. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science. 2001;293:306-311.

Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. Mol. Cell Biol. 2000;20:1628-1638.

Mahajan NP, Earp HS. An SH2 domain-dependent, phosphotyrosine-independent interaction between Vav1 and the Mer receptor tyrosine kinase: a mechanism for localizing guanine nucleotide-exchange factor action. J. Biol. Chem. 2003;278:42596-42603.

Martin M, Schifferle RE, Cuesta N, Vogel SN, Katz J, Michalek SM. Role of phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by *Porphyromonas gingivalis* lipopolysaccharide. J. Immunol. 2003;171:717-725.

McDonald PP, Fadok VA, Bratton D, Henson PM. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-β in macrophages that have ingested apoptotic cells. J. Immunol. 1999;163:6164-6172.

Ninomiya-Tsuji, J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K. The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in IL-1 signaling pathway. Nature. 1999;398:252-256.

O'Brien BA, Huang Y, Geng X, Dutz JP, Finegood DT. Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. Diabetes. 2002;51:2481-2488.

Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-κB subunits. Immunity. 2002;16:257-270.

Poligone B, Weaver DJ Jr, Sen P, Baldwin AS Jr, Tisch R. Elevated NF-κB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. J. Immunol. 2002;168:188-196.

Pulendran B, Palucka K, Banchereau J. Sensing pathogens and tuning immune responses. Science. 2001;293:253-256.

Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. J. Exp. Med. 1998;188:2175-2180.

Roos A, Xu W, Castellano G, et al. A pivotal role for innate immunity in the clearance of apoptotic cells. Eur. J. Immunol. 2004;34:921-929.

Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: Exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J. Exp. Med. 2000;191:423-433.

Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat. Rev. 2002;2:965-975.

Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J. Exp. Med. 2002;196:1079-1090.

Scott RS, McMahon EJ, Pop SM, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001;411:207-211.

Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu. Rev. Immunol. 2003;21:685-711.

Stuart LM, Lucas M, Simpson C, Lamb J, Savill J, Lacy-Hulbert A. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. J. Immunol. 2002;168:1627-1635.

Tanaka M, Fuentes ME, Yamaguchi K, et al. Embryonic lethality, liver degeneration, and impaired NF-κB activation in IKK-β deficient mice. Immunity. 1999;10:421-429.

Todt JC, Hu B, Curtis JL. The receptor tyrosine kinase MerTK activates phospholipase Cγ2 during recognition of apoptotic thymocytes by murine macrophages. J. Leukoc. Biol. 2004;75:705-713.

Weaver DJ, Poligone B, Bui T, Abdel-Motal UM, Baldwin AS Jr, Tisch R. Dendritic cells from NOD mice exhibit a defect in NF-κB regulation due to a hyperactive IκB kinase. J. Immunol. 2001;167:1461-1468.

Wu L, D'Amico A, Winkel KD, Suter M, Lo D, Shortman K. RelB is essential for the development of myeloid-related CD8α^- dendritic cells but not of lymphoid-related CD8α^+ dendritic cells. Immunity. 1998;9:839-847.

Yu Q, Kovacs C, Yue FY, Ostrowski MA. The role of the p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphoinositide-3-OH kinase signal transduction pathways in CD40 ligand-induced dendritic cell activation and expansion of virus-specific CD8+ T cell memory responses. J. Immunol. 2004;172:6047-6056.