TYPE 1 DIABETES AND TOLEROGENIC DENDRITIC CELLS: TOWARD THE PREVENTION AND CURE OF THE DISEASE.

By

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To my parents who have always believed and supported me.

I love you.

To Carlo, my future husband

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ABSTRACT

Type 1 Diabetes (T1D) is an autoimmune disease characterized by beta cell destruction leading to high blood glucose. Type 1 Diabetes (T1D) results from poorly defined interactions between susceptibility genes, the environment, and the immune system. It is initiated by a combination of susceptibility genes that create a setting in which an environmental trigger can launch the autoimmune process. The immune system attacks and destroys the insulin-producing beta cells of the pancreas, causing the complete loss of insulin production.

Dendritic cells are a specialized lineage of cells for presenting antigen to naive or quiescent cells, having immunomodulatory properties. Dendritic cells are derived from hemopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature dendritic cells. These cells are characterized by high endocytic activity and low T-cell activation potential. Immature dendritic cells constantly sample the surrounding environment for pathogens. This is probably done through many molecular pathways like the pattern recognition receptors (PRRs) and the Toll-like receptors (TLRs). Once they have come into contact with a presentable antigen, they become activated into mature dendritic cells and present the antigen at their cell surface using MHC molecules. Simultaneously, they upregulate cell-surface receptors that act as co-receptors in T-cell activation such as CD80, CD86 and others, enhancing their ability to activate T-cells.

Modulating dendritic cell function can modulate the immune system balance. Inducing tolerogenic dendritic cells can be a way to prevent and/or cure immune disorders. Tolerogenic dendritic cells are less effective in T cell activation. A lower T cell response to specific antigens may be the key to cure some diseases, Type 1 Diabetes included.

The balance of the immune system is a complex mechanism involving many molecules and cells, some of them probably still unknown. In a well functioning immune system there are not only T cells able to fight against antigens but also T cells able to regulate the activity of the other T cell subsets, the regulatory T cells. Tolerogenic dendritic cells have less power to activate T cells against antigens, while having the power to stimulate regulatory T cells making them instrumental in the balance of tolerance in two ways: lowering T cell stimulation and stimulating higher T cell suppression by the regulatory

T cells. This leads to a lower immune response to antigens. Indeed, the generation of tolerogenic dendritic cells may be a key for the prevention and cure in Type 1 Diabetes.

Many chemicals (aspirin, vitamin D, Aloe Vera) are able to modulate dendritic cells activity and their T cells interaction.

PDL1, the Programmed Death Ligand 1 or B7H1 is a new member of the B7 family that can interact with PD1, a putative negative regulator for immune function, encoded by Pdcd1. PDL1 may be a key in Type 1 Diabetes.

Pathogenesis of Type 1 Diabetes involves genetic, environmental and immunological factors. As many factors are involved, many points could be targets for therapy. Targets involved in blocking and/or modulating the immune response should be explored for the protection of the residual beta cell mass and the regeneration of the insulin producing cells. Many studies have been performed focusing on individual pathogenetic steps by many scientists around the world. While it is extremely useful to study each step separately for a better understanding of the mechanisms of the disease, only a combo therapy having as targets each one of the altered mechanisms could lead to a cure for Type 1 Diabetes. So, as Type 1 Diabetes is a complex disease involving many different altered molecular mechanisms, only a combo therapy could have significance in finding a cure for Type 1 Diabetes.

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SECTION I – Introduction.

1 INTRODUCTION AND BACKGROUND

History, Medical Significance and Epidemiology

Type 1 Diabetes (T1D) is an autoimmune disease characterized by beta cell destruction leading to high blood glucose. It is a complex disease where hyperglycemia, polyuria, polydipsia, hyperphagia and loss of weight are the main clinical signs.

The word *diabetes* comes from the greek δια βαινω (= going through) and indicates an increased volume of liquids going through the kidney (polyuria). The word *mellitus* comes from the latin word mel, mellis (= honey) and indicates the presence of glucose in the urine (glycosuria).

No one knows when man first identified diabetes as a disease, but we do know Egyptians talked about it in the Erbers papyrus (1500 b.C.). Additional support for the disease was provided in papers of about the same age from Ayur Veda which described insects being attracted by the sweet flavor of some people's urine. The first professional description of this disease was made by **Hippocrates** who identified a way to make diagnosis of the disease by urine tasting. Celsus, Galenus and Avicennae studied diabetes as well, but Apollonius of Memphis (230 b.C.) was the first one to use the word *diabetes*. Paulus of Aegina defined *dypsacus* to mean kidney weakness and excessive loss of body fluids. He prescribed drinking a mix of herbs, seafood, red wine and some fruit along with applying a vinegar mixture to the stomach as therapy. Aetius (45-117 a.C.) gave opium to his patients while Hali Abbas (900-1037 a.C,.) believed diabetes was caused by increased body fluid temperature.

The first scientist to measure the hydric balance and to establish a body fluids loss in diabetic people was Cardona (1501-1576 a.C.). John Rollo (1798 a.C.) proved hyperglycemia and Claude Bernard (1813-1878 a.C.) thought glycogenolysis had a role in the disease. L. Traube (1816-1876) observed the disappearance of glycosuria when diabetics were given a diet without carbohydrates.
Maybe, the biggest step leading us to the modern studies on Type 1 Diabetes were made by Mehring and Minkowski's experiments proving a correlation between diabetes and

pancreatectomy in dogs (1889 a.C.) and the discovery of beta cells by Langherans (1892 a.C.). In 1907, Zuelzer published results demonstrating the presence of an antidiabetic hormone in alcoholic extracts of the pancreas. Unfortunately, even though several diabetic patients benefited from the administration of the extracts, investigations were not sufficient to convince others. Apparently, Zuelzer himself was discouraged in continuing these treatments because of toxic reactions in the treated patients. Special reference must also be made to the more recent work of Paulesco, who prepared extracts having beneficial effects on the level of sugar and urea in the blood of diabetic animals. The American scientists Banting and Best (1921 a.C.), Nobel Prize winners in 1923, on the other hand, changed the life prognosis of diabetic people by discovering insulin.

Figure 1.1. Hippocrates

Figure 1.2. Best and Banting

* *Figures 1.1 and 1.2 have been downloaded from the internet (www.google.it)*

In 1979 the National Diabetes Data Group (NDDG) proposed for the first time a classification of diabetes, which was adopted in 1980 by the World Health Organization (WHO) who modified it in 1985. In 1997 the American Diabetes Association (ADA) proposed a new classification by physiopathological characteristics of diabetes.

Tab. I- Diabetes Classification

NDDG Classification	***ADA Classification***
1. Type 1 Diabetes (IDDM)	1. Type 1 Diabetes (T1DM)
2. Type 2 Diabetes (NIDDM)	2. Type 2 Diabetes a) with obesity b) without obesity
3. Others types of Diabetes	3. Others types of Diabetes
4. Impaired Glucose Tolerance	4. Gestational Diabetes (GDM)*
5. Gestational Diabetes (GDM)	

* it includes both gestational impaired glucose tolerance and gestational diabetes

Type 1 Diabetes is a complex disease. It is initiated by a combination of susceptibility genes that create a setting in which an environmental trigger can launch the autoimmune process. The immune system attacks and destroys the insulin-producing beta cells of the pancreas, causing the complete loss of insulin production (Green and Flavell 1999, Thomas and Kay 2000). Without insulin, the uptake of glucose into insulin-dependent cells, adipose tissue and skeletal muscle, is decreased. Because these key tissues cannot use glucose, the glucose circulating in the blood increases, leading to an increase in the uptake of glucose into insulin-independent cells such as nerves, glomeruli of the kidney, and the lens and retina of the eye. The excess of glucose is converted into sorbitol that cannot easily be transported out of the cells and causes a build-up of substances that can lead to long-term complications such as blindness, kidney failure, and nerve damage (Williams and Pickup, 1999).

Including both Type 1 and Type 2 Diabetes, the prevalence of diabetes in the United States in 2005 was 20.8 million people—7.0% of the population— with 14.6 million officially diagnosed and 6.2 million undiagnosed. The total prevalence of diabetes

among people aged 20 years or older in the United States in 2005 was 20.6 million, which is 9.6% of all people in this age group; and 10.3 million for the group aged 60 years and older, which is 20.9% of all people in this age group. Specifically, 10.9 million men or 10.5% of all men aged 20 years or older and 9.7 million women, or 8.8% of all women aged 20 years or older have diabetes (USA National Diabetes Fact Sheet 2005).

The total prevalence of diabetes among people aged 20 years or older in the United States in 2005 was 13.1 million for non-Hispanic whites, or 8.7% of this group, 3.2 million of non-Hispanic blacks, or 13.3% of this group. After adjusting for population age differences, non-Hispanic blacks are 1.8 times as likely to have diabetes as non-Hispanic whites (National Diabetes Fact Sheet 2005). The prevalence of diagnosed diabetes in people under 20 years of age in the United States in 2005 is about 176,500 people which represents 0.22% of all people in this age group. About one in every 400 to 600 children and adolescents have Type 1 Diabetes (National Diabetes Fact Sheet 2005). In the United States, 1.5 million new cases of diabetes were diagnosed in people aged 20 years or older in 2005 (National Diabetes Fact Sheet 2005).

The peak onset of T1D is twelve years of age in females and fourteen years of age in males, although the disease can manifest itself at any age from birth through adulthood (Williams and Pickup, 1999). Type 1 Diabetes does not have a cure. It can only be managed by diet, exercise and daily injections of insulin. There is a marked variation in the incidence of disease between populations, with high frequencies in Finland and Sweden and low frequencies in areas such as China and Venezuela. Migrant studies illustrate that the disease incidence of migrants resembles that of their host country. The incidence of Type 1 Diabetes varies also by season, with a decline in incidence during the summer and an increase in the winter. Type 1 Diabetes affects 1.27 million people in the European region, over one million Americans and more than 4.9 million people worldwide (www.idf.org), amounting to 0.09% of the world population. Diabetes is the fourth cause of death in the world and responsible for complications such as cardiovascular events, blindness, kidney failure, amputations, neuropathy and foot disease. In the United States, diabetic retinopathy causes 12,000 to 24,000 new cases of blindness each year. 44,400 people with diabetes began treatment for end-stage kidney disease and a total of 153,730 people with end-stage kidney disease due to diabetes

were living on chronic dialysis or with a kidney transplant in the U.S. and Puerto Rico in 2002. About 60% to 70% of people with diabetes have mild to severe forms of nervous system damage. The results of such damage include impaired sensation or pain in the feet or hands, slowed digestion of food in the stomach, carpal tunnel syndrome, and other nerve problems. More than 60% of non traumatic lower-limb amputations occur in people with diabetes. Almost one-third of people with diabetes have severe periodontal disease with loss of attachment of the gums to the teeth measuring 5 millimeters or more. Poorly controlled diabetes before conception and during the first trimester of pregnancy can cause major birth defects in 5% to 10% of pregnancies and spontaneous abortions in 15% to 20% of pregnancies. Poorly controlled diabetes during the second and third trimesters of pregnancy can result in excessively large babies, posing a risk to both mother and child. Uncontrolled diabetes often leads to biochemical imbalances that can cause acute life-threatening events, such as diabetic ketoacidosis and hyperosmolar (nonketotic) coma. People with diabetes are more susceptible to many other illnesses and, once they acquire these illnesses, often have worse prognoses. For example, they are more likely to die with pneumonia or influenza than people who do not have diabetes.

Costs of the disease are elevated. Direct costs involve the care and management of diabetes for both individuals and the health care system. Indirect costs occur on several levels; to the individuals as increased health and car insurance, to society for loss of productivity, and intangible costs to the individuals and their families related to stress, life expectancy, quality of life, pain, anxiety and other psychosocial costs. The estimated diabetes costs in the United States in 2002 was $132 billion total (direct and indirect) cost, $92 billion for direct medical costs and $40 billion (disability, work loss, premature mortality) for indirect costs.

Clinical Diagnosis

The National Diabetes Data Group (NDDG) first proposed criteria of diagnosis for diabetes and impaired glucose tolerance. The American Diabetes Association (ADA) established the present criteria. Normal fasting blood glucose should be <110 mg/dl. A blood sugar >110 mg/dl and <125 mg/dl indicates a condition of impaired glucose tolerance while a diagnosis of diabetes can be made when the fasting blood glucose is >126 mg/dl twice. A diabetes diagnosis is also certain when the blood sugar is 200 mg/dl two hours after a glucose load while a glycaemia >140 mg/dl and <200 mg/dl two hours after a glucose load underlines the impaired glucose tolerance condition.

Tab II- Criteria of Diagnosis

	Fasting Blood Glucose	***2 h after a Glucose Load***
Normal	<110 mg/dl	<140 mg/dl
Impaired Glucose Tolerance	110 > <125 mg/dl	140 > <200 mg/dl
Diabetes	>126 mg/dl (twice)	200 mg/dl

The prognosis of Type 1 Diabetes has been recently changed by insulin and increased availability of medical assistance. The mortality for acute complications such as ketoacidosis has been reduced and the quality and life expectancy have been increased, however, the late complications of the disease are still an unsolved issue. Increased understanding in the mechanisms of disease leads to a better diagnosis and prognosis.

The clinical manifestations of Type 1 Diabetes depend on numerous factors, including the age of onset. It starts from dehydration, hyperglycaemia, glycosuria and insulin dependence of absolute insulin deficiency in neonatal diabetes to ketoacidosis of childhood and adolescence to metabolic failure without ketoacidosis in adults.

Classic Type 1 Diabetes affects children and teenagers and has an acute onset with ketoacidosis. There are usually three phases: onset, remission and chronic disease.

The onset is usually characterized by ketoacidosis:

1. polyuria and polydipsia
2. weakness and cramps
3. vision disorders
4. anorexia, nausea, vomiting and abdominal pain
5. Kussmaul's respiration
6. hypothermia
7. consciousness status
8. vulvovaginitis and balanoposthitis
9. loss of weight
10. oligomenorrhoea or amenorrhoea
11. hepatic steatosis

Lab tests results:

1. blood glucose >250mg/dl
2. ketones in the urine (>80 mg/dl)
3. blood pH <7.35
4. normal pO2 and decreased pCO2
5. C-peptide low or not detected
6. ICA or IAA or GAD positive

About 50% of patients have a "honeymoon" (remission phase). This phase lasts usually less than 12 months and is characterized by a spontaneous improvement of the clinical presentation with need of insulin <0.1 UI/Kg, normal HbA1c and C-peptide >1.0 ng/dl.

The chronic disease is characterized by clinical symptoms due to the variability of blood glucose (high and low blood sugar) and the general condition of the patient. We need to underline the concerns about the short and long term complications due to diabetes, retinophaty, diabetic foot, infections, kidney diseases, cardiovascular diseases, coma….etc, that can be expressed in many different ways in different patients and can overlap in the same patients giving an infinite number of clinic presentations.

Natural History of Type 1 Diabetes and Therapy

Genetic susceptibility is the first step toward Type 1 Diabetes. However, susceptibility genes alone are not sufficient to cause the disease. One or more unknown environmental triggers initiate the disease. Many possible candidates have been proposed, such as viral infection, cow's milk, and chemical toxins (Akerblom and Knip, 1998). The environmental trigger administered at the proper time leads to the initiation of the autoimmune process that begins with autoreactive lymphocyte invasion into the islets of Langerhans known as insulitis. Insulitis can persist for months to years before the beta cells are completely destroyed. Before the appearance of clinical diabetes, circulating autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), islet tyrosine phosphatases (IA2), and islet cytoplasmic autoantibodies (ICA) can be detected in the peripheral blood (Skyler et al., 2001). When beta cell destruction reaches a certain threshold, an abnormal insulin response to IV glucose tolerance (IVGT) is observed. When 25-50% of the beta cell mass is lost, an abnormal response to oral glucose tolerance (OGT) is detected. Beta cell mass continues to be compromised until the loss of insulin production leads to the clinical onset of Type 1 Diabetes with increased thirst, frequent urination, and weight loss (Williams and Pickup, 1999).

Molecular Mechanisms of Type 1 Diabetes

In consideration of the medical significance of Type 1 Diabetes and its impact in social health worldwide, it is necessary to understand the molecular mechanisms underling diabetes pathogenesis for prevention and cure of the disease. Human experimentation is difficult and rightly limited by both ethical and safety issues, so animal models have been developed such as the nonobese diabetic (NOD) mouse. Type 1 Diabetes (T1D) is an autoimmune disease characterized by beta cell destruction. Genetic, immunological and environmental factors are all involved in the disease process, however even with the general understanding that a genetic predisposition needs environmental factors as trigger for immunological effects more details about specific molecular mechanisms still need to be explained.

Genetics of Type 1 Diabetes

The evidence of genetic involvement in Type 1 Diabetes comes from the frequency of the disorder: it is higher in relatives of diabetic patients than in the general population. The frequency of the disease is also 15 times more common in the siblings of diabetic

patients than in the general population. Many studies have focused on identifying the genes that contribute to Type 1 Diabetes susceptibility in humans (reviewed in She and Marron 1998, Buzzetti et al. 1998, Pugliese 2001). Many genes contribute to disease susceptibility and most of the genetic risk is focused on the MHC class II molecules. IDDM1 has been identified as the major susceptibility locus and is located in the human leukocyte antigen (HLA) region on chromosome 6p21.3 that contains more than 128 genes (Undlien et al., 2001). Several genes within this region have been associated with diabetes susceptibility. Three are members of the human leukocyte antigen (HLA) class II molecules, i.e. DRB1, DQB1, and DQA1. In Caucasian populations, the DRB1*04, DRB1*03, and DQB1*0302 alleles confer susceptibility to Type 1 Diabetes, while protective effects have been consistently observed for the DQB1*0602 allele. Several researchers have proposed a model in which the proteins encoded by the susceptibility alleles DR4, DR3, and DQ8 whose normal function is to bind extracellular protein for presentation by antigen presenting cells (APC) have reduced capability of binding peptide. This leads to incomplete thymic selection and an increase in the peripheral T cell repertoire for self-peptides (Ridgway and Fathman, 1999). Evidence for one or two other susceptibility genes have also been found in the HLA region (Undlien et al. 2001, Wong et al. 1999). IDDM2 is located in the promoter region of the insulin gene. The short class I variable number of tandem repeats (VNTR) locus has been associated with diabetes susceptibility (Bell et al, 1984). Regulation of thymic insulin gene expression is the mechanism by which the VNTR is believed to confer susceptibility (Pugliese et al., 1997).

While genes are very important in the genetic susceptibility of Type 1 Diabetes, they are not sufficient to cause the disease. In fact, the disease concordance between monozygotic twins is 40% suggesting that there must be other components in diabetes development. Gene expression is a direct reflection of the mRNA population in a given tissue that is available for translation into proteins and although monitoring gene expression profiles will not determine the specific function of a gene, it is the first step in answering this question, in understanding the disease and individuating subjects at risk of disease for acts of prevention. The initiation of the autoimmune cascade and the resulting molecular and cellular changes culminating in clinical disease occur in genetically susceptible individuals in the very early years of life. These changes are difficult to document because we do not know *a priori* which genes are involved.

Recent advances in functional genomics have made possible the simultaneous analyses of tens of thousands of genes. The application of genetic studies and microarray technology to the screening of newborns such as the PANDA study in the USA or the DIABFIN study in Italy have revealed a large number of genetic profiles involved in Type 1 Diabetes that maybe useful for the screening of the general population and for acts of prevention. In fact, these genes and profiles may serve as biomarkers for identifying the subset of subjects that will progress to T1D. However, a great deal of research still needs to be done before these markers can be used in a clinical setting.

Environmental Factors and Type 1 Diabetes

It is commonly accepted that Type 1 Diabetes (T1D) is the result of an autoimmune process, which leads to progressive damage of islet beta cells, culminating with their destruction. It has been suggested that a genetic predisposition requires the interaction with still unknown environmental factors for triggering the autoimmune process [1]. Among the environmental factors operating early in life and indicated to have a possible pathogenetic role are: cow's milk introduction within the first 3 months of age [2], early exposure to gluten in the diet [3], vitamin D deficiency [4] and viral infections [5]. In recent years, due to the increasing frequency of T1D around the world, studies have been designed to characterize risk factors, which leads to better prediction and ultimately design strategies for preventing the disease; among them, the DAISY in the U.SA. [6], the BABYDIAB in Germany [7], the DIPP in Finland [5], the DIABFIN [8] and PREVEFIN [4] in Continental Italy, and the TRIGR worldwide [*9*].

The DIABFIN project, which started in 1999 in Continental Italy and was recently extended to Sardinia, aimed to identify newborns with high (HR) and medium (MR) HLA risk genotypes for T1D in the general population. The HLA DRB1*03/DRB1*04-DQB1*0302 was considered to be the HR genotype, whereas the three combinations of DRB1*04/DRB1*X-DQB1 not 0602-3/DQB1 0302 (where X is not DRB1*03 nor DRB1*04, and DRB1*04 is not 0403), DRB1*04/DRB1*04-DQB1 0302 (where DRB1*04 is not 0403) and DRB1*03/*03 were of MR, just like the DRB1*0405-DQA1*0301-DQB1*0302. The DRB1*0405-DQA1*0301-DQB1*0302 in combination to the DRB1*03-DQB1*0201 has been considered as HR. All the remaining genotype combinations were classified as at low risk (LR) for developing T1D, including DRB1*0901-DQA1*0301-DQB1*0303 which is a genotype of susceptibility in the

Asian population but not in the Caucasian population.

In the study scientists analyzed potential risk factors operating during pregnancy and tried to correlate them with the presence of different HLA risk genotypes for T1D in a population of newborns analysed at the time of delivery. Length of gestation was significantly associated with the HLA risk categories being lowest in the HR group and highest in the LR group. Therefore, the higher the HLA risk for T1D, the shorter the gestation period.

The hypothesis of scientists is that a neonate born a few days early, in addition to carrying genetic susceptibility to T1D, is slightly immature at the time of birth and therefore more susceptible to harmful environmental factors. For instance, an immature gut mucosa may be more sensitive to large molecular weight proteins such as cow's milk. The proteins in cow's milk, which pass through the immature intestinal mucosa, may generate an immune response, cross-reacting with islet B-cell antigens. The first days of life are also important in the maturation of thymic T cells. This mechanism is particularly relevant for CD4+CD25+ regulatory T cells [10], which mature in the thymus and migrate from the gland to the periphery around the time of birth [11]. With a shorter gestation period, there would be impaired maturation of these regulatory cells during the last part of gestation and in the early neonatal period [12].

Immunopathology

Type 1 Diabetes (T1D) results from poorly defined interactions between susceptibility genes, the environment, and the immune system. Studies have shown that a large number of susceptibility genes are implicated in T1D. These susceptibility genes are probably required risk factors for the vast majority of T1D patients; however, many genetically predisposed subjects may not develop clinical diabetes. The progression from genetic predisposition to β-cell autoimmunity is a critical, but poorly understood process. The initiation of the autoimmune cascade and the resultant molecular and cellular changes culminating in clinical disease could occur in genetically susceptible individuals in the very early years of life triggered by environmental factors (viruses, breast feeding, cow's milk proteins, early birth...etc...).

Type 1 Diabetes (T1D) becomes clinically apparent as the end result of cellular immune mediated destruction of the insulin producing pancreatic β-cells. Its features include

mononuclear cell infiltration of islets (insulitis), production of islet-cell specific autoantibodies, and β -cell antigen-specific T-cell responses. Cumulative evidence indicates that beta cell antigens, macrophages, dendritic cells, B-lymphocytes, and T lymphocytes are involved in the complicated pathogenic process of this disease [13]. Macrophage and/or dendritic cells are the first type of cells to infiltrate the pancreatic islets [14]. These infiltrating macrophages are reported to secrete interleukin-2 (IL-2), which activates Th1 type $CD4^+$ T cells, these activated $CD4^+$ T cells in turn secrete IL-2 and interferon-γ which assists in the recruitment and activation of $CD8^+$ T cells [15]. Other macrophage-derived factors responsible are oxygen-free radicals [16] and cytokines such as IL-1, tumor necrosis factor-β and IFN-γ. They are believed to be cytotoxic to beta cells [17] [18]. B cells have a role as antigen presenting cells involved in regulating diabetogenic T cells [19].

Some studies suggest that the initial insult is mediated by CD8+ cytotoxic T lymphocytes but another view holds that Type 1 Diabetes is initiated by $CD4^+$ T cells. Several reports have documented defects in immune regulation by regulatory T (Treg) cells ($CD4^+CD25^+$ and iNKT) in T1D patients and several other autoimmune diseases. However, contradictory observations have been reported for both $CD4^+CD25^+$ and iNKT populations The controversial findings from different studies can be explained by different factors. First, many of the studies had small sample sizes so that the studies could be under-powered with the results reflecting random (experimental or biological) variation. Second, the selection of study subjects differed; study populations vary largely so that heterogeneity is unavoidable. Third, some of the assays such as the suppression assay used for Treg function analysis are highly variable, even in the same laboratory. Studies have shown a pivotal role of another population of cells: dendritic cells. Dendritic cells uniquely orchestrate the delicate balance between T cell immunity, Th1 and Th2 and an imbalance favoring immunogenic rather than tolerogenic DC is believed to contribute to the development of autoimmune diseases such as Type 1 Diabetes (T1D).

Beta-cells Destruction and Regeneration

Type 1 Diabetes results from a lack of insulin production. Genetic, immunological and environmental factors lead to the beta-cell destruction. Recovery of the beta-cell mass and function could be a cure for this disease. Transplants of beta cells have been performed with partial good results. Introduction of new islets solve the lack of insulin

production in the short term, but later the same mechanisms of disease lead once again to destruction of the new cells. Indeed, only the regeneration of the insulin producing cells and the block of the autoimmune cascade could lead to a cure for Type 1 Diabetes.

Imaging the Pancreatic Beta Cell

Diabetes results when there is an inadequate beta-cell mass whether by destruction (Type 1 Diabetes) or inadequate compensation for increased functional need (Type 2 Diabetes). Non-invasive assessment of beta-cell mass would provide an important tool for both therapeutic interventions and better understanding of the natural history of the disease.

A few years ago, a small group of talented scientists met to discuss the significance of imaging the pancreatic islet in vivo and, despite the high risk of failure, NIH (National Institutes of Health), JDRFI (Juvanile Diabetes Research Foundation International) and other funding agencies across the world have sponsored a large variety of projects focused on imaging the pancreatic beta-cells.

The imaging of the islets can provide important data to scientists and clinicians. The beta cell mass is dynamic with compensatory changes (both expansion and involution) to maintain glucose homeostasis. Changes can be in number or volume of cells and islets. In both rodents and humans the beta-cell mass is related to body weight and BMI (Body Mass Index). The imaging of beta cells would give us a nice help in understanding such changes. As islet transplantation becomes a potential clinical modality for restoring normoglycaemia in Type 1 Diabetic patients, there is a critical need for non-invasive assessment of the fate of the transplanted grafts. A significant loss occurs even after the transplant due to immunological and non immunological events so monitoring of islet mass using non invasive methods would significantly help in studying post transplantation events. Scientists have developed a non invasive technique to detect human islets labelled with a magnetic resonance imaging contrast agent and transplanted under the kidney capsule in mice. Sweet, Cook and Greenbaum from the University of Washington, Seattle (WA) and the Benaroya Research Institute, Seattle (WA) defined a theoretical framework and an in vivo screening assay that establish testable criteria for quantifying the potential of candidate agents to serve as in vivo beta cell imaging agents. They showed their data at the Imaging the Pancreatic

Beta Cell in Health and Disease Third Workshop in 2006. To estimate the requisite association of candidate beta cell imaging agents they calculated the beta cell contribution to a PET signal using an equation that accounted for the small relative population of beta cells with respect to non beta cells, exocrine tissue, extracellular and vascular space and an equation describing equilibrium ligand binding. Hara, Adeboje and Waight from the University of Chicago (IL) used a mouse insulin I promoter (MIP) green fluorescent protein (GFP) transgene to genetically tag pancreatic beta cells. They have been able to study the distribution of beta cells throughout the entire pancreas in situ and to visualize every beta cell within the pancreas and construct a three-dimensional (3D) model showing the distribution.

Beautiful progress had been made in cellular imaging and scientists are still speedily walking toward their goals but the imaging of beta cells is not an easy task. Islets are only about 1-2 percent of the pancreas and are small in size. They range in diameter from 20 μm to 400 μm and have very little to differentiate them from surrounding tissue. Beta cells have a common origin with other islet endocrine cells, the acinar cells and the pancreatic ductal cells. So, many potential markers of beta cells may be expressed in these other pancreatic tissues. Those and other issues make the scientists job harder, but as modern science and technologies advance, better imaging would provide better support to scientists and clinicians.

Dendritic Cells Surface Molecules

Dendritic cells are a specialized lineage of cells for presenting antigen to naive or quiescent cells, having immunomodulatory properties. Dendritic cells are stellate cells that were first described in the skin by a medical student, Paul Langherans in 1868 in Germany. It took almost a century to understand, with Ralph Steinman first and Zanvil Cohn later, the dendritic cell's role in the immune system. Recently, dendritic cells were considered to have a pivotal role in the balance of tolerance and in the pathogenesis of immune disorders. DCs are widely distributed in the body to better differentiate antigens. There are different types of DCs and each one has different strategies of function but a common behaviour.

Dendritic cells are derived from hemopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature dendritic cells. These cells are

characterized by high endocytic activity and low T-cell activation potential. Immature dendritic cells constantly sample the surrounding environment for pathogens. This is probably done through many molecular pathways like the pattern recognition receptors (PRRs) and the Toll-like receptors (TLRs). Once they have come into contact with a presentable antigen, they become activated into mature dendritic cells and present the antigen at their cell surface using MHC molecules. Simultaneously, they upregulate cell-surface receptors that act as co-receptors in T-cell activation such as CD80, CD86 and others, enhancing their ability to activate T-cells.

Dendritic cells are sentinel cells exploring the body for foreign invaders. They are antigen capturing cells, antigen presenting cells and immune system stimulating cells. The high levels of major histocompatibility complex (MHC) at the dendritic cell surface allow T cell recognition. This recognition allows scientists to test these cells in the mixed leukocyte reaction (MLR), the well-known clinical assay for identifying the compatibility of tissue transplants between donors and recipients.

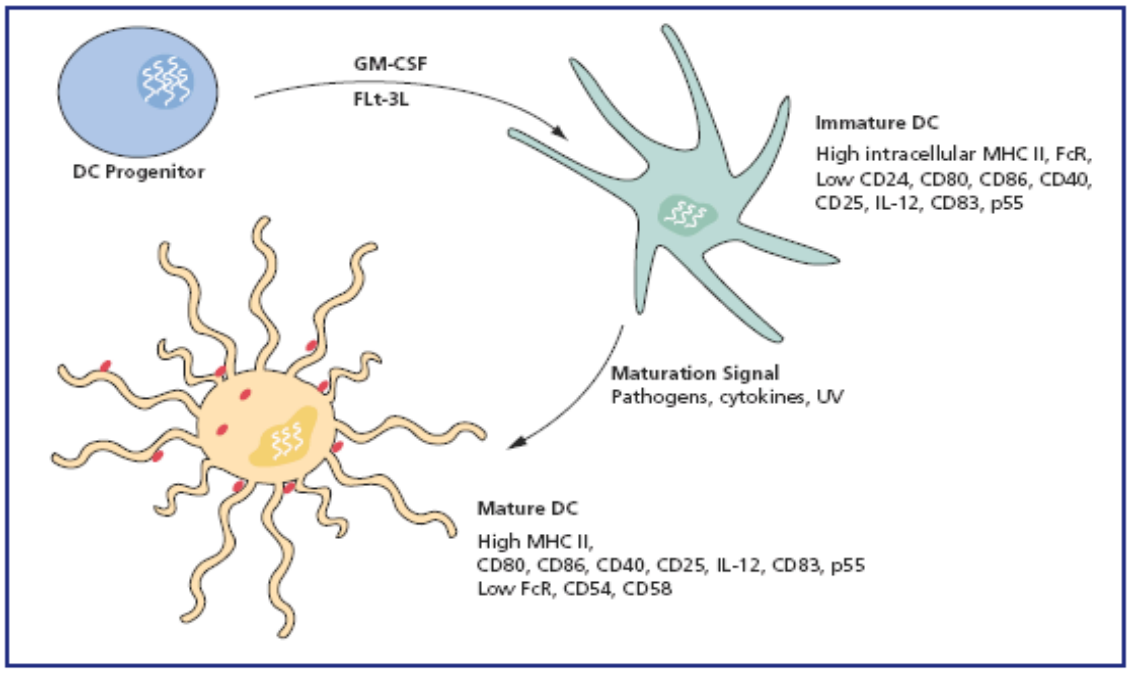
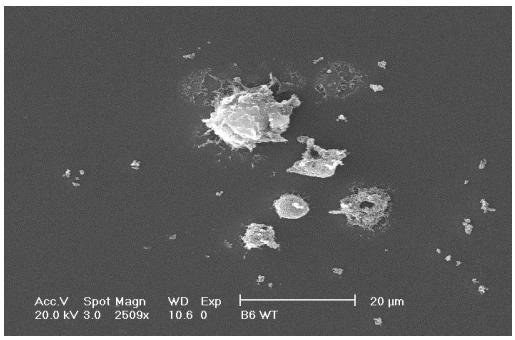
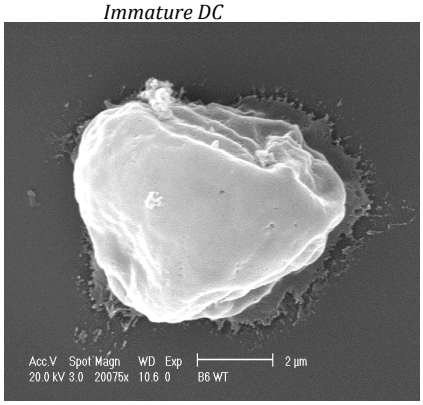


Fig.1.3- Maturation of dendritic cells. Dendritic cell (DC) progenitors from the bone marrow migrate to lymphoid and non-lymphoid tissues where they respond to maturation signals to fully develop. Maturation of DCs from "immature" to the "mature" stage involves changes in surface expression of antigens involved in antigen presentation and stimulation (from BD Bioscience web site)

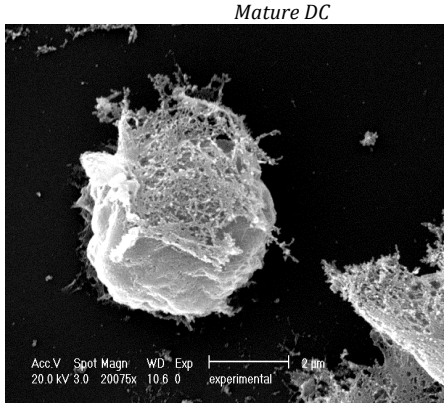
Fig. 1.4 Dendritic cells deriveded from cultures of bone marrow from a B6 mouse



Immature DC



Mature DC



Electronic microscopy- Images taken at the Medical College of Georgia, Augusta, GA; USA by Dan Eisenman and Anna Lisa Montemari

Immunomodulation for Prevention and Cure

Modulating dendritic cell function can modulate the immune system balance. Inducing tolerogenic dendritic cells can be a way to prevent and/or cure immune disorders. Tolerogenic dendritic cells are less effective in T cell activation. A lower T cell response to specific antigens may be the key to cure some diseases, Type 1 Diabetes included.

The balance of the immune system is a complex mechanism involving many molecules and cells, some of them probably still unknown. In a well functioning immune system there are not only T cells able to fight against antigens but also T cells able to regulate the activity of the other T cell subsets, the regulatory T cells. Tolerogenic dendritic cells have less power to activate T cells against antigens, while having the power to stimulate regulatory T cells making them instrumental in the balance of tolerance in two ways: lowering T cell stimulation and stimulating higher T cell suppression by the regulatory T cells. This leads to a lower immune response to antigens. Indeed, the generation of tolerogenic dendritic cells may be a key for the prevention and cure in Type 1 Diabetes.

Programmed Death Ligand 1

PDL1, the Programmed Death Ligand 1 or B7H1 is a new member of the B7 family that can interact with PD1, a putative negative regulator for immune function, encoded by Pdcd1. (Trends in immunology review, The PD1-PDL pathway in immunological tolerance. Taku Okazaki and Tasuku Honjo). This PD1 function was initially suggested because PD1 knockout ($Pdcd1^{-/-}$) mice developed spontaneous lupus-like glomerulonephritis and arthritis (Nishimura H 1999, Immunity 11, 141-151 Development of lupus-like autoimmune diseases by disruption of the PD1 gene encoding an ITIM motif carrying immunoreceptor). When the knockout mice are backcrossed to different strains of mice, different target organs are affected by autoimmune events. (Science 291, 319-322, 2001) (Nat Med 9, 1477-1483, 2003) (Proc Natl Acad Sci USA 102, 11823-11828, 2005) (Cancer Res 65, 1089- 1096)

Combo Therapy Significance in Type 1 Diabetes Cure

Pathogenesis of Type 1 Diabetes involves genetic, environmental and immunological factors. On a genetic predisposition to the disease, environmental factors activate the immune system against the beta cells leading to a lack of insulin production. As many factors are involved, many points could be targets for therapy. All the environmental and genetic factors regulating the immune system in this disease should be explored for possible prevention.

Targets involved in blocking and/or modulating the immune response should be explored for the protection of the residual beta cell mass and the regeneration of the insulin producing cells. Many studies have been performed focusing on individual pathogenetic steps by many scientists around the world. However in my opinion, while it is extremely useful to study each step separately for a better understanding of the mechanisms of the disease, only a combo therapy having as targets each one of the altered mechanisms could lead to a cure for Type 1 Diabetes. So, as Type 1 Diabetes is a complex disease involving many different altered molecular mechanisms, only a combo therapy could have significance in finding a cure for Type 1 Diabetes.

2 MATERIALS AND METHODS

NOD Mouse as a Model of Type 1 Diabetes

The nonobese diabetic (NOD) mouse is an excellent experimental animal model for Type 1 Diabetes. This strain was introduced in 1980 from a laboratory in Japan (Makino et al., 1980), and was derived from the Jcl:ICR strain. The NOD mouse has been extensively studied in Type 1 Diabetes. NOD mice are healthy at birth but from three to ten weeks of age, they spontaneously develop insulitis, lymphocyte infiltration in the pancreas. Overt diabetes occurs from 14 to 30 weeks of age. Susceptibility to IDDM in NOD mice is both polygenic and environmental. Housing conditions, health status, and diet exert a strong effect on penetrance. The disease develops in 80-90% of the female mice and 10-40% of the male mice (Delovitch and Singh, 1997).

There are many similarities between the development of T1D in NOD and T1D in humans. In both cases, multiple susceptibility loci are involved in the predisposition toward disease development, with the main component of susceptibility being contributed by the MHC region. Specifically the beta chain of the class II HLA molecules in both NOD and humans share an amino acid substitution at Position 57 at which the absence of aspartic acid leads to disease susceptibility (Atkinson and Leiter, 1999). Another similarity shared by humans and the NOD mouse is that the genetic susceptibility of both is controlled by multiple regions throughout the genome. Of the twenty regions identified in humans and the sixteen in the NOD mouse, only seven have been shown to be syntenic. They are Idd1 to *I*DDM1; Idd2 to IDDM3; Idd5 to IDDM6, IDDM7, IDDM12, IDDM13; and Idd14 to IDDM15 (Leiter, 1998). The next two similarities are in the manifestation of the disease. Both humans and NOD mice exhibit a long pre-diabetic phase followed by spontaneous development of the disease (Thivolet, 2001). Also the development of insulitis has been shown to occur in both humans and NOD mice (Atkinson and Leiter, 1999). Finally, destruction of the pancreatic beta cells is accomplished by autoreactive T lymphocytes in both humans and NOD mice (Toyoda and Formby, 1998).

The differences exhibited in disease development between humans and NOD mice must also be taken into consideration. One important difference is that NOD mice have been

inbred to obtain genetic homogeneity, while humans represent a genetically diverse population. NOD mice lack expression of the I-E gene that is homologous to the HLA-DR gene that plays an important role in genetic susceptibility in humans. Polymorphisms in the 5' region of the insulin gene (IDDM2) play a role in genetic susceptibility in humans. However, the NOD mouse has two non-linked copies of the insulin gene, both of which are expressed, but neither demonstrates a comparable polymorphism relating to disease susceptibility. Beyond the genetics, differences also exist in the disease pathogenesis. NOD mice exhibit a resistance to ketoacidosis. Although insulitis occurs in both humans and NOD mice, the type of insulitis differs between these two species (Atkinson and Leiter, 1999). A difference is also found in the number of T cells that are located in the peripheral blood and lymphoid tissues. NOD mice appear to have an increased percentage when compared to humans (Atkinson, 1998). Other discrepancies are shown by the gender bias of disease development in NOD mice that is not seen in humans, and the fact that NOD mice will only develop T1D when housed in a specific-pathogen free (SPF) environment (Atkinkson and Leiter, 1999).

Despite the numerous differences between T1D in NOD mice and humans, the core components of the disease are constant. The genetic homogeneity of the NOD mice implies that the disease manifested in this model most likely represents one specific case of Type 1 Diabetes seen in humans (Atkinson and Leiter, 1999). Thus, the information derived from studying the NOD mouse model can be applied to human disease pathogenesis with the caveat that the NOD will not supply all of the answers for deciphering human Type 1 Diabetes. However, the information gathered from this model may be the key needed to unlock the basics of disease development.

Other Mouse Strains: NOD.SCID

In this model, the scid mutation has been transferred onto a diabetes-susceptible Non-Obese Diabetic background. Animals homozygous for the scid mutations experience a block in T and B-cell lymphocyte development. Mice homozygous for the severe combined immune deficiency spontaneous mutation (Prkdc^{scid}, commonly referred to as scid) are characterized by an absence of functional T cells and B cells, lymphopenia, hypogammaglobulinemia, and a normal hematopoietic microenvironment. Normal antigen-presenting, myeloid, and NK cell functions are strain dependent. Scid mice carry a DNA repair defect and a defect in the rearrangement of genes that code for antigen-specific receptors on lymphocytes. Most homozygotes have no detectable IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA. Thymus, lymph nodes, and splenic follicles are virtually devoid of lymphocytes.

Scid mice accept allogeneic and xenogeneic grafts, making them an ideal model for cell transfer experiments. Some scid mice spontaneously develop partial immune reactivity. Scid mice that have serum Ig levels greater than 1 ug/ml are considered "leaky." Scid leakiness is highly strain dependent, increases with age, and is higher in mice housed under non-SPF conditions. In general, scid leakiness is high on the C57BL/6J and BALB/cBy genetic backgrounds, low on the C3H/HeJ background, and even lower on the NOD/ShiLtSz background. NOD/ShiLtSz-Prkdc^{scid} mice are both insulitis- and diabetes-free throughout life and serve as a diabetes-free controls for comparison to NOD/ShiLtJ mice. Thymic lymphomas occur with high frequency, however, and life span typically is limited to only 8.5 months under specific pathogen-free conditions. In addition to being an excellent host for xenografts, NOD.CB17-Prkdc^{scid}/J mice may be useful for delineation of the role of T cell subsets in autoimmune diabetes and also as a source for insulitis-free islets. (www.jax.org, The Jackson Laboratory web site).

Bone Marrow Derived Dendritic Cells

Female NOD mice 8-10 weeks old were euthanized, the legs removed and bone marrow flushed from the femurs using a 22G needle after pealing off skin and muscle using media (RPMI+mercaptoethanol+non essential aminoacid solution+sodium piruvate+FBS+antibiotics 20 ml per 3x106 cells). Cells were cultured for 7-10 days

with 10 ng/ml GM-CSF and 10 ng/ml IL-4. Cultures were refreshed with new media and GM-CSF plus IL-4 at day 4 and day 7. Aspirin, vitamin D or other agents were added in the cultures on day 4 and again on day 7 at the dose indicated above (IL10 = 20ng/ml) (Aspirin 2.5 mM) (Vit. D from a stock 1.2 $x10^{-14}$ M, 10µL/ML from a solution of 10µL/ML of vitamin D). To compare stimulated and unstimulated dendritic cells, 1 µ g/ml of LPS was added to some plates 24 hours before the harvast. This protocol has been adapted from Lutz et al. Journal of Immunological Methods 223 (1999) 77-92.

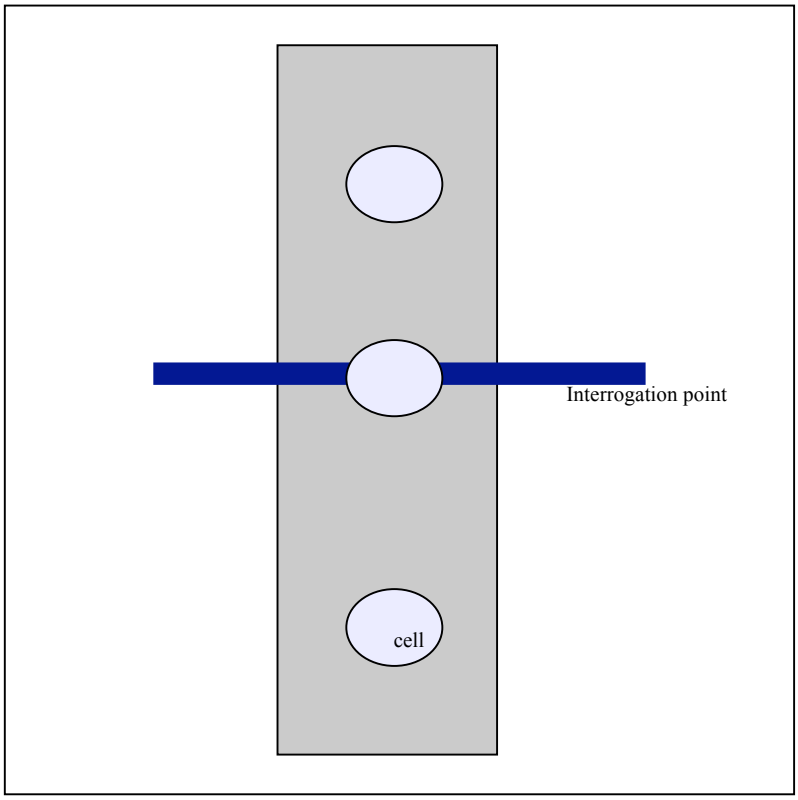
T cell Isolation Technique

Spleens from mice were disrupted between two glass slides in 5 mL of media (PBS 1X Cellgro+3% FBS+2mM EDTA) and put in 15 mL tubes. Five mL of media was added to each tube. After spinning at 500 G/ 5 min / 4 ºC, the supernatant was discarded and the pellet resuspended in 2 mL of 1X RCL buffer, stored for 5 minutes at RT and then resuspended in media. After spinning at 500 G/ 5 min /4 ºC the supernatant was discarded and the pellet resuspended in 40 µL of MACS buffer per 10^7 total cells. After incubating for 10 minutes at 4 ºC with Biotin-Antibody Cocktail, 30 µ L of MACS buffer and 20 µL of Anti-Biotin Microbeads per 10^7 total cells were added and samples incubated for 15 minutes at 4ºC. After incubation, the cells were washed by adding 10-20 times the labeling volume and centrifuged at 500 G for 10 minutes. The supernatant was discarded and the pellet resuspended in 500 µL of MACS buffer per 10^8 total cells. The cells were then applied to a MACS Column that separates out the T cells using negative selection by magnetic separation or magnetic separation was applied using AUTOMACS.

Flow Cytometry Technique

Flow cytometry is a useful technique for characterizing and analyzing cells. As its name means (cyto= cells, metry= measurement, flow= stream) it measures simultaneously multiple physical characteristics of the cells as they move in a fluid stream. The technique can tell us information about cell size, granularity and fluorescence intensity. The instrument uses fluidic, optic and electronic systems working together to determine how cells scatter incidence laser light and emit fluorescence as they pass through the interrogation point.

Fig. 2.1



The fluidic system, usually a saline solution, transports the cells to the interrogation point. Lasers, lenses and filters are the optic system and it excites and collects cells. When cells pass through the interrogation point they scatter light and any molecules present on the cells fluoresce. Detectors in the electronic system convert detected light into electronic signals that are further processed and sent to the computer. The characteristics of each cell are based on its light scattering and fluorescent properties.
Some instruments are equipped for sorting and have an electronic system able to start the sorting decisions.

One million cells per each sample have been used. CD11c APC-labeled, CD80 PE-labeled, CD86 PE-labeled or PDL1 PE-labeled from BD Bioscience at the house recommendations doses have been incubated for 15 minutes on ice, after FC-blocking. Cells have been washed with PBS and centrifuged at 500 G for 5 minutes. Supernatant have been discharged and cells resuspended in 400 µl 2% formalin. APC Isotype control and PE Isotype control have been used as control.

Mixed Lymphocyte Reaction Technique

Spleen T cells were purified from 6-10 week old B6 female mice or NOD female mice using the Pan T cell Isolation Kit (Miltenyi Biotec) which yields >90% CD3+ T cells. T cells were then plated in 96 well plates with 10^5 T cells/well. Stimulatory DC were added at a 1:1, 1:2 and 1:4 DC: T cell ratio. The reaction was incubated at 37^0 C for 72h. From 12 to 16 hours before the harvesting, 1 µl/well of H^3 was added and the reaction incubated at 37^0C until the harvest when the plate was either read using the Cell Harvester and the Trilux Scintillation Counter or stored at -20 ^{0}C. All the experiments were carried out according to Good Laboratory Practice guidelines and have been approved by the Safety Radiation Office of Medical College of Georgia.

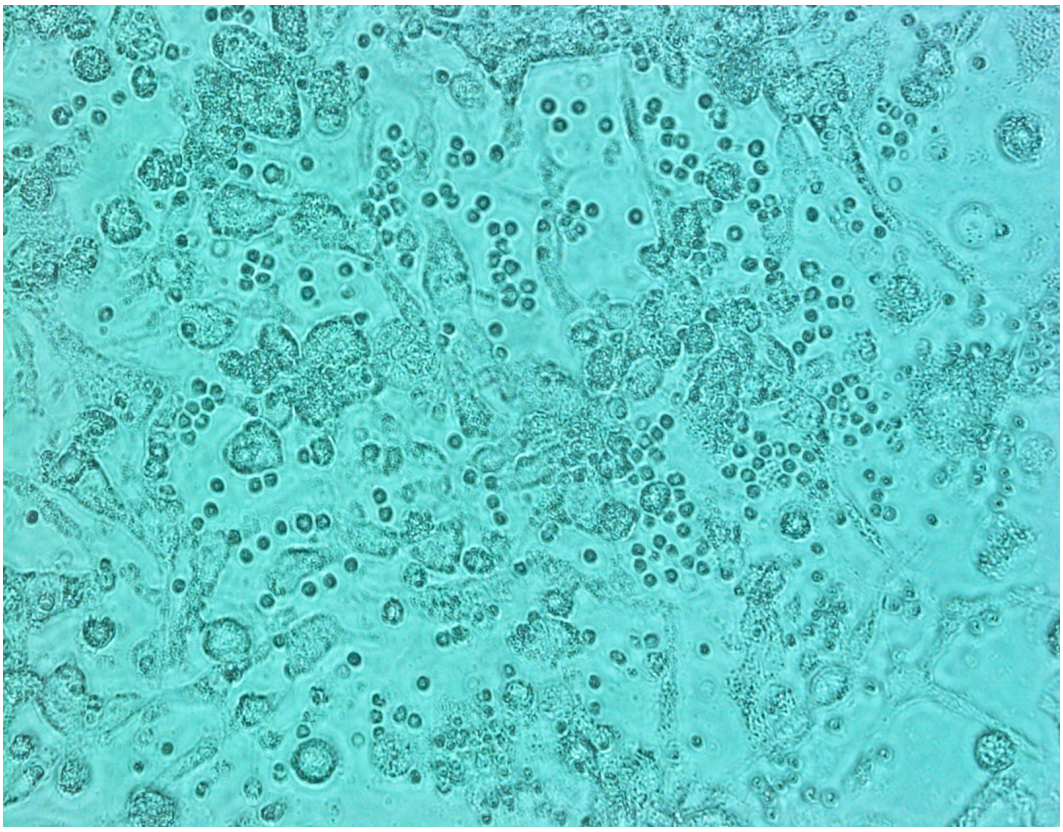


Fig. 2.2- NOD-DC1 cell line and murine T cells in a mixed lymphocytes reaction. 20X image taken at the Medical College of Georgia by Anna Lisa Montemari

RNA Extraction Technique

After 10 days of culture, BMDCs were stored in 350µl RLT buffer and 3.5µl β-mercaptoethanol for less than $5x10^6$ cells at -80°C until RNA extraction could be performed using the RNeasy Mini Kit from QIAGEN. Samples were melted on ice and the lysate was pipetted directly onto a QIAshredder spin column placed in a 2 ml colletion tube and centrifuged for 2 minutes at maximum speed. The flow-through was kept and 350µl of 70% ethanol was added to the homogenized lysate and well mixed by pipetting. Up to 700 µl of sample was applied to an RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 seconds at ≥10000 rpm. The flow-through was discarded and 700 µl of Buffer RW1 was added to the RNeasy column. The sample was centrifuged for 15 seconds at ≥10000 rpm to wash the column. Once again the flow through was discarded and 500 µl Buffer RPE was added to the RNeasy column and centrifuged for 2 minutes at ≥10000 rpm to dry the RNeasy silica-gel membrane. To elute, the RNeasy column was transferred to a new 1.5 ml RNase-free collection tube and 50µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and centrifuged for 1 minute at ≥10000 rpm for eluition. The RNA was then stored at -80°C.

The RNA concentration was measured using the Agilent 2100 Bioanalyzer RNA 6000 Nano Assay, Technologies, Inc. Reagents and samples were allowed to equilibrate to room temperature for 30 minutes before use and RNA samples and ladder denatured at 70 ^{0}C for 2 minutes before use. RNA 6000 Nano dye concentrate was vortexed for 10 seconds and spun down and 1 µl of the dye was added to a 65µl aliquot of the filtered gel and vortexed again. After decontaminating the Bioanalyzer electrodes with 350 µl RNaseZAP for 1 minute and 350 µl RNase-free water for 10 seconds, a new RNA Nano chip was placed on the Chip Priming Station. Nine microliters of the gel-dye mix was dispensed in the chip and the Chip Priming Station locked for 30 seconds with the plunger at 1 ml. After opening the Chip Priming Station, 9 µl of the gel-dye mix were pipetted in the chip. Five microliters of the RNA 6000 Nano Marker were pipetted in the ladder well and samples loaded in the chip. After placing the chip in the adapter of the vortex mixer, it was vortexed for 1 minute at the IKA vortexer set-point of 2400 rpm. The chip was then loaded into the receptacle of the Agilent 2100 bioanalyzer, the software started and sample RNA concentration was measured.

Semiquantitative RT-PCR Technique

Specific primers were designed for each gene using the OLIGO 4.1 program. A new software PERLPRIMER was used to design the primers for the most recent RT-PCR experiments. All primers were shipped from IDT (Integrated DNA Technologies, Inc., USA). RNA (2 µg) from BMDCs was converted to cDNA using polyT primers. Briefly, RNA and polyT primers were incubated at 80°C for 10 minutes and later with reverse transcriptase at 42°C for 2 hours. cDNAs were stored at -80°C until used. A series of RT-PCR reactions were conducted with 2 µl of RT product in a 20µl reaction volume. All samples were normalyzed using β-actin. PCR products were run on 1% agarose gels and a picture of the gel was taken to document the bands. The intensity of the bands was compared and a ratio calculated using β-actin.

CY Model

Cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide monohydrate) is a synthetic antineoplastic drug chemically related to the nitrogen mustards. Many authors use it to accelerate and synchronize the development of overt diabetes in NOD mice [Autoantigen-specific protection of non-obese diabetic mice from cyclophosphamide-accelerated diabetes by vaccination with dendritic cells. Krueger T, Wohlrab U, Klucken M, Schott M, Seissler J. Diabetologia (2003) 46: 1357-1365]. However, the specific mechanism that causes diabetes in CY treated mice remains unknown [21].

Cytoxan from Myers-Bristol Squibb have been used at the dose 200mg/kg diluited in sterile PBS to induce diabetes in our mice.

NOD.SCID Mouse Model and Type 1 Diabetes Co-transfer Model

The co-transfer model in NOD mice is an accepted model to study the protective properties of subsets of cells in Type 1 Diabetes. Many researchers have used it to verify what kind of cells, treatments and conditions play a role in diabetogenesis. Briefly, T cells from diabetic NOD spleens are transferred into NOD.SCID mice to transfer diabetes to recipients. [22] [23] [24] [25]

In our model we injected 5 x 10^6 T cells to transfer diabetes to recipients.

Testing our BMDCs we injected 5 x 10^5 BMDCs twice in our recipients.

NOD DC1 Cell Line

Authors of the paper Diabetologia 2003, 46: 1357-1365 established a culture system for the long-term expansion of dendritic cells from NOD mouse splenocytes. These cells have characteristic features of myeloid dendritic cells, possess full functional activity and can be used as adjuvant for autoantigenspecific immunization. The cells were plated into cell culture petri dishes. The cells were fed every 2 to 3 days and sub-cultures were made every 2 to 3 weeks. Cells were harvested using a cell scraper and transferred onto new plates. Dendritic cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 2 mmol/l glutamine, 50 µmol 2β-mercaptoethanol (ME), 100 U/ml penicillin and 100 µg/ml streptomycin, 10 ng/ml GM-CSF (BD Biosciences, San Diego, Calif., USA) and 30% culture supernatant from NIH/3T3 fibroblasts. Our NOD DC1 cells were generously provided by Dr. Seissler, Germany and then cultured in our lab. The culture protocol has been adapted from Seissler, Diabetologia 2003,46: 1357-1365.

NIH/3T3 fibroblasts

NIH/3T3 cells were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, 100µg/ml streptomycin. Supernatants of confluent cells were collected and filtered using a 0.2 µm filter (Corning Incorporated, NY, USA) and stored at -20 ^{0}C up to 4 weeks. The NIH/3T3 fibroblast were generously provided by Dr. Seissler, Germany and then cultured in our lab. The culture protocol has been adapted from Seissler, Diabetologia 2003, 46: 1357-1365.

DC2.4 Cell Line

DC2.4 cells, which were previously characterized as an immature murine DC line (H-2b; Shen, Z., Reznikoff, G., Dranoff, G., and Rock, K. L. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J. Immunol., 158: 2723 2730, 1997), were generously provided by Dr. K. L. Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA) and were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 µM nonessential amino acid, 50 µM 2-ME, and antibiotics.

1α, 25 (OH) 2 Vitamin D3

The active form of vitamin D, 1,25-dihydroxyvitamin D3 [1,25- (OH) 2D3], is a secosteroid hormone that binds to the vitamin D receptor (VDR), a member of the superfamily of nuclear receptors for steroid hormones. 1α, 25(OH) 2 vitamin D3 plays a pivotal role in bone metabolism. Many studies have also shown that it is an important regulator of the immune system and it acts through the vitamin D receptor (VDR) [27].
1α, 25 (OH) 2 vitamin D3 is important in direct and indirect regulation of $CD4^+$ T cells. It inhibits T cell proliferation and production of the cytokines IL-2, TNF-alpha, and IFN-gamma in cell cultures stimulated with T cell mitogens. In $CD4^+$ T cells, 1α, 25 (OH) 2 vitamin D3 inhibited Th1 cell development and cytokine production and enhanced Th2 cell expansion. This led to an increase in the production of IL-4 in mice, which halted the progression of autoimmune diseases [28]. Vitamin D can block DC maturation and activation of naive T cells by immature DC. Therefore, vitamin D by inhibiting DC maturation can induce tolerance [29]. This tolerance comes from immature DC's inability to present antigen to $CD8^+$ T cells.
For our experiments we used 200 μl/20 ml from a mix of 20 μl 1.2 x 10^{-4} M in 2 ml.

Aspirin

Salicylates are widely distributed in plants and aspirin is one of the most used drugs in the world. Aspirin is one of the oldest known drugs. Around 400 bC Hippocrates recommended a brew of leaves from the willow tree (*Cortex salicis*), a rich source of salicylates, for the relief of pain in childbirth. The attention of chemists seems to have been first drawn to salicylates after the Reverend Edward Stone wrote, in 1763, to the President of the Royal Society in London. Stone reported how he had used increasing doses of a powder prepared from the white willow tree to cure 'fever in over 50 patients suffering from various agues'.

Salicylic acid seems to have been first synthesised from carbolic acid around 1859 by a German chemist, and it came to be widely used for the relief of pain and fever. In 1897 Felix Hoffman, a chemist working in a laboratory owned by Friedrich Bayer in Elberfeld, Germany, formulated a pure and stable form of acetyl salicylic acid by mixing acetic and salicylic acids. Bayer patented the name and started to market the product in 1899. It was a huge success and sales grew rapidly. Aspirin was soon

recommended in the medical press for use with fever, migraine, the pain of inoperable cancer, rheumatoid arthritis, gout, rheumatic fever, acute tonsillitis, corns and warts. In 1997, the National Library of Medicine listed over 23,000 papers on aspirin, and it has been estimated that currently a paper on aspirin is published on average every two hours. (30)

The anti-inflammatory action of the drug comes from PG synthesis inhibition. Recently, many studies have shown a large spectrum of pharmacological mechanisms, including NF-kB inhibition and several molecular pathways involving inflammation and immunological actions [31] [32] [33] [34]. However, many mechanisms and functions of the drug in the immune system are still being studied.

Aspirin is now accepted as an important weapon in the prevention of heart disease. After the first study by Elwood and Cochrane was reported in the British Medical Journal (1974), larger trials involving 20,000 US doctors showed that aspirin reduced the risk of coronary thrombosis. A single dose of 300 mg is recommended for patients in the acute stages of a heart attack followed by a daily dose of 75-100 mg. A similar low dose treatment regime is recommended for patients with angina, a history of heart problems or who have undergone coronary by-pass surgery.

Some form of dementia affects about one in four people aged 70 years or above. There is some evidence that aspirin may help prevent both the condition resulting from impaired blood flow and the most serious form of dementia, Alzheimer's disease. The latter is believed to be an inflammatory condition similar to arthritis. Aspirin is a highly effective anti-inflammatory drug and a preliminary study found a lower than expected incidence in patients with rheumatoid arthritis, who frequently have to take aspirin over a prolonged period. So, aspirin is a cheap and potent drug that has changed many disease prognoses.
For our experiments we used 900 μl/20ml from a mix of 2 tablets in 45 ml of media.

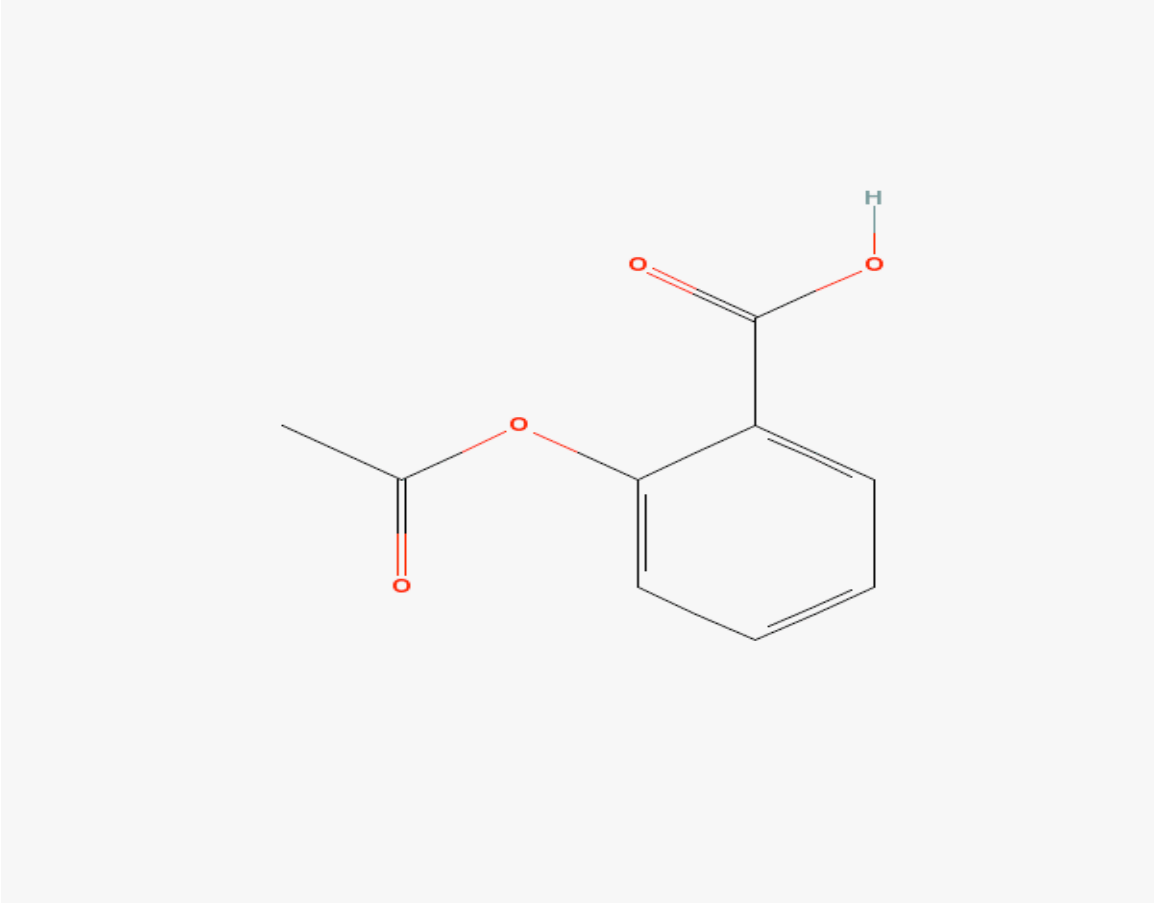


Fig.2.3 Aspirin Chemical Structure

Fig. 2.4 The discovery of aspirin: a reappraisal Walter Sneader

BMJ VOLUME 321 23–30 DECEMBER 2000

Aloe Vera

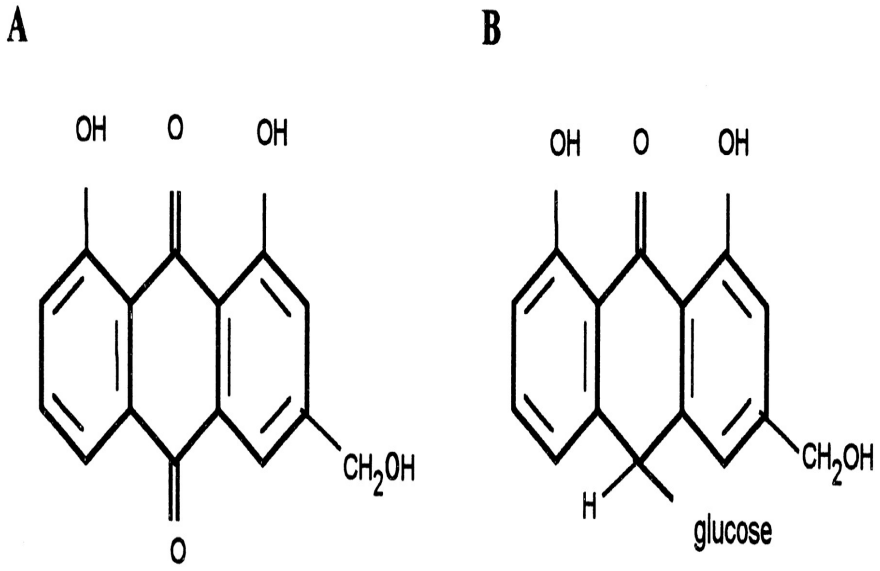
Aloe Vera is a plant from the Aloe family (400 different species), a tropical plant which is easily grown in hot and dry climates and widely distributed in Asia, Africa and other tropical areas. The use of aloe vera is being promoted for a large variety of conditions. Aloe is frequently cited as being used in herbal medicine since the beginning of the first century AD. It is mentioned in the Bible, John 19:39–40 "*And there came also Nicodemus, which at the first came to Jesus by night, and brought a mixture of myrrh and aloes....*". Early records of *A. vera* use appear in the Ebers Papyrus in the Dioscorides' De Materia Medica and in the Pliny the Elder's Natural History.

Oral administration of aloe vera in mice is effective on wound healing, can decrease the number and size of papillomas and reduce the incidence of tumors and leishmania parasitemia by >90% in the liver, spleen, and bone marrow. It can be useful for genital herpes, psoriasis, human papiloma virus, seborrheic dermatitis, aphthous stomatitis, xerosis, lichen planus, frostbite, burn, wound healing and inflammation. Even though clinical effectiveness of oral and topical aloe vera is not sufficiently explored as yet. [35]

However, studies supported the idea Aloe could have a role against cancer and can modulate the immune system [36].

Fig.2.5

Chemical structure of AE (1,8-dihydroxy-3-hydroxymethyl-9,10-anthracenedione; A) and aloin (10–1',5'-anhydroglucosyl-aloe-emodin-9-anthrone; B).



Pecere, T. et al. Cancer Res 2000; 60:2800-2804

IL10

Human IL-10 (hIL-10) is a cytokine that modulates diverse immune responses. Although in mice IL-10 is predominantly a Th-2-derived cytokine, in man it is produced by both Th-2 and Th-1 cells and has antiinflammatory properties. It reduces MHC and co-stimulatory molecule expression, reduces pro-inflammatory cytokine release and increases IL-lRa expression. [37]

Immunomodulatory effects of IL-10 on APCs [38] were described soon after the discovery of this cytokine in the late 1980s [39]. Early work had shown that IL-10 prevented the generation of human DCs from CD14+ precursor cells *in vitro*, if applied together with GM-CSF and IL-4 or IL-13 from beginning of cell culture.[40]

G-CSF

Granulocyte colony-stimulating factor (G-CSF) is usually administered to patients in malignant and non-malignant disease to mobilize hematopoietic stem cell from the bone marrow into the blood stream [41] [42]. T cells from blood or spleen cells of G-CSF treated mice show decreased ability to induce GVHD in allogenic recipients [43] [44]. G-CSF induces mobilization of DC2 in humans [45] and promotes the generation of human T regulatory type 1 cells and the generation of regulatory DC [46].

Treatment with G-CSF protects NOD mice from developing spontaneous diabetes. G-CSF triggered marked recruitment of dendritic cells (DCs), particularly immature CD11c (lo) B220 (+) plasmacytoid DCs, with reduced costimulatory signal expression and higher interferon-alpha but lower interleukin-12p70 release capacity than DCs in excipient-treated mice. G-CSF recipients display accumulation of functional CD4 (+) CD25 (+) regulatory T-cells that produce transforming growth factor-beta1 (TGF-beta1) and actively suppress diabetes transfer by diabetogenic effector cells in secondary NOD-SCID recipients. G-CSF ability to promote key tolerogenic interactions between DCs and regulatory T-cells is demonstrated by enhanced recruitment of TGF-beta1-expressing CD4 (+) CD25 (+) cells after adoptive transfer of DCs isolated from G-CSF-relative to vehicle-treated mice into naive NOD recipients. Results suggest that G-CSF, a promoter of tolerogenic DCs, may be evaluated for the treatment of human Type 1 Diabetes, possibly in association with direct inhibitors of T-cell activation. Scientists

also provide a rationale for a protective role of the endogenous G-CSF produced during infections in early diabetes. [47]

Insulin Peptides

Immunization with insulin, inactive insulin, or a peptide from the B chain of insulin (B9-23) has all been able to prevent or delay the onset of Type 1 Diabetes (T1D) in the NOD mouse model. [48] [49]. The protective effect is maintained despite the route of administration whether oral, intranasal, intravenous, or subcutaneous. [50] [51] Many possible mechanisms of action have been proposed including induction of tolerance to insulin, restoration of defective T cell function, and alteration of lymphocyte function by signaling through the insulin receptor. However, the most popular theory is that insulin treatment induces Th2 cells, which correct the imbalance in the immune system that promotes autoimmunity.

IGRP *peptides*

Because of the crucial role that CD8+ T cells and their class I MHC-restricted targets play in disease progression to T1D, the knowledge of the antigenic peptides recognized by pathogenic CD8+ T cells is extremely important. Efforts to study the peptide targets of CD8+ T cells have resulted in the identification of epitopes derived from several antigens, including the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). Early work showed that the TCR -chain expressed by a high frequency of CD8+ T cells infiltrating the islets of NOD mice was shared with the pathogenic 8.3 CD8+ T cell clone. 8.3-like CD8+T cells are specific for an H2Kd-restricted epitope of islet- specific glucose-6-phosphatase catalytic subunit-related protein (IGRP206 –214) and are detected with H2Kd (Kd) tetramers complexed with NRP mimotopes such as the high avidity NRP-A7 and NRP-V7 analogues (Wong et al., 2006). IGRP is an islet-specific protein expressed in pancreatic cells, and shares 50% identity with the catalytic subunit of the liver enzyme glucose-6-phosphatase (Martin et al., 2001). IGRP206 –214 differs from the homologous residues of murine glucose-6-phosphatase (KYCLITIFL) at six of nine positions. Despite its homology to glucose - 6 - phosphatase, no catalytic activity has been demonstrated for IGRP (Di Lorenzo et al., 2003). It is predicted to be an ER-resident protein that spans the membrane nine times (Arden et al., 1999). The human IGRP gene maps to a diabetes susceptibility locus on chromosome 2, IDDM7

(Pociot et al., 2002).

DIAPEP277

Antigen specific suppression using Diapep 277 is a new and interesting preventive approach to stop progression towards beta cell destruction. The strategy underlying the Diapep 277 approach in LADA is that of antigen specific suppression. Hsp60- specific autoimmunity precedes the onset of clinical hyperglycemia in the NOD mouse and the low-dose streptozotocin (STZ) model. The diabetogenic T-cells recognize an hsp60 peptide epitope corresponding to the positions 437-460, called p277. This peptide contains two cystein residues that are highly sensitive to oxidation. The peptide is fully cross-reactive with the original p277 and has the same biological activity. Phase II trials have begun in recent-onset type 1 patients (children and adults) and early data has shown that stimulated C-peptide can be preserved at 1 year after diagnosis compared to control patients.

Proinsulin peptide

Insulin and its precursor molecule, proinsulin, are known islet cell-specific autoantigens involved in T1D. Much evidence suggests a role for these autoantigens in the pathogenesis of T1D in humans, NOD mice and Bio-Breeding rats. Delovitch et al., in 2001 demonstrate that whereas T cells from peripheral lymphoid tissues (e.g., spleen) of perinatal NOD mice do not react against insulin or insulin B chain 9 –23, these T cells do respond strongly to proinsulin with the same kinetics as the GAD65-specific T cell response. The decamer peptide p24 –33 of mouse pro-insulin was found to be an immunodominant epitope in these NOD mouse T cell responses.

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is a neuropeptide released by both innervation and immune cells, particularly T helper (Th) 2 cells, in response to Ag stimulation and under inflammatory autoimmune conditions. VIP elicits a broad spectrum of biological functions, including immunomodulation, predominantly acting as a potent anti-inflammatory factor and a suppressive agent for Th1 responses [52] [53]. Therefore, VIP has emerged as a promising therapeutic factor for the treatment of autoimmune and

inflammatory diseases, including rheumatoid arthritis (RA), ulcerative colitis, uveoretinitis, and experimental autoimmune encephalomyelitis (EAE) [54].

The neuropeptide vasoactive intestinal peptide (VIP) is a potent immunosuppressive agent, affecting both innate and adaptive immunity. Recently, scientists have shown that VIP affects bone marrow-derived DCs (BMDCs) differently, depending on the DC maturation state. Immature DCs treated with VIP up-regulate CD86 expression, stimulate T cell proliferation and promote Th2-type responses while inhibiting the Th1-type proinflammatory response. In contrast, VIP down-regulated CD80 and CD86 expression of LPS-matured DCs and inhibit their capacity to activate allogeneic or syngeneic T cells in vivo and in vitro [Delgado, M., A. Reduta, V. Sharma, D. Ganea. 2004. VIP/PACAP oppositely affect immature and mature dendritic cell expression of CD80/CD86 and the stimulatory activity for $CD4^+$ T cells. J. Leukocyte Biol. 75: 1122-1130].

In a study, [55] scientists reported that VIP induced CD11clowCD45Rbhigh DCs that do not up-regulate CD40, CD80, or CD86, and secrete high amounts of IL-10 upon LPS stimulation. The VIP/PACAP-generated DCs induce Ag-specific Tr1-like Tregs in vitro and in vivo, and these Tregs are capable of transferring suppression to naive hosts.

Statins

Statins inhibit an enzyme crucial to cellular cholesterol synthesis, 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase. The resulting decrease in intracellular cholesterol leads to a compensatory increase in cholesterol uptake via low density lipoprotein (LDL) receptors and concomitant decrease in plasma cholesterol.
Kwak et al. demonstrated that statins inhibit the interferon-γ (IFN-γ)–induced expression of class II major histocompatibility complexes (MHCII) on antigen-presenting cells (APC), and thus identified a new mechanism by which statins may modulate immune responses. They were able to reverse the downregulation of MHCII by adding Lmevalonate (the product of uninhibited HMG-CoA reductase) to cells.

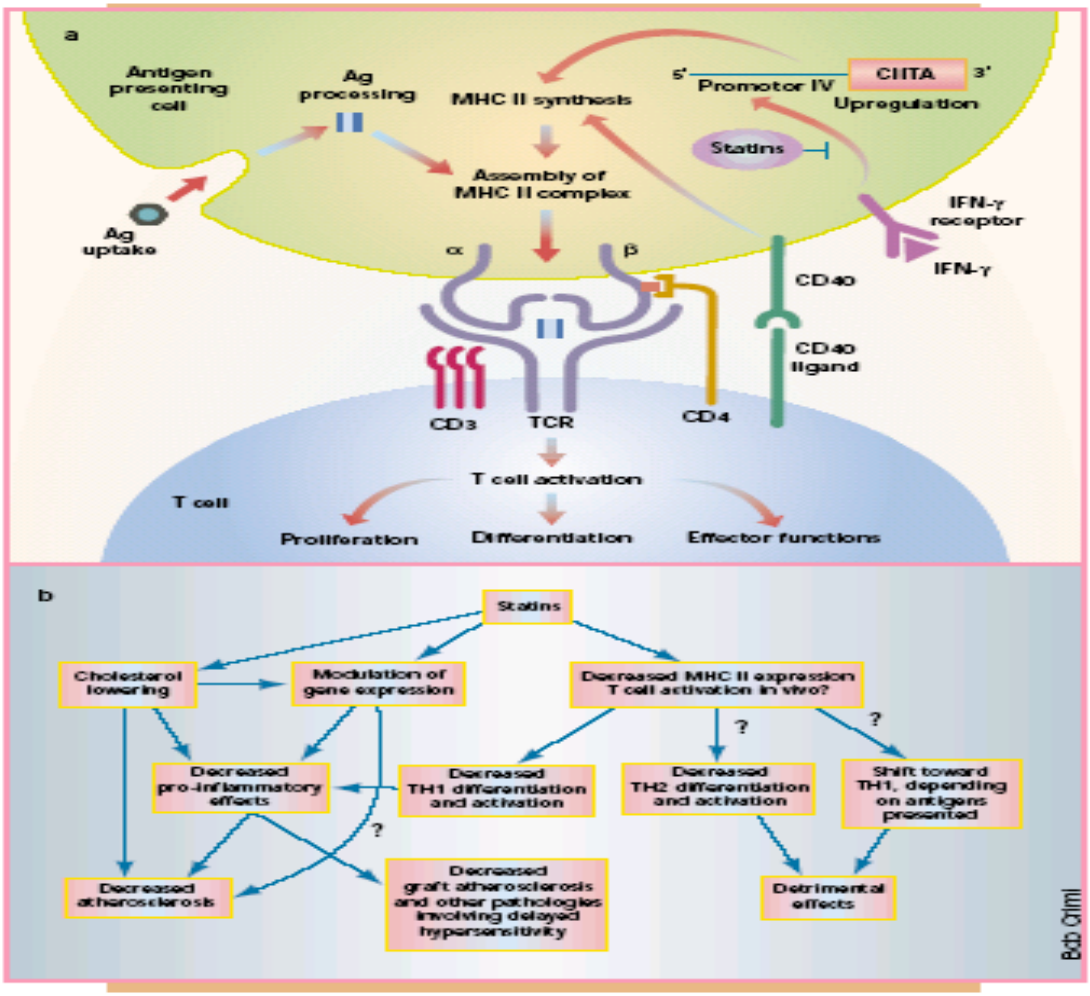


Fig. 2.6 Statin modulation of the immune response. ***a***,Extracellular antigens taken up by APC are processed into peptides fitting into the peptide-binding cleft formed by the α- and β-chains of MHCII, assembled with MHC into a heterotrimeric complex, and transported to the cell surface. IFN-γ, the major macrophage-activating cytokine, induces MHC expression by macrophages and other APCs. It does this by activating the class II transactivator (CIITA) through a transcriptional regulatory element known as promoter IV. Kwak *et al*.3 demonstrate that statins inhibit this pathway and lead to reduced CIITA and subsequently reduced MHC expression. However, MHCII expression on macrophages can also be induced by direct cell to cell contact, such as through CD40 CD40 ligand interactions. APC that constitutively express MHCII are not affected by statin treatment. Tcell activation results from the joint interaction of the T-cell receptor (TCR) with MHCII and the antigenic peptide. Interaction of the T-cell co-receptor CD4 with MHCII is also required for T-cell activation. CD3 and other proteins contribute to transduction of the TCR signal. ***b***, Statins reduce atherosclerosis by lowering plasma cholesterol and decreasing inflammatory processes. The ability of statins to downregulate expression of MHCII may lead to decreased TH1 differentiation and activation *in vivo* and thus inhibit the release of pro-inflammatory cytokines. This suggests that statins may be beneficial in reducing graft atherosclerosis and treating other chronic inflammatory conditions. The immunomodulatory effects of statins could also be detrimental, however, because a reduced MHCII expression may inhibit anti-inflammatory effects resulting from TH2 activation, or lead to a shift towards a TH1-type immune response, depending on the antigens presented.

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Immunosuppressive Drugs and Rapamycin (FDA web site: revised 6/1/2005)

Cyclosporine

Cylcosporine is an oral and parenteral immunosuppressive agent. Cyclosporine is a cyclic polypeptide consisting of 11 amino acids, and it is produced by the fungus *Beauveria nivea*. Cyclosporine is used to prevent allograft rejection; the drug has revolutionized transplantation by improving transplant survival, reducing hospitalization, and reducing patient morbidity. Cyclosporine is also effective in various autoimmune conditions such as uveitis, psoriasis, Type I Diabetes, rheumatoid arthritis, inflammatory bowel disease, and certain nephropathies. While many of these uses are still experimental, cyclosporine is FDA-approved for the treatment of refractory rheumatoid arthritis and psoriasis. Adverse reactions of cyclosporine treatment and the relapse of the conditions upon discontinuation of therapy limits cyclosporine use in autoimmune diseases. The FDA originally approved cyclosporin in 1983. Cyclosporine induces immunosuppression by inhibiting the first phase of T-cell activation. The first phase of T-cell activation causes transcriptional activation of immediate and early gene products (e.g., interleukins - IL-2, IL-3, and IL-4, tumor necrosis factor alpha and interferon gamma) that allow T-cells to progress from the G_0 to G_1 phases.

Cyclosporine binds to an immunophilin termed cyclophilin. Immunophilins (e.g., cyclophilin and FK binding proteins) are immunosuppressant-binding proteins that are distributed in all cellular compartments and play an important role in protein regulation. The cyclosporine-cyclophilin complex then binds to and inhibits the calcium-calmodulin activated phosphatase calcineurin. The calcineurin enzyme catalyzes critical dephosphorylation reactions necessary for early lymphokine gene transcription, and subsequent early activation of T-cells. Calcineurin inhibition results in blockade of signal transduction of the nuclear factor of activated T-cells (NF-AT). The blockade of signal transduction results in failure to activate NF-AT regulated genes. NF-AT activated genes include those required for B-cell activation including interleukin (IL)-4 and CD40 ligand, and those required for T-cell activation including IL-2 and interferon gamma. Cyclosporine does not affect suppressor T-cells or T-cell independent, antibody-mediated immunity. Cyclosporine is administered orally, intravenously or ophthalmically. Cyclosporine is extremely hydrophobic. Because of the unpredictability of oral absorption, it is difficult to convert between oral and parenteral doses. First-pass

metabolism, mode of administration, formulation, and drug interactions all affect cyclosporine absorption. Following ophthalmic administration, blood concentrations of cyclosporine were below the quantization limit of 0.1 ng/ml.

Cyclosporine is a substrate and inhibitor of P-glycoprotein, which is an energy-dependent drug-efflux pump located in the intestinal epithelium and the blood brain barrier. There appears to be overlap between inhibitors and/or substrates of cytochrome P450 (CYP) 3A4 and P-glycoprotein. The P-glycoprotein efflux of cyclosporine from intestinal cells back into the gut lumen allows for CYP3A4 metabolism prior to absorption, thus limiting cyclosporine availability. When cyclosporine is administered with inhibitors of both CYP3A4 and P-glycoprotein (e.g., diltiazem, erythromycin, or ketoconazole) increased cyclosporine bioavailability leads to increased cyclosporine concentrations. The absolute bioavailability of cyclosporine administered as cyclosporine (Nonmodified) is highly variable; the bioavailability is estimated to be < 10% in liver transplant patients and can range from 7.4—92.2% in renal transplant patients.

The oral absorption of cyclosporine (Nonmodified) is limited by the relatively narrow window for absorption in the proximal small intestine, the potential for pre-systemic metabolism in the gut, reliance on pancreatic enzymes and bile in the gut to achieve adequate dispersion, and the variable affects of food. The time to maximum concentration varies widely, both within-patient and between patients. In general, absorption of cyclosporine (Nonmodified) is not affected by a light meal, but may be increased with a high-fat meal or grapefruit juice. The status of the GI tract may decrease absorption as well; conditions associated with decreased absorption include diarrhea, decreased small bowel length, and concurrent administration of drugs that increase GI motility. Due to the large differences in bioavailability in cyclosporine (Nonmodified), patients titrated to the same trough levels could be exposed to different amounts of the cyclosporine as measured by area under the time-concentration curve (AUC).

The physical properties of the cyclosporine (Modified) formulation (i.e., microemulsion) make the absorption of cyclosporine less dependent on bile, food, and other factors that assist dispersion and subsequent absorption of lipophillic substances

from the GI tract. Agents which influence pre-systemic metabolism (e.g., grapefruit juice) may still influence cyclosporine (Modified) absorption. The absolute bioavailability of cyclosporine (Modified) has not been determined in adults. Following oral administration, the Tmax for cyclosporine (Modified) ranges from 1.5—2 hours. Food decreases the absorption of cyclosporine (Modified). As compared to cyclosporine (Nonmodified), the AUC of cyclosporine (Modified) is linear within the therapeutic dosage range. Intersubject variability of cyclosporine exposure (AUC) ranges from about 20—50% when administered as cyclosporine (Modified) or cyclosporine (Nonmodified). There is less intrasubject variation in AUC with cyclosporine (Modified), despite random changes in food intake, bile secretion, or time of concentration measurement. Intrasubject variability of AUC in renal transplant patients is 9—21% for cyclosporine (Modified) and 19—26% for cyclosporine (Nonmodified). Cyclosporine is distributed widely throughout the body, crosses the placenta, and is found in breast milk. Preferential uptake of cyclosporine occurs in the liver, pancreas, and adipose tissue, while it penetrates the CNS poorly.

In blood, the distribution of cyclosporine is concentration dependent; as the hemocrit rises, the cyclosporine concentration in plasma decreases. Approximately 22—47% of cyclosporine is found in plasma, 4—9% in lymphocytes, 5—12% in granulocytes, and 41—58% in erythrocytes. At high drug concentrations the binding to lymphocytes and erythrocytes becomes saturated. In plasma, cyclosporine is approximately 90% bound to lipoproteins. In addition, the binding of cyclosporine to erythrocytes and lipoproteins is temperature dependent. As the temperature increases, binding to lipoproteins increases; however, binding to erythrocytes increases as the temperature decreases.

Other medications that may affect the binding of cyclosporine to lipoproteins may modify the clinical response to cyclosporine. Cyclosporine is metabolized extensively by the CYP3A enzyme system in the liver and to a lesser extent in the gastrointestinal tract and kidney. Agents that affect the CYP3A system may significantly alter the metabolism of cyclosporine. At least 25 metabolites of cyclosporine have been identified, some of which are biologically active. Although most cyclosporine metabolites show only 10—20% of the immunosuppressive activity of the parent drug, they do contribute to toxicity. The major metabolites of cyclosporine are M1, M9, and M4N, resulting from oxidation at the 1-beta, 9-gamma, and 4-N-desmethylated

positions. The percentage of dose present as M1, M9, or M4N is similar when either cyclosporine (Modified) or cyclosporine (Nonmodified) is administered. At steady state, concentrations and AUCs of cyclosporine metabolites may exceed that of cyclosporine. Mean AUCs for blood concentrations of these metabolites are 70%, 21%, and 7.5% respectively, of blood cyclosporine concentrations. The elimination half-life of cyclosporine is highly variable. In patients with normal hepatic function the average half-life ranges from 16—27 hours, but can vary from 10—40 hours. Elimination of cyclosporine and its metabolites is principally through the bile and feces. Cyclosporine undergoes enterohepatic recycling. Only 6% of the cyclosporine dose is excreted renally, of which 0.1% is excreted as unchanged cyclosporine. Although cyclosporine blood levels are widely used to assist dosing, accurate interpretation is hampered by variation in absorption, variation in protein binding, sampling error, type of assay, cross-reactivity of metabolites, enterohepatic recycling of drug and drug interactions. In patients with hepatic disease or disorders of biliary excretion, the half-life of cyclosporine could be prolonged. Neither renal failure nor dialysis alters cyclosporine clearance significantly. In pediatric patients, cyclosporine (Modified) also demonstrates an increased bioavailability as compared to cyclosporine (Nonmodified). In 7 liver transplant patients age 1.4—10 years, the absolute bioavailability of cyclosporine (Modified) was 43% (range 30—68%) compare to 28% (range 17—42%) for cyclosporine (Nonmodified). Data derived from elderly patients and young adults show no significant differences in pharmacokinetic parameters. [56]

Tacrolimus

Tacrolimus is a macrolide immunosuppressive agent that is derived from the fungus *Streptomyces tsukubaensis,* originally found in a soil sample taken from the base of Mt. Tsukuba in Japan. Tacrolimus has been studied in patients receiving heart, kidney, liver, lung, pancreas, small bowel or bone marrow transplants. Tacrolimus has been shown to be effective in graft rejection prophylaxis and in the management of acute and steroid- or cyclosporine-resistant transplant rejection. Tacrolimus is considered an alternative to cyclosporine immunosuppression. Tacrolimus has been shown to be 10—100 times more potent than cyclosporine. A review of clinical trials in liver and kidney transplantation suggests comparable patient and graft survival rates between patients

receiving cyclosporine and those receiving tacrolimus, and a consistent statistically significant advantage for tacrolimus with respect to acute rejection rate. Tacrolimus has also been used for the treatment of refractory or chronic graft rejection. Tacrolimus was approved by the FDA for the prevention of liver transplant rejection in April 1994. A topical formulation of tacrolimus for the treatment of atopic dermatitis was FDA-approved for use in adults and children in December 2000. On February 15, 2005, the FDA announced the addition of a Black Box warning to the professional label for tacrolimus topical, instructing prescribers to use only after failure of other eczema treatments due to a possible increased cancer risk.

Tacrolimus induces immunosuppression by inhibiting the first phase of T-cell activation. The first phase of T-cell activation causes transcriptional activation of immediate and early proteins (e.g., interleukin (IL)-2, IL-3, IL-4, granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon gamma) that allow T-cells to progress from the G_0- to G_1-phase. Tacrolimus binds to an immunophilin termed FK binding protein (FKBP), specifically FKBP12. Immunophilins (cyclophilin and FK binding proteins) are immunosuppressant-binding proteins that are distributed in all cellular compartments and play an important role in protein activation. The tacrolimus-FK binding protein complex binds to and inhibits the phosphatase activity of calcineurin. The calcineurin enzyme catalyzes critical dephosphorylation reactions necessary for early lymphokine gene transcription. Calcineurin inhibition results in blockade of signal transduction by the cytosol component of the nuclear factor of activated T-cells (NF-AT), which results in a failure to activate NF-AT regulated genes. NF-AT activated genes include those required for B-cell activation (e.g., IL-4 and CD40 ligand) and those required for T-cell activation (e.g., IL-2, TNF-alpha, and interferon gamma). Reduced circulating levels of T-cell activators result in inhibition of T-cell proliferative responses to antigens and mitogens including mixed lymphocyte reactivity and cytotoxic T-cell generation. Compared to cyclosporine, tacrolimus is about 100-times more potent in inhibiting T-cell proliferative responses.

In atopic dermatitis, topical tacrolimus acts to inhibit inflammation primarily by inhibiting T-cells. Tacrolimus may also bind to cell surface steroid receptors, inhibit the release of mast cell mediators, down-regulate IL-8 receptors, and decrease intracellular

adhesion molecule-1 and E-selectin lesional blood vessel expression. These activities lead to decreased antigen recognition and down-regulation of the entire inflammatory cascade leading to a clinical response. Topical tacrolimus does not inhibit collagen synthesis and, therefore, does not cause skin atrophy as seen with corticosteroid therapy. Tacrolimus is administered orally, parenterally, and topically. Following oral administration, tacrolimus absorption is poor and variable. The absolute bioavailability of tacrolimus varies from 17—22%. The presence of food can alter both the rate and extent of tacrolimus absorption. In healthy volunteers, the mean AUC and Cmax are decreased 37% and 77%, respectively, following a high-fat meal. A high carbohydrate meal also decreases the mean AUC and Cmax 28% and 65%, respectively. The time of the meal in relation to the dose also affects tacrolimus bioavailability in healthy volunteers. Tacrolimus is a substrate and inhibitor of P-glycoprotein, which is an energy-dependent drug-efflux pump located in intestinal epithelium and the blood brain barrier. There appears to be overlap between inhibitors and/or substrates of cytochrome P450 (CYP) 3A4 and P-glycoprotein. The P-glycoprotein efflux of tacrolimus from intestinal cells back into the gut lumen allows for CYP3A4 metabolism prior to absorption, thus limiting tacrolimus availability. When tacrolimus is administered with inhibitors of both CYP3A4 and P-glycoprotein (e.g., diltiazem, erythromycin, or ketoconazole) increased tacrolimus bioavailability leads to increased concentrations. The absolute bioavailability of tacrolimus applied topically is unknown. Following topical administration of single or multiple doses of the 0.1% ointment to adults, peak tacrolimus blood concentrations ranged from undetectable to 20 ng/ml. Most patients had peak blood concentrations < 5 ng/ml. In pediatric patients, application of the 0.1% ointment resulted in peak blood concentrations below 1.6 ng/ml. There is no evidence that tacrolimus accumulates systemically following topical administration. The lowest tacrolimus blood concentration that may elicit systemic effects is unknown.

Due to high lipophilicity, tissue distribution of tacrolimus following oral or parenteral therapy is extensive. Tacrolimus crosses the placenta creating fetal cord plasma concentrations that are 35% of the maternal plasma concentration. The drug is also excreted in breast milk with concentrations similar to those in plasma. Protein binding is approximately 99%. Tacrolimus is mainly bound to albumin and alpha$_1$-acid glycoprotein. Erythrocytes bind 75—80% of the drug resulting in whole blood concentrations that are 10 to 30 times higher than plasma concentrations. The

distribution of tacrolimus between erythrocytes and plasma is dependent upon tacrolimus concentration, hematocrit, and temperature.

Metabolism of tacrolimus is mainly by demethylation and hydroxylation via the hepatic cytochrome P450 enzyme 3A4. The formation of 8 possible metabolites has been proposed. The major metabolite identified *in vitro* is 13-demethyl tacrolimus, which *in vitro* has been reported to have the same activity as tacrolimus. The elimination half-life of tacrolimus in liver transplant patients is about 12 hours. Less than 1% of the dose is excreted unchanged in the urine.

Systemic tacrolimus administration to patients with hepatic dysfunction is associated with increased whole blood concentrations, prolonged half-life, and reduced clearance. The half-life of tacrolimus in patients with mild hepatic impairment ranges from 28—141 hours. In a study of 6 patients with severe hepatic dysfunction (mean Pugh score > 10), the mean tacrolimus clearance was substantially lower, irrespective of the route of administration. Pediatric transplant patients have a greater clearance of tacrolimus as compared to adults resulting in the need for higher doses relative to body weight in pediatric patients to achieve appropriate whole blood concentrations. Black patients may also require increased doses to achieve therapeutic whole blood levels. [57] [58]

Numerous trials have studied the use of tacrolimus in patients receiving pancreas transplants alone, simultaneous pancreas/kidney transplants, and pancreas transplants after kidney transplants. These studies have shown that tacrolimus is effective both as part of primary immunosuppressive therapy and in patients who do not tolerate cyclosporine or develop refractory rejection. For rejection prophylaxis dosages of 4—10 mg/day PO in divided doses have been used to maintain tacrolimus whole blood concentrations at an average of 12 ng/ml. In patients who switched to tacrolimus for rejection or rescue therapy the median dose was 10mg/day PO in divided doses with a median whole blood concentration of 11 ng/ml.

In some patients, immunosuppression with daclizumab, sirolimus, and low-dose

tacrolimus 1 mg PO twice daily was begun immediately prior to islet transplantation. The tacrolimus dose was adjusted to maintain a whole blood concentration at 12 hours of 3—6 ng/ml. No corticosteroids were used for immunosuppression. All patients remained free of the need for exogenous insulin and no episodes of acute rejection have been observed with median follow-up of 11.9 months. [59] [60]

Mycophenolic acid (MPA)

Mycophenolic acid (MPA) is an immunosuppressive drug. Mycophenolic acid was isolated in 1898 from a *Penicillium* culture; however, it was not studied as an immunosuppressive agent until the 1970s. Mycophenolate mofetil (MMF, RS-61443), a prodrug for MPA, and mycophenolate sodium, a delayed-release tablet of MPA are used in conjunction with cyclosporine and corticosteroids for the prevention of rejection in patients with a renal allograft. Twice daily oral administration of mycophenolate sodium 720 mg and MMF 1000 mg were found to be therapeutically equivalent. Similar percentages of patients that received MMF or mycophenolate sodium developed delayed graft function and drug-related adverse events. Also, the percentage of *de novo* and maintenance renal transplant patients that developed a fungal or viral infection was similar for patients that received MMF as compared with patients that received mycophenolate sodium. In one study, the rate of rejection in renal allograft recipients during therapy with MMF was lower than during therapy with azathioprine both given in combination with cyclosporine and corticosteroids. However, use of MMF for treatment of acute renal allograft rejection has shown no statistically significant benefit as compared with standard therapy. Nephrotoxicity appears to be less with MMF than with cyclosporine, and lower doses of cyclosporine can be used when mycophenolate therapy is added, thereby reducing the risk of cyclosporine-induced nephrotoxicity. Mycophenolic acid administration has been successful in patients with rheumatoid arthritis, including patients resistant to methotrexate. Mycophenolate mofetil was approved by the FDA on May 3, 1995. Intravenous and oral suspensions were approved in late 1998. Mycophenolate sodium delayed-release tablets were FDA-approved in February 2004.

Mycophenolic acid (MPA) inhibits lymphocyte purine synthesis by reversibly and noncompetitively inhibiting the enzyme, inosine monophosphate dehydrogenase (IMPDH). IMPDH is an important enzyme in the *de novo* synthesis of purines and is the

rate-limiting step in converting inosine monophosphate (IMP) to guanosine monophosphate (GMP), an important intermediate in the synthesis of lymphocyte DNA, RNA, proteins, and glycoproteins. T- and B-lymphocytes, unlike other cells, cannot synthesize GMP sufficiently through the salvage pathway. The cytostatic effect on lymphocytes is thus, greater than the effect on other cell types. Mycophenolic acid's inhibition of IMPDH prevents the formation of GMP, which decreases guanosine triphosphate (GTP) and deoxy-GTP that are necessary substrates for DNA, RNA, and protein synthesis. Subsequently, MPA inhibits lymphocyte proliferation and the formation of adhesion molecules in response to antigenic or mitogenic stimulation. Adhesion molecules are usually present on the surface of activated T cells.

In comparison with other immunosuppressive agents, MPA has several potential advantages. First, *in vitro* studies show that MPA blocks the secondary antibody responses mediated by memory B cells. Secondly, in contrast to azathioprine and methotrexate, which have a nonselective effect on DNA synthesis in all cell types, MPA has a selective effect on lymphocyte proliferation. Next, MPA is not incorporated into DNA and does not cause chromosome breaks. Lastly, MPA inhibits the proliferation of human B lymphocyte cell lines transformed by the Epstein-Barr virus (EBV); cyclosporine inhibits T-cell mediated surveillance of EBV-transformed B lymphocytes but does not block B-lymphocyte replication.

The overall effects of MPA as revealed by clinical studies show that MPA is at least as potent as azathioprine when used in combination with cyclosporine and corticosteroids for immunosuppression. Mycophenolate mofetil (MMF) is administered orally or intravenously whereas mycophenolate sodium is only administered orally. Following oral administration, MMF is rapidly and extensively absorbed. Following both intravenous and oral administration, MMF is immediately hydrolyzed to form free mycophenolic acid (MPA), the active compound. During intravenous infusions the parent drug MMF can be measured; however, 5 minutes after the infusion is stopped the MMF concentration is not detectable. In healthy volunteers, peak plasma concentrations of MPA occur within 36—42 minutes. In contrast, the peak plasma concentrations of MPA are attained between 1.5 and 2.75 hours after administration of mycophenolate sodium, which is highly soluble in the neutral pH conditions of the intestine.

Mycophenolate sodium is insoluble in acidic milieu (pH <5). Although the systemic exposure of MPA is similar when mycophenolate is taken with or without food, peak concentrations are decreased by 33—40% when administered with food. Similar systemic MPA exposure is provided by oral administration of mycophenolate sodium 720 mg and MMF 1000 mg, which contain near equimolar MPA content. There is little correlation with area under the concentration curve (AUC) and dose.

Greater than 98% of MPA is bound to albumin. Most of the free MPA is conjugated in the liver by glucuronyl transferase to form to the inactive metabolite, mycophenolic acid glucuronide (MPAG). The minor acyl glucuronide metabolite has similar pharmacologic activity as compared with MPA. At steady state, the AUC ratio of MPA:MPAG:acyl glucuronide is approximately 1:24:0.28. MPAG is excreted into the bile and can be deconjugated by gut flora. The resulting MPA can be reabsorbed, which can result in a second peak of MPA plasma concentration 6—12 hours after the initial dose. The mean elimination half-life of MPA is approximately 17.9 hours following oral administration of the capsules, 13—17 hours following oral administration of the delayed-release tablets, and approximately 16.6 hours following intravenous administration. Therapeutic drug monitoring (TDM) for mycophenolate is not currently recommended, as there is an absence of needed data. Data suggest the possible utility of TDM.

Greater exposure to MPAG, which can be a source of MPA, is likely in patients with renal impairment. The systemic exposure of MPAG and of free MPA is increased over the first 12 hours after dosing in patients with renal dysfunction. Additionally, the MPA free fraction is increased. Data suggest that MPAG may compete with MPA for albumin binding sites. Hemodialysis or peritoneal dialysis does not significantly remove either MPA or MPAG. Pharmacokinetic data from patients with hepatic disease are limited. Systemic exposure to MPA after 450 mg/m^2 of mycophenolate sodium was 18% higher in children 5—16 years of age as compared with adults. [61] [62] [63] [64] [65] [66] [67]

Glucocorticoids

Glucocorticoids are important adjuncts to immunosuppressive therapy. Of all the agents employed, prednisone has effects that are easiest to assess, and in large doses it is

usually effective for the reversal of rejection. In general, 200 to 300 mg prednisone is given immediately prior to or at the time of transplantation, and the dosage is reduced to 30 mg within a week. The side effects of the glucocorticoids, particularly impairment of wound healing and predisposition to infection, make it desirable to taper the dose as rapidly as possible in the immediate postoperative period. Customarily, methylprednisolone, 0.5 to 1.0 g intravenously, is administered immediately upon diagnosis of beginning rejection and continued once daily for 3 days. When the drug is effective, the results are usually apparent within 96 h. Such "pulse" doses are not effective in chronic rejection. Most patients whose renal function is stable after 6 months or a year do not require large doses of prednisone; maintenance doses of 10 to 15 mg/d are the rule. Many patients tolerate an alternate-day course of steroids without an increased risk of rejection. A major effect of steroids is on the monocyte-macrophage system, preventing the release of interleukin (IL)-6 and IL-1. Lymphopenia after large doses of glucocorticoids is primarily due to sequestration of recirculating blood lymphocytes to lymphoid tissue.

Azathioprine

Azathioprine, an analogue of mercaptopurine, was for two decades the keystone to immunosuppressive therapy in humans. This agent can inhibit synthesis of DNA, RNA, or both. Because cell division and proliferation are a necessary part of the immune response to antigenic stimulation, suppression by this agent may be mediated by the inhibition of mitosis of immunologically competent lymphoid cells, interfering with synthesis of DNA. Alternatively, immunosuppression may be brought about by blocking the synthesis of RNA (possibly messenger RNA), inhibiting processing of antigens prior to lymphocyte stimulation. Therapy with azathioprine in doses of 1.5 to 2.0 mg/kg per day is generally added to cyclosporine as a means of decreasing the requirements for the latter. Because azathioprine is rapidly metabolized by the liver, its dosage need not be varied directly in relation to renal function, even though renal failure results in retention of the metabolites of azathioprine. Reduction in dosage is required because of leukopenia and occasionally thrombocytopenia. Excessive amounts of azathioprine may also cause jaundice, anemia, and alopecia.

Sirolimus

Sirolimus is an immunosuppressive agent. Sirolimus is a macrocyclic lactone produced by *Streptomyces hygroscopicus*. Sirolimus is a white to off-white powder and is insoluble in water, but freely soluble in benzyl alcohol, chloroform, acetone, and acetonitrile. Sirolimus inhibits T lymphocyte activation and proliferation that occurs in response to antigenic and cytokine (Interleukin [IL]-2, IL-4, and IL-15) stimulation by a mechanism that is distinct from that of other immunosuppressants. Sirolimus also inhibits antibody production. In cells, sirolimus binds to the immunophilin, FK Binding Protein-12 (FKBP-12), to generate an immunosuppressive complex. The sirolimus: FKBP-12 complex has no effect on calcineurin activity. This complex binds to and inhibits the activation of the mammalian Target Of Rapamycin (mTOR), a key regulatory kinase. This inhibition suppresses cytokine-driven T-cell proliferation, inhibiting the progression from the G1 to the S phase of the cell cycle. Studies in experimental models show that sirolimus prolongs allograft (kidney, heart, skin, islet, small bowel, pancreatic-duodenal, and bone marrow) survival in mice, rats, pigs, and/or primates. Sirolimus reverses acute rejection of heart and kidney allografts in rats and prolongs the graft survival in presensitized rats. In some studies, the immunosuppressive effect of sirolimus lasts up to 6 months after discontinuation of therapy. This tolerization effect is alloantigen specific. In rodent models of autoimmune disease, sirolimus suppresses immune-mediated events associated with systemic lupus erythematosus, collagen-induced arthritis, Type I Diabetes, autoimmune myocarditis, experimental allergic encephalomyelitis, graft-versus-host disease, and autoimmune uveoretinitis.

Sirolimus is a substrate for both cytochrome P450 IIIA4 (CYP3A4) and P-glycoprotein (P-gp). Sirolimus is extensively metabolized by the CYP3A4 isozyme in the intestinal wall and liver and undergoes counter-transport from enterocytes of the small intestine into the gut lumen by the P-gp drug efflux pump. Sirolimus is potentially recycled between enterocytes and the gut lumen to allow continued metabolism by CYP3A4. Therefore, absorption and subsequent elimination of systemically absorbed sirolimus may be influenced by drugs that affect these proteins. Inhibitors of CYP3A4 and P-gp increase sirolimus concentrations. Inducers of CYP3A4 and P-gp decrease sirolimus concentrations.

Sirolimus is extensively metabolized by O-demethylation and/or hydroxylation. Seven major metabolites, including hydroxy, demethyl, and hydroxydemethyl, are identifiable in whole blood. Some of these metabolites are also detectable in plasma, fecal, and urine samples. Glucuronide and sulfate conjugates are not present in any of the biologic matrices. Sirolimus is the major component in human whole blood and contributes to more than 90% of the immunosuppressive activity.

Sirolimus is indicated for the prophylaxis of organ rejection in patients aged 13 years or older receiving renal transplants. It is recommended that Rapamune be used initially in a regimen with cyclosporine and corticosteroids. In patients at low to moderate immunologic risk, cyclosporine should be withdrawn 2 to 4 months after transplantation and the Rapamune dose should be increased to reach recommended blood concentrations.
The safety and efficacy of cyclosporine withdrawal in high-risk patients have not been adequately studied and it is therefore not recommended. This includes patients with Banff grade III acute rejection or vascular rejection prior to cyclosporine withdrawal, those who are dialysis-dependent, or with serum creatinine > 4.5 mg/dL, black patients, re-transplants, multi-organ transplants, patients with high panel of reactive antibodies.

The safety and efficacy of Rapamune have not been established in pediatric patients less than 13 years old, or in pediatric (<18 years) renal transplant recipients considered at high immunologic risk. Increased susceptibility to infection and the possible development of lymphoma and other malignancies, particularly of the skin, may result from immunosuppression. Hypersensitivity reactions, including anaphylactic/anaphylactoid reactions, have been associated with the administration of sirolimus. As usual for patients with increased risk for skin cancer, exposure to sunlight and UV light should be limited by wearing protective clothing and using a sunscreen with a high protection factor. Increased serum cholesterol and triglycerides, that may require treatment, occurred more frequently in patients treated with Rapamune compared with azathioprine or placebo controls.

Renal function should be closely monitored during the administration of Rapamune in combination with cyclosporine since long-term administration can be associated with deterioration of renal function. Appropriate adjustment of the immunosuppression

regimen, including discontinuation of Rapamune and/or cyclosporine, should be considered in patients with elevated or increasing serum creatinine levels. Caution should be exercised when using other drugs which are known to impair renal function. In patients at low to moderate immunologic risk continuation of combination therapy with cyclosporine beyond 4 months following transplantation should only be considered when the benefits outweigh the risks of this combination for the individual patients. In clinical trials, Rapamune has been administered concurrently with corticosteroids and with the some formulations of cyclosporine. The efficacy and safety of the use of Rapamune in combination with other immunosuppressive agents has not been determined.

OKT3

Basiliximab functions as an IL-2 receptor antagonist by binding with high affinity (Ka = 1 x 1010 M-1) to the alpha chain of the high affinity IL-2 receptor complex and inhibiting IL-2 binding. Basiliximab is specifically targeted against IL-2Ra, which is selectively expressed on the surface of activated T-lymphocytes. This specific high affinity binding of Simulect to IL-2Ra competitively inhibits IL-2-mediated activation of lymphocytes, a critical pathway in the cellular immune response involved in allograft rejection. Single-dose and multiple-dose pharmacokinetic studies have been conducted in patients undergoing first kidney transplantation. The pharmacokinetics of Simulect has been assessed in 39 pediatric patients undergoing renal transplantation.

Complete and consistent binding to IL-2Ra in adults is maintained as long as serum Simulect levels exceed 0.2 µg/mL. As concentrations fall below this threshold, the IL-2Ra sites are no longer full bound and the number of T-cells expressing unbound IL-2Ra returns to pretherapy values within 1-2 weeks.

The duration of clinically relevant IL-2 receptor blockade after the recommended course of Simulect is not known. When basiliximab was added to a regimen of cyclosporine, USP (MODIFIED) and corticosteroids in adult patients, the duration of IL-2Ra saturation was 36 ± 14 days (mean ± SD), similar to that observed in pediatric patients (36 ± 14 days) When basiliximab was added to a triple therapy regimen consisting of cyclosporine, USP (MODIFIED), corticosteroids, and azathioprine in adults, the

duration was 50 ± 20 days and when added to cyclosporine, USP (MODIFIED), corticosteroids, and mycophenolate mofetil in adults, the duration was 59 ± 17 days.

Simulect is indicated for the prophylaxis of acute organ rejection in patients receiving renal transplantation when used as part of an immunosuppressive regimen that includes cyclo-sporine, USP (MODIFIED) and corticosteroids. The efficacy of Simulect for the prophylaxis of acute rejection in recipients of other solid organ allografts has not been demonstrated. Severe acute (onset within 24 hours) hypersensitivity reactions including anaphylaxis have been observed both on initial exposure to Simulect and/or following re-exposure after several months. No dose adjustment is necessary when Simulect is added to triple immunosuppression regimens including cyclosporine, corticosteroids and either azathioprine or mycophenolate mofetil.

Daclizumab

Daclizumab is an immunosuppressive, humanized IgGl monoclonal antibody produced by recombinant DNA technology that binds specifically to the alpha subunit (~55 alpha, CD25, or Tat subunit) of the human high-affinity interleukin-2 (IL-2) receptor that is expressed on the surface of activated lymphocytes. Daclizumab is a composite of human (90%) and murine (10%) antibody sequences. The human sequences were derived from the constant domains of human IgGl and the variable framework regions of the Eu myeloma antibody. The murine sequences were derived from the complementarity-determining regions of a murine anti-Tat antibody.

Daclizumab saturates the Tat subunit of the IL-2 receptor for approximately 90 and 120 days post transplant, respectively in pediatric and adult patients. The duration of clinically significant IL-2 receptor blockade after the recommended course of ZENAPAX is not known. ZENAPAX is indicated for the prophylaxis of acute organ rejection in patients receiving renal transplants. It is used as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids.

There are no incremental increases in adverse reactions using Daclizumab with cyclosporine, mycophenolate mofetil, ganciclovir, acyclovir, azathioprine, and corticosteroids. Very limited experience exists with the use of ZENAPAX

concomitantly with tacrolimus, muromonab-CD3, antithymocyte globulin, and antilymphocyte globulin.

Long-term studies to evaluate the carcinogenic potential of ZENAPAX have not been performed.

The safety and effectiveness of ZENAPAX have been established in pediatric patients from 11 months to 17 years of age. The safety profile of ZENAPAX in pediatric transplant patients was shown to be comparable with that in adult transplant patients with the exception of the following adverse events, which occurred more frequently in pediatric patients (>15% difference in incidence): diarrhea, postoperative pain, fever, vomiting, aggravated hypertension, pruritus, and infections of the upper respiratory tract and urinary tract.

Agent	Pharmacology	Mechanisms	Side Effects
Glucocorticoids	Increased bioavailability with hypoalbuminemia and liver disease; prednisone/prednisolone generally used	Binds cytosolic receptors and heat shock proteins. Blocks transcription of IL-1,-2,-3,-6, TNF-α, and IFN-γ	Hypertension, glucose intolerance, dyslipidemia, osteoporosis
Cyclosporine (CsA)	Lipid-soluble polypeptide, variable absorption, microemulsion more predictable	Trimolecular complex with cyclophilin and calcineurin →block in cytokine (e.g., IL-2) production; however, stimulates TGF-β production	Nephrotoxicity, hypertension, dyslipidemia, glucose intolerance, hirsutism/hyperplasia of gums
Tacrolimus (FK506)	Macrolide, well absorbed	Trimolecular complex with FKBP-12 and calcineurin →block in cytokine (e.g., IL-2) production; may stimulate TGF-β production	Similar to CsA, but hirsutism/hyperplasia of gums unusual, and diabetes more likely
Azathioprine	Mercaptopurine analogue	Hepatic metabolites inhibit purine synthesis	Marrow suppression (WBC > RBC > platelets)
Mycophenolate mofetil (MMF)	Metabolized to mycophenolic acid	Inhibits purine synthesis via inosine monophosphate dehydrogenase	Diarrhea/cramps; dose-related liver and marrow suppression is uncommon
Sirolimus	Macrolide, poor oral bioavailability	Complexes with FKBP-12 and then blocks p70 S6 kinase in the IL-2 receptor pathway for proliferation	Hyperlipidemia, thrombocytopenia

Fig.2.7: Table from the FDA web site

VELCADE

VELCADE (bortezomib) for Injection is an antineoplastic agent. Bortezomib is a modified dipeptidyl boronic acid. The product is provided as a mannitol boronic ester, which, in reconstituted form, consists of the mannitol ester in equilibrium with its hydrolysis product, the monomeric boronic acid. The drug substance exists in its cyclic anhydride form as a trimeric boroxine.

The chemical name for bortezomib, the monomeric boronic acid, is [(1R)-3-methyl-1-15[[(2S)-1-oxo-3-phenyl-2-[(pyrazinylcarbonyl) amino]propyl]amino]butyl]boronic acid. Bortezomib is a reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome in mammalian cells. The 26S proteasome is a large protein complex that degrades ubiquitinated proteins. The ubiquitin-proteasome pathway plays an essential role in regulating the intracellular concentration of specific proteins, thereby maintaining homeostasis within cells. Inhibition of the 26S proteasome prevents this targeted proteolysis, which can affect multiple signaling cascades within the cell. This disruption of normal homeostatic mechanisms can lead to cell death**.** Experiments have demonstrated that bortezomib is cytotoxic to a variety of cancer cell types *in vitro*.

Bortezomib causes a delay in tumor growth *in vivo* in non-clinical tumor models, including multiple myeloma. Women of childbearing potential should avoid becoming pregnant while being treated with VELCADE. Bortezomib was not teratogenic in nonclinical developmental toxicity studies in rats and rabbits at the highest dose tested (0.075 mg/kg; 0.5 mg/m2 in the rat and 0.05 mg/kg; 0.6 mg/m2 in the rabbit) when administered during organogenesis. These dosages are approximately half the clinical dose of 1.3 mg/m2 based on body surface area.

Pregnant rabbits given bortezomib during organogenesis at a dose of 0.05mg/kg (0.6 175mg/m2) experienced significant post-implantation loss and decreased number of live fetuses. Live fetuses from these litters also showed significant decreases in fetal weight. The dose is approximately 0.5 times the clinical dose of 1.3 mg/m2 based on body surface area.

Among the adverse effects there are orthostatic/postural hypotension, nausea, diarrhea, constipation, and vomiting, thrombocytopenia, peripheral neuropathy (including peripheral sensory neuropathy and peripheral neuropathy aggravated) (37%), pyrexia (36%) and anemia (32%), pneumonia. Two deaths were reported: one case of

cardiopulmonary arrest and one case of respiratory failure. Carcinogenicity studies have not been conducted with bortezomib. The safety and effectiveness of VELCADE in children has not been established.

FTY720

FTY720 is the first agent in a new class of drugs called Sphingosine 1-Phosphate Receptor (S1P-R) agonists. FTY720 reversibly redirects lymphocytes away from the graft, preventing T-cells from damaging the graft (Yanagawa Y, Sugahara K, Kataoka H et al. J Immunol. 1998, 160: 5493-5499).

Pre-clinical research has shown that FTY720 exerts its unique lymphocyte-homing action without changing the host's ability to respond to other infectious antigens. The FTY720 clinical trial programme includes evaluation for both prevention of acute rejection and graft loss in renal transplantation. FTY720 has been administered concomitantly with Neoral at different exposures and Certican in the Phase II clinical trials.

FTY720 is a novel transplantation drug that, after phosporylation, acts as sphingosine-1-phosphate receptor (S1P-R) agonist, thereby reducing the recirculation of lymphocytes to blood and peripheral tissues, including inflammatory lesions and graft sites. FTY720 does not impair T-lymphocyte proliferation or cytokine production in vitro. FTY720 has shown excellent tolerability in Phase I clinical studies

Beta-cell Protective Drugs: Vitamin E and Nicotinamide

Nicotinamide, a vitamin of the B group, has in vitro actions capable of interfering with the pathogenetic process leading to IDDM. Since 1987, several studies have evaluated nicotinamide as a means of protecting beta cells from end-stage destruction in insulin-treated patients with newly diagnosed IDDM. There are no adverse effects of the therapy and clinical trials are on going in Type 1 Diabetes new onset patients.

Vitamin E is found naturally in some foods and available as a dietary supplement. Vitamin E exists in eight chemical forms (alpha-, beta-, gamma-, and delta-tocopherol and alpha-, beta-, gamma-, and delta-tocotrienol) that have varying levels of biological activity but alpha- (or α-) tocopherol is the only form that is recognized to meet human

requirements. Vitamin E is a fat-soluble antioxidant that stops the production of ROS formed when fat undergoes oxidation. Alpha-tocopherol inhibits the activity of protein kinase C, an enzyme involved in cell proliferation and differentiation in smooth muscle cells, platelets, and monocytes. Interesting studies on animals have been carried out and I would like to underlines the methods in Vitamin E somministration in one of these studies: "All animals received long-term (before and after disease onset) supplementation with *vitamin E* administered at the same doses and according to the same regimen. Vitamin E (natural _-tocopherol) was dissolved in sunflower oil and administered at a dosage of 0.134 mg/day (0.171 IU/day for a 30-gm mouse), which is equivalent to a dosage of 400 IU/day for humans, as previously recommended. We administered vitamin E by gavage every other day (0.268 mg in 100 _l), starting on day 21 of life (1 week before the onset of arthritis) and continuing for 6 weeks. The placebo group underwent the same procedure, but received sunflower oil alone. Mice were given conventional oral food and water ad libitum". (Arthritis Rheum. 2002 Feb; 46(2): 522-32)

Beta-cells Regeneration Agents: Hepatocyte Growth Factor, GLP-1, Epidermal Growth Factor and Gastrin

Hepatocyte growth factor (HGF) according to some recent papers, looks to promote beta-cell regeneration in vitro (Biochem Biophys Res Commun. 2005 Jun 8; 333(1): 273-282).

Glucagon-likepeptide-1 (GLP-1), a gut hormone derived from enteroendocrine L cells, inhibits cell death in freshly isolated human islets cultured *in vitro* for 5 days. The antiapoptotic effects of GLP-1 have previously been demonstrated in diabetic rodents, islet cell lines, purified rat β -cells, and heterologous cells expressing the GLP-1 receptor. GLP-1 reduces food intake, inhibits gastric emptying and glucagon secretion, and enhances glucose-dependent insulin secretion, actions that promote lowering of glycemia and restoration of a normal metabolic milieu. Furthermore, activation of GLP-1 receptor signaling expands islet mass via stimulation of islet neogenesis and induction of cell proliferation in both young and old, normal and diabetic animals in multiple different experimental paradigms.

Recent experiments using a combination of cell lines and transgenic mice have suggested that GLP-1 increases cell survival via cAMP-dependent stimulation of cAMP response element binding protein (CREB) activity, and subsequent enhancement of the insulin receptor substrate (IRS)-2 growth factor-regulated pathway. (Endocrinology 144(12): 5145–5148)

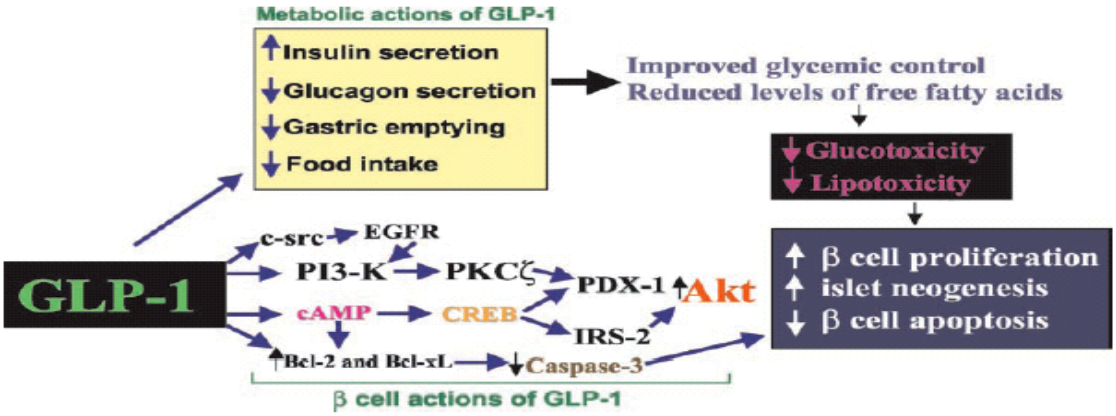


Fig.2.8 GLP1 actions diagram from the paper Endocrinology 144(12) 5145-5148)

Exendin-4, a potent GLP-1R agonist, induces pancreatic and duodenal homeobox gene-1 expression in human fetal islet cell cultures and promotes functional maturation and proliferation of human islet cell cultures transplanted under the rat kidney capsule.

The combination of *EGF* and *gastrin* induces islet β-cell neogenesis from pancreatic exocrine duct cells in rodents. β -cell numbers are increased in cultures with EGF+gastrin (+118%) and with EGF (+81%) but not with gastrin (-3%) or control media (-62%). After withdrawal of EGF and gastrin and a further 4 weeks in control media, β-cell numbers continued to increase only in cultures previously incubated with both EGF and gastrin (+232%). EGF+gastrin increase cytokeratin 19-positive duct cells and gastrin alone or in combination but not EGF alone increase PDX-1 (pancreatic and duodenal homeobox factor-1) as well as insulin and C-peptide in the CK19-positive duct cells. EGF+gastrin increase β-cell and insulin content in human islets implanted in NOD-scid.

Immunosuppression in Islet Transplants

The immunosuppressive protocols utilized in past years include monoclonal or polyclonal T-cell antibodies such as ALG, ATG, OKT3, or anti-CD4 -based induction immunosuppression, and triple-drug maintenance therapy based on glucocorticoids, cyclosporine or tacrolimus, and azathioprine or mycophenolate mofetil. Unfortunately, most of the immunosuppressive drugs such as glucocorticoids and calcineurin inhibitors are known to exert toxic effects on the islet b cells or to induce insulin resistance.

The anti-rejection protocols commonly used for solid organ transplantation may have detrimental consequences on islet graft fate. The International Islet Transplant Registry reported that a total of 405 adult islet allogeneic transplants were performed from 1983 through 1998 (see White, et. al.). Analysis of 200 C-peptide negative Type 1 Diabetic patients transplanted from 1990 through 1997 showed a cumulative 1-year patient survival of 96%, and graft survival assessed by measurable basal C-peptide levels above 0.5 ng/ml of 35%, with insulin independence at 1 year in only 8% of the recipients. The rate of insulin independence was higher in patients receiving multiple-donor islet preparations (19%), when compared to those receiving single-donor preparations (11%), most likely reflecting the need for a conspicuous islet graft mass higher than 6,000 islet equivalents per kilogram (IE/kg) of the recipient's body weight.

The results reported by Shapiro et al. in the NEJM in July 2000 on the successful series of cadaveric human islet cell transplants in Type 1 Diabetic patients treated with a glucocorticoid-free immunosuppression protocol, including a short course of anti-IL2 receptor antibody, and maintenance therapy based on rapamycin and low dose of tacrolimus, has been thought to represent a major breakthrough in the field of islet transplantation. In this study, all patients received islets isolated from more than one donor. Two to three grafts were generally needed in order to obtain an islet mass sufficient to achieve insulin independence (11,547 ±1,604, mean ± SD, IE/kg of recipient's body weight). Although infusion of islets from a single donor did not result in insulin independence in any of the patients studied, all the patients had improved glycemic control, reduced insulin requirements, and absence of hypoglycemic episodes soon after receiving the first graft. Two-year follow-up data has shown continued glycemic control in a number of these subjects. A possible explanation of the need for multiple donors in this study is the destruction of a substantial mass of islets

immediately after transplantation. Early loss of implanted islets has been proposed to be due to their susceptibility to microenvironment alteration with consequent functional impairment and loss to apoptosis.

Type 1 Diabetes Prevention: in Vivo Combo Therapy in NOD

Type 1 Diabetes is a complex disease. Many cells and many events are involved in the mechanism of the disease. There is a genetic predisposition to the disease. Many environmental triggers are supposed to have a role in the pathogenesis. When the disease is triggered, the immune system destroys the beta cells. The target of prevention and cure of Type 1 Diabetes is basically a three steps strategy: 1) preserving the residual pancreatic mass 2) stopping the autoimmune process 3) regenerating the beta cell mass. In targeting a cure for Type 1 Diabetes all three steps should be pursued.

The idea is to apply the three-step strategy using NOD mice 10-14 weeks old. A period of time characterized by the administration of an immunosuppressive treatment aimed at stopping the autoimmune process should be followed by or simultaneously administered with a beta cell protective treatment, a beta cell regenerative treatment and an antigen treatment aimed at switching the immune condition towards a tolerogenic immune condition.

The question is which chemicals are the best treatment for this strategy and how long should the treatments be administered. Also, what are the best doses of each chemical? For each chemical candidate to be in the three-step strategy, I tried to analyze the reasons why it should be included in the protocol and/or the reasons it should not be included.

Cyclosporine: It's a classical immunosuppressive drug used in human transplant. There have been clinical trials using cyclosporine in children with Type 1 Diabetes.

Tacrolimus: It's an immunosuppressive drug used in human transplant similar to cyclosporine but it is a more recent drug. It has different adverse effects and is more effective than cyclosporine in some transplants.

Mycophenolate: It's an immunosuppressive drug now used in many centers in place of the azathioprine.

Glucocorticoids: They are potent immunosuppressive agents usually included in protocols of treatment for transplants. Their effect on the immune system is strong and fast, however they induce hyperglycemia and are toxic to beta-cells. Because the immunosuppression window will be short, it may be possible to use these agents at the beginning of the treatment just to jump start the immunosuppression and then switch to other immunosuppressive agents as soon as possible. Diabetic subjects will need to increase the insulin dosage at the time of treatment.

Azathioprine: It is an immunosuppressive drug used in human transplants.

Sirolimus (Rapamune): It is an immunosuppressive drug used in human transplants.

OKT3: It induces good immunosuppression, however its use, especially long-term, is limited by the induction of anti-mouse Ab in humans.

Basiliximab (Simulect) and Daclizumab: These chemicals are genetically engineered monoclonal antibodies that do not induce anti-mouse Ab in humans. They are approved for prophylaxis of acute rejection in the immediate posttransplant period.

Velcade, FTY720, anti-integrins, PDL1-Ig, DSG, CTLA4 Ig, statins, TGF-beta, TNF-alpha, CCR5, CXCR3: All of these chemicals have proved to be immunosuppressive or have modulative effects on the immune system and many of them are used in transplants.

DC treatment: Many studies show the effectiveness of this treatment to slow down the diabetes onset in mice. I have seen this result in my own experiments. (see Chapter 3).

Vitamin D: According to the Mathieu's paper Endocrinology, 2005 Apr; 146(4): 1956-64, vitamin D treatment in NOD mice gives the following results:

TABLE 1. Effect of *in vivo* treatment with 1,25-$(OH)_2D_3$ on insulitis score and insulin content in pancreases of diabetes-prone NOD mice

	Mean insulitis score	*P* value	Insulin content (pmol/mg pancreas)	*P* value
Control (wk)				
4	0.11 ± 0.14		28.14 ± 10.13	
8	1.18 ± 0.48		7.22 ± 7.84	
10	2.06 ± 0.54		4.17 ± 3.06	
1,25-$(OH)_2D_3$ (wk)				
4	0.00 ± 0.00	NS	30.41 ± 28.99	NS
8	0.53 ± 0.39	NS	35.53 ± 22.23	≤0.05
10	0.55 ± 0.42	≤0.01	17.45 ± 19.17	≤0.001

NOD mice were treated either with vehicle or 1,25-$(OH)_2D_3$ by ip injection every other day from weaning until 4, 8, and 10 wk of age (n = 8 animals). Each pancreas was subjected to a blinded analysis for all islets (a mean of ≥10 islets per pancreatic sample). Scoring was done as described in *Materials and Methods*. Pancreatic insulin content was determined by ELISA.

NS, Not significant.

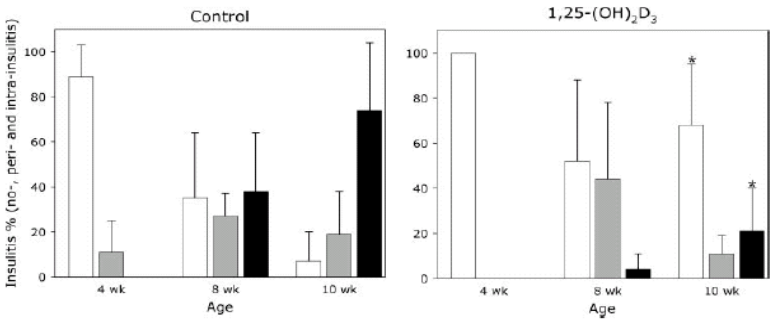


FIG. 1. Effect of *in vivo* treatment with 1,25-$(OH)_2D_3$ on severity of islet infiltration in pancreases of diabetes-prone NOD mice. NOD mice were treated with either vehicle or 1,25-$(OH)_2D_3$ by ip injection every other day from weaning until 4, 8, and 10 wk of age (n = 8 animals). Each pancreas was subjected to a blinded analysis for all islets (mean of 10 or more islets per pancreatic sample). *Bars* indicated the percentage of islet infiltration: *white bars*, no insulitis; *gray bars*, periinsulitis; *black bars*, intrainsulitis. *, $P \leq 0.05$ *vs.* age-related vehicle-treated NOD mice.

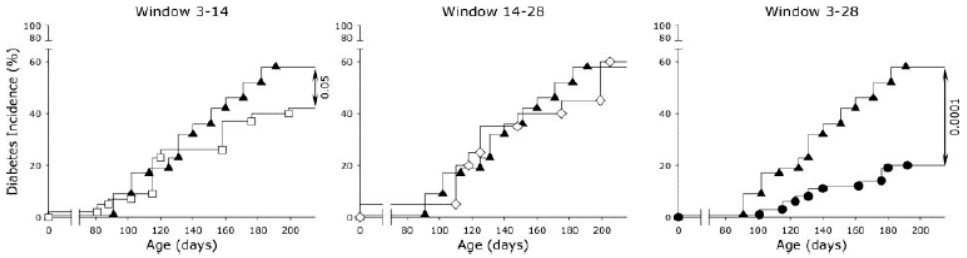


FIG. 7. Effect of *in vivo* treatment with 1,25-$(OH)_2D_3$, applied in different time windows, on diabetes incidence in diabetes-prone NOD mice. Female NOD mice were treated with either vehicle (peanut oil) until 28 wk of age (▲, n = 90) or 1,25-$(OH)_2D_3$ (5 μg/kg body weight) by ip injection every other day from weaning until 28 wk of age (●, n = 69) or 14 wk of age (□, n = 43) or from 14 until 28 wk of age (◇, n = 20). Mice with blood glucose levels of more than 200 mg/dl at 28 wk of age were scored as having diabetes. Significance is expressed compared with vehicle-treated NOD mice.

Fig. 2.9- Images of the results of the paper Endocrinology. 2005 Apr; 146(4):1956-64.

The previous images demonstrate the validity of this protocol, especially during the 3-28 week window of treatment. They used the same protocol as in other papers, and they had similar results. They used 1,25(OH)2 D3 in peanut oil 5ml/Kg/day i.p. (50ml).

Other authors are using oral administration of vitamin D in peanut oil or a special food with vitamin D either added or vitamin D-free. In order to give vitamin D orally, the researcher has to use needles to put the drug into the mouse's mouth. Or special food, with and without vitamin D, needs to be purchased. Because of this and because it's not possible to exclude metabolism defects during the oral absorption of a drug, using i.p. injections is safer for the aim of this project. Also, the results shown in Fig 2-9 lends support for the use of Mathieu's protocols.

G-CSF: G-CSF is used in humans, although it's used for clinical conditions other than diabetes. In Kared et al.'s paper (Diabetes Jan 2005), the authors used Neupogen Amgen 200mg * Kg-1*day-1 in 5% dextrose in sterile water subcoutaneous injection 5 consecutive days at week 4, week 8, week 12 and week 16 to prevent insulitis in mice.

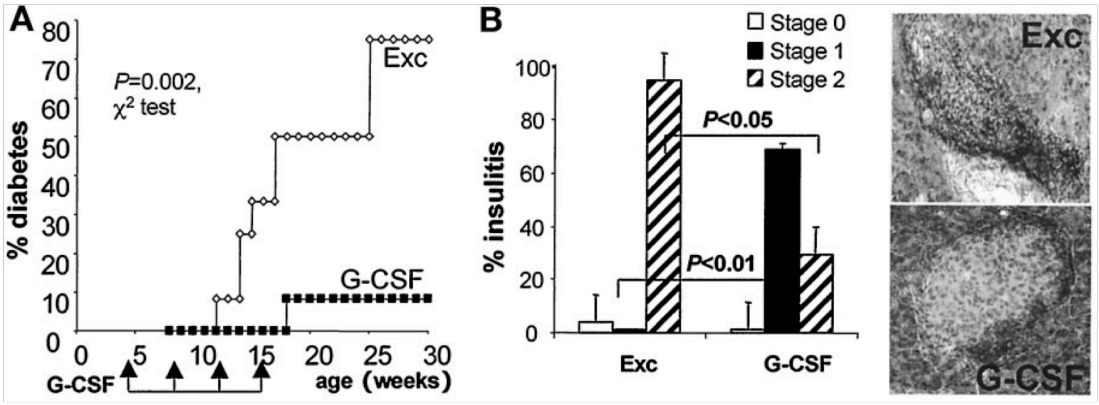


Fig. 2.10. Figure from Kared's paper, Diabetes Jan 2005: Treatment with G-CSF prevents spontaneous diabetes and destructive insulitis. A: NOD mice (n=12 per group) were treated with G-CSF (200 g/kg) or excipient for 5 consecutive days every 4 weeks starting at age 4 weeks. G-CSF significantly reduced diabetes incidence. B: Insulitis was scored at age 25 weeks (means SD of islet counts from three to six organs per group). Representative hematoxylin- and eosin-stained pancreatic sections are shown.

Insulin and proinsulin peptide: Many papers show insulin peptide prevents diabetes and in my current lab, coworkers have succesfully used this protocol. So, it could be interesting to utilize it in a new protocol.

IGRP peptides: Papers show IGRP peptides are involved as antigens in Type 1 Diabetes.

DIAPEP277: Because many molecules have been identified as antigens in Type 1 Diabetes, and because it's not possible to include so many molecules in this study, I will seriously consider using DIAPEP277, which is already used in clinical trials.

Nicotinamide: Papers and clinical trials show the positive effect of nicotinamide on beta-cell protection. Clinical trials are ongoing.

Hepatocyte growth factor (HGF): It promotes beta-cell regeneration in vitro, however its use in humans isn't common.

GLP-1, EGF and gastrin: Studies show their effectiveness in beta-cell regeneration, however these chemicals are expensive and not commonly used in human therapies.

The majority of these agents are FDA approved drugs (except some of them: example: HGF and insulin/IGRP peptides), and they are used in children even if they are only approved for clinical conditions other than Type 1 Diabetes. A balance of advantages/adverse effects should be carefully considered in order to find a truly applicable therapy for Type 1 Diabetes in humans and also to make FDA approval easier to obtain.

Possible Protocols of Treatment:

1) Corticosteroids once or twice (+ insulin)+ Cyclosporine and Sirolimus + DIAPEP277 (as used in ongoing protocols) + Vit D + nicotinamide + HGF
2) Corticosteroids once or twice (+ insulin) + tacrolimus and sirolimus + insulin peptide + G-CSF + Vit D + nicotinamide + HGF

3) Corticosteroids once or twice (+ insulin)+ daclizumab + cyclosporine + DIAPEP277 + Vit D + nicotinamide + HGF
4) Corticosteroids once or twice (+ insulin)+ daclizumab + cyclosporine + DIAPEP277 + Vit D + G-CSF + nicotinamide + HGF
5) OKT3 + sirolimus + DIAPEP277 + Vit D + nicotinamide
6) Corticosteroids once or twice (+ insulin)+ cyclosporine or tacrolimus + azathioprine or mycophenolate + DIAPEP 277 + vit D + nicotinamide + HGF
7) Sirolimus + Daclizumab + insulin peptide + vit D + nicotinamide + G-CSF + HGF
8) Rapamycin + low dose of tacrolimus+ G-CSF + Vit D + DIAPEP277 + insulin peptide + nicotinamide + GLP1 + EGF+gastrin
9) Daclizumab + sirolimus + low dose of tacrolimus + G-CSF + Vit D + DIAPEP277 + insulin peptide + nicotinamide + GLP1 + EGF+gastrin
10) DSG + G-CSF + Vit D + DIAPEP277 + insulin peptide + nicotinamide + GLP1 + EGF+gastrin

The final protocol that came from the previous considerations is as follows:

We used 12 week sold NOD female mice from Jackson Labs. Mice housing was patogen-free and all the animal experiments were carried out according to the IACUC guidelines and approved by the Animal Use Committee at Medical College of Georgia. We gave them 1mg/Kg of rapamycin i.p. (in 500ml deionized water containing 1g sodiumcarboxymethylcellulose and 1.25 g Tween 80), 5 mg/Kg i.p. of vitamin D (in peanut oil), 0.045 mg per mouse of vitamin E by oral route, 500 mg/kg i.p. of nicotinamide (in sterile PBS) every other day for 2 weeks. At this point mice were 14 weeks old. We stopped the treatment. We waited 2 weeks and we immunized mice using insulin peptides following the protocol already in use in Dr. She's lab. (16 weeks old mice). We basically immunized mice using B9-23 insulin peptide. We injected the mix of peptides equally distribuiting the volume in 5 subcoutaneous injections per mouse (in two axyllary points, in two inguinal points and on the neck). The control group received veichles without drugs, basically, i.p. injection of deionized water containing sodiumcarboxymethylcellulose and Tween 80 without rapamycin, i.p. injection of peanut oil without vitamin D, oral peanut oil without vitamin E, i.p.

injection of sterile PBS without nicotinamide and subcoutaneous injections of Freud's adjuvant without insulin peptides.

3 EXPERIMENTS

Initial Hypothesis

Genetics, environment and immunology are involved in diabetogenesis. Its features include mononuclear cell infiltration of islets (insulitis), production of islet-cell specific autoantibodies, b-cell antigen-specific T-cell responses, and dendritic cell interaction. Early studies have shown that individuals (both relatives and the general population) at risk for T1D can be identified by using a combination of genetic, metabolic and immunologic (autoantibodies against islet-cell antigens) markers. It has also been shown that a large number of susceptibility genes are implicated in T1D. These susceptibility genes are probably required risk factors for the vast majority of T1D patients; however, many genetically predisposed subjects may not develop autoantibodies (Ab) or clinical diabetes. The progression from genetic predisposition to β-cell autoimmunity is a critical, but poorly understood process. The hypothesis is that the initiation of the autoimmune cascade and the resultant molecular and cellular changes culminating in clinical disease occur in genetically susceptible individuals in the very early years of life. These changes are difficult to document and many events and cells are involved. To easily understand all the cascades and the involved events, scientists need to focus on single cell populations and events. However, only linking all the events will allow us to understand all the molecular and cellular mechanisms of the disease. Focusing on a few events allows us to better understand the process of the disease. In line with this consideration, I wanted to focus my studies on dendritic cells, their interaction with T cells, their molecular changes and their role in Type 1 Diabetes and in the prevention and cure of the disease.

Dendritic cells are potent antigen presenting cells. They are widely distributed throghout the body and they have potent immunostimulatory properties. Although their principal function is the activation of T cells, their anatomical distribution suggests they have different strategies of function and they represent a control point for the onset of immunity. Circulating precursor DCs enter the periphery as immature DCs, where they capture antigens. Following the antigen capture the immature DCs leave the tissues and migrate to the lymphoid organs where they mature and display antigen-derived peptides on their MHC molecules which in turn select out rare circulating antigen-specific T

cells. The activated T cells further induce final DC maturation which support lymphocyte expansion and differentiation. In Type 1 Diabetes, mature DCs directly and indirectly induce T cell activation and pancreatic beta cell damage. Modulating DC maturation and function and inducing tolerance would lead us to modulate T cell activation and, hopefully, the pancreatic beta cell loss.

The goals of this study include the generation of engineered tolerogenic dendritic cells able to modulate the immune response in Type 1 Diabetes; identifying molecules that will aid in the characterization of immature versus mature dendritic cells; understanding of how these molecules may be involved in Type 1 Diabetes development; and modulating these molecules by drug treatment or genetic overexpression using vectors to generate engineered tolerogenic dendritic cells able to aid in the prevention/cure of type 1 diabetes in mice, with application in humans as the next step.

Bone Marrow Dendritic Cell Purity

Checking dendritic cell purity was the first step of this project. Cd11c is considered to be a pan marker for murine dendritic cells. Female NOD mice 8-10 weeks old were euthanized, the legs removed and bone marrow flushed from the femurs using a 22G needle after pealing off skin and muscle using media (RPMI+mercaptoethanol+non essential aminoacid solution+sodium piruvate+FBS+antibiotics 20 ml per $3x10^6$ cells). Cells were cultured for 10 days with 10 ng/ml GM-CSF and 10 ng/ml IL-4. Cultures were refreshed with new media and GM-CSF plus IL-4 at day 4 and day 7. To compare stimulated and unstimulated dendritic cells, 1 µg/ml of LPS was added to some plates 24 hours before the harvest. This protocol has been adapted from Lutz et al. Journal of Immunological Methods 223 (1999) 77-92.

At day 10 cells were harvested, washed using sterile buffer, counted and resuspeded in buffer at $1x10^6$ cells/ml. Following the company directions, samples were stained with CD11c antibody APC-labeled at 1 µl / ml and isotype control APC-labeled, incubated on ice for 15 minutes, washed, fixed in 2% formalin and run along with control samples using the FACSCALIBUR.

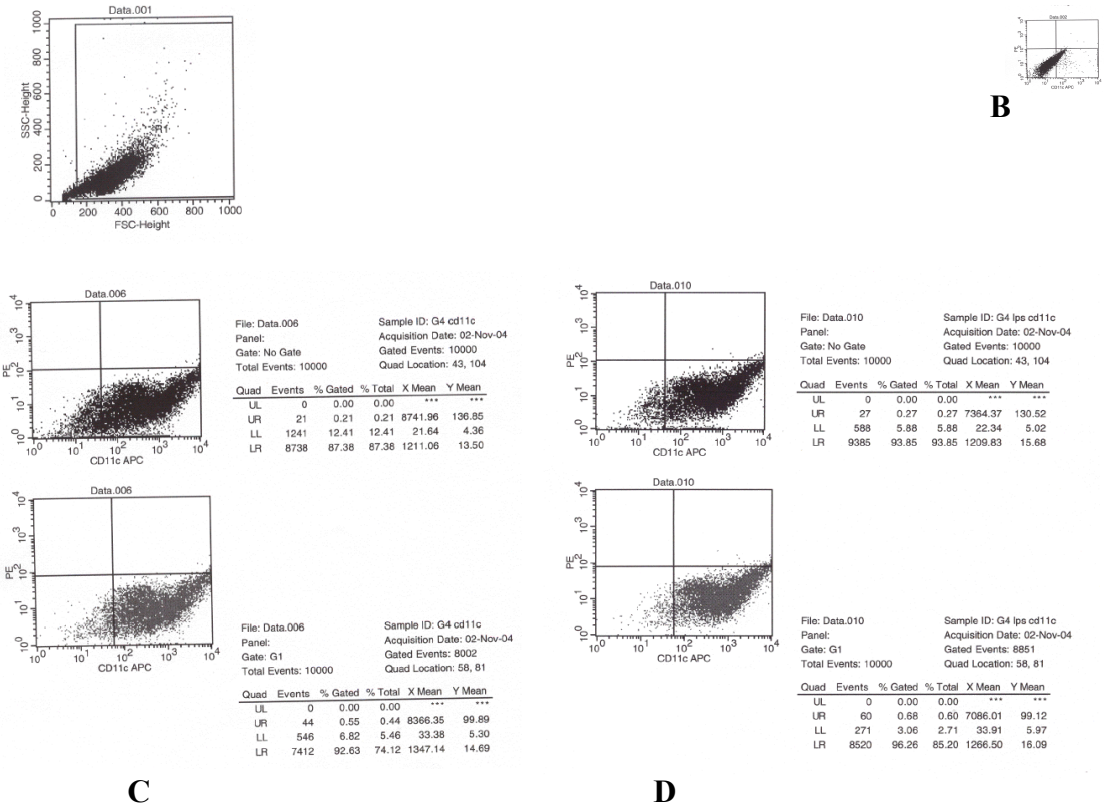


Fig. 3.1

CD11c + cells from NOD mouse bone marrow cultures with (D) and without (C) LPS stimulation.

A: total cells **B: isotype control**

As figure 3.1 shows, bone marrow cultures from NOD mice, with and without LPS stimulation, produce a high percentage of cd11c+ cells (>90% in fig. 3.1). Drug treatments of the bone marrow cultures don't change the cd11c expression. The percentage of CD11c+ cells decreases smoothly in vitamin D treated bone marrow cultures but still remains around 80%. In figures 3.2 and 3.3, we can see flow cytometry images of aspirin treated (figure 3.2) and vitamin D treated (figure 3.3) bone marrow cultures, showing the percentage of cd11c+ cells.

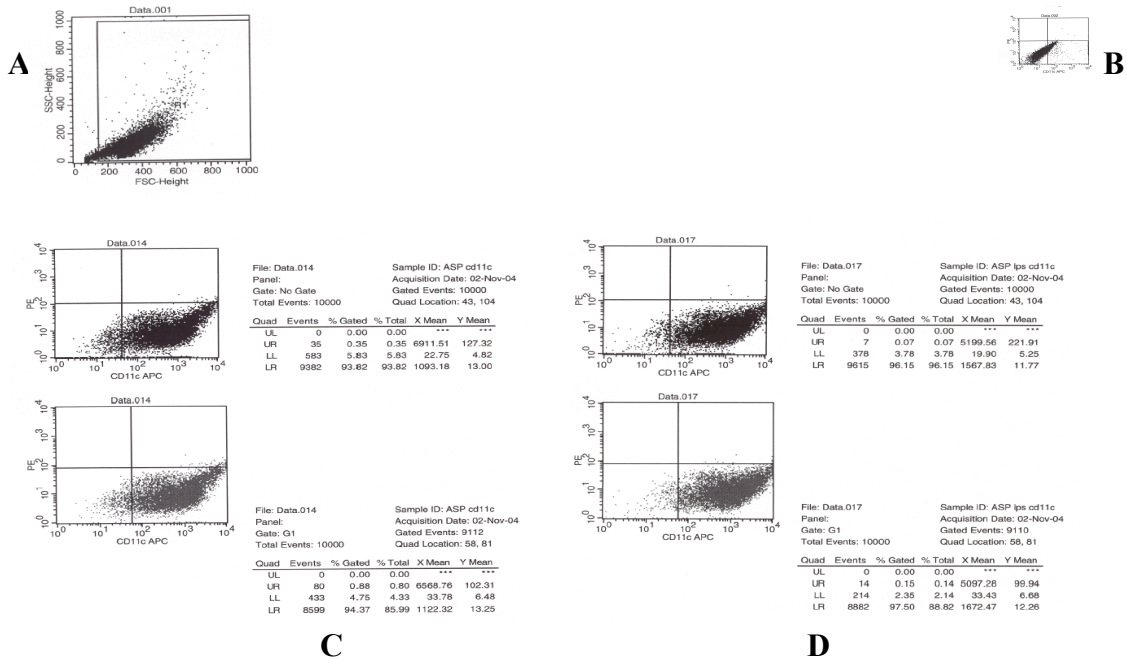


Fig. 3.2

CD11c + cells from aspirin treated NOD mouse bone marrow cultures with (D) and without (C) LPS stimulation.

A: total cells B: isotype control

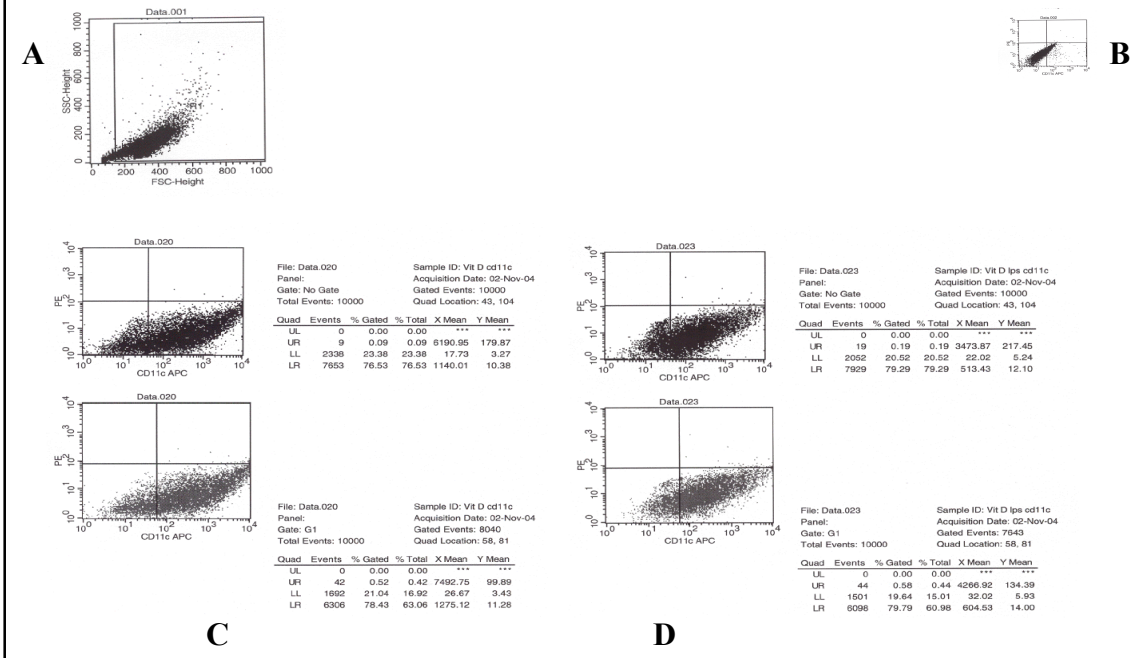


Fig. 3.3

CD11c + cells from vitamin D treated NOD mouse bone marrow cultures with (D) and without (C) LPS stimulation.

A: total cells B: isotype control

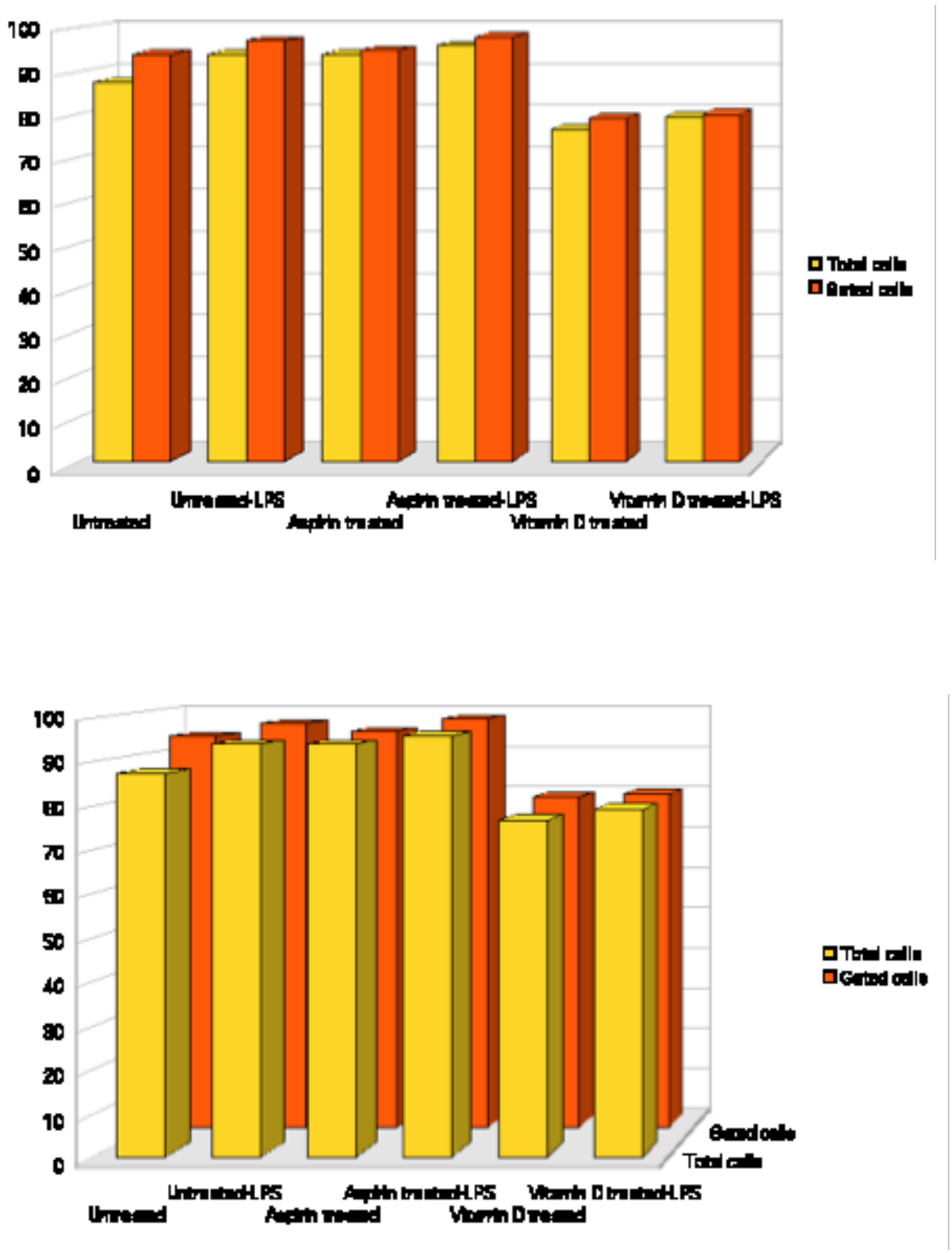


Fig. 3.4 – Diagrams showing the percentage of cd11c+ cells from treated/untreated NOD mouse bone marrow cultures

Bone Marrow Surface Molecules

Type 1 Diabetes is an autoimmune disease where, simplifying a complex immunological process in a few words, T lymphocytes attack the pancreatic beta cells leading to insulin producing cell loss and increased blood sugar. To be able to proliferate and attack the pancreas, T cells need to be fully actived by two signals. A first signal is an antigen specific signal while the second signal is a co-stimulatory signal. Both signals are started by antigen presenting cells. Dendritic cells are antigen presenting cells and molecules on their surface interact with molecules on the T cell surface, establishing a molecular communication between the two populations of cells. This communication leads to an intracellular cascade of molecular events in the T cells ending in the activation of the T cells themselves.

The antigen specific signal is provided by the MHC molecules on the APC cells, the costimulatory signal is provided by the costimulatory molecules. The most well-known costimulatory molecules are CD80 and CD86. These two co-stimulatory molecules are also widely used as markers to detect the first maturation stage of dendritic cells.

Murine bone marrow dendritic cells express different levels of CD80 and CD86 expression depending on the time of harvast and culturing protocol. Our NOD mouse bone marrow dendritic cells express good levels of CD80 and CD86 molecules.

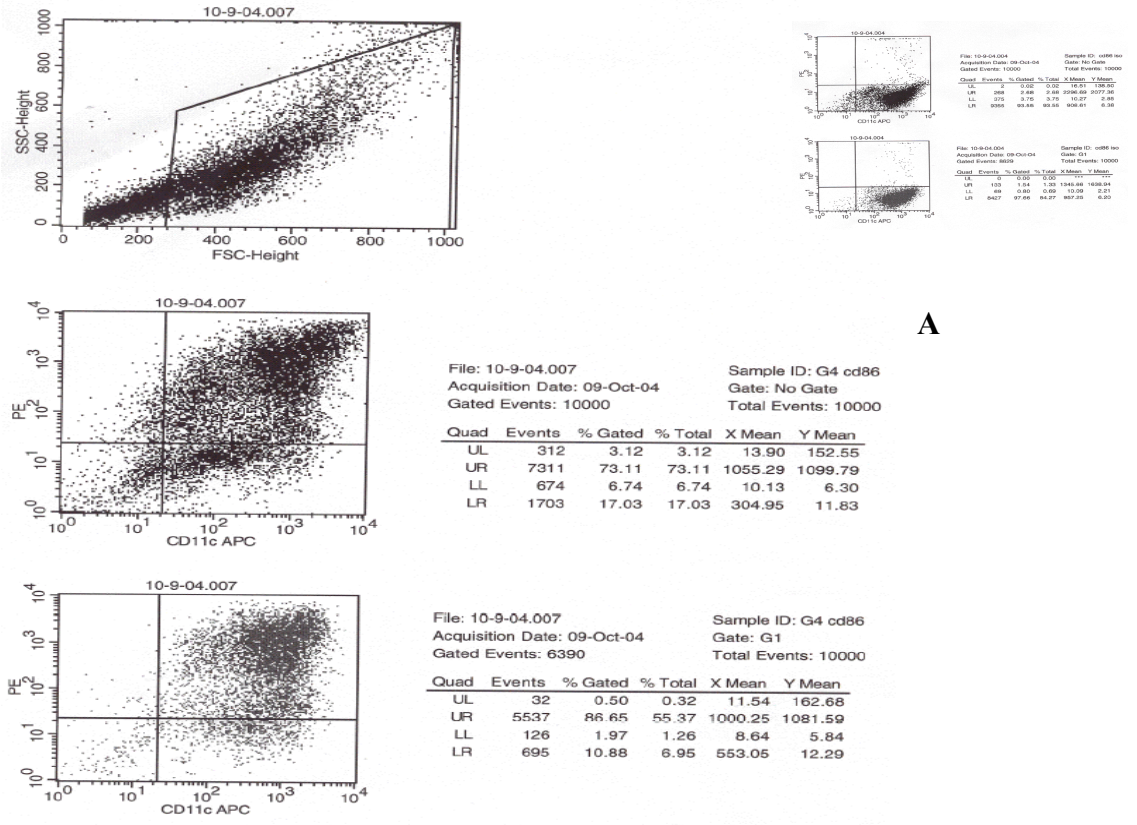
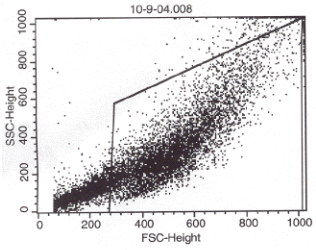
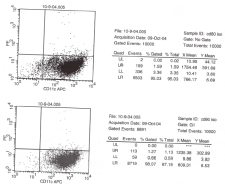


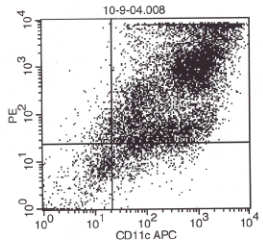
Fig. 3.5- CD86 expression on bone marrow DCs from the NOD mouse

A= Isotype control





A



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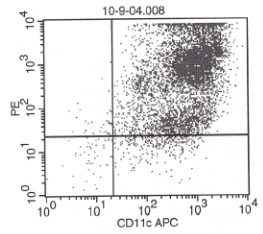
Total Events: 10000

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	217	2.17	2.17	14.39	187.68
UR	8286	82.86	82.86	1042.86	1727.22
LL	621	6.21	6.21	10.31	7.25
LR	876	8.76	8.76	163.93	14.72



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Gated Events: 6653

Sample ID: G4 cd80
Gate: G1
Total Events: 10000

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	38	0.57	0.38	13.73	100.80
UR	6255	94.02	62.55	1018.89	1647.88
LL	47	0.71	0.47	9.53	9.34
LR	313	4.70	3.13	296.33	14.87

Fig. 3.6- CD80 expression on bone marrow DCs from the NOD mouse

A= Isotype control

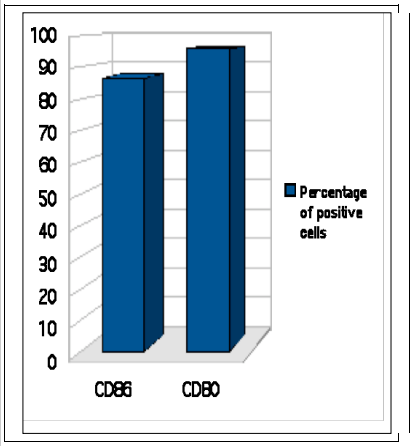


Fig. 3.7- Percentage of CD86+cells and CD80+ cells from NOD mouse bone marrow cultures

These results are consistent with Morel's paper, Clinical Immunology Vol. 98 No. 1 January, pp.133-142, 2001, where authors demonstrate NOD BMDCs cultured in GM and IL4 have a more mature phenotype than NOD BMDCs cultured in GM alone. High expression of costimulatory molecules is a sign of a more mature phenotype. Our NOD BMDCs show good expression of both costimulatory molecules, CD80 and CD86 (fig. 3.8) showing that we were able to generate NOD BMDCs with a mature phenotype.

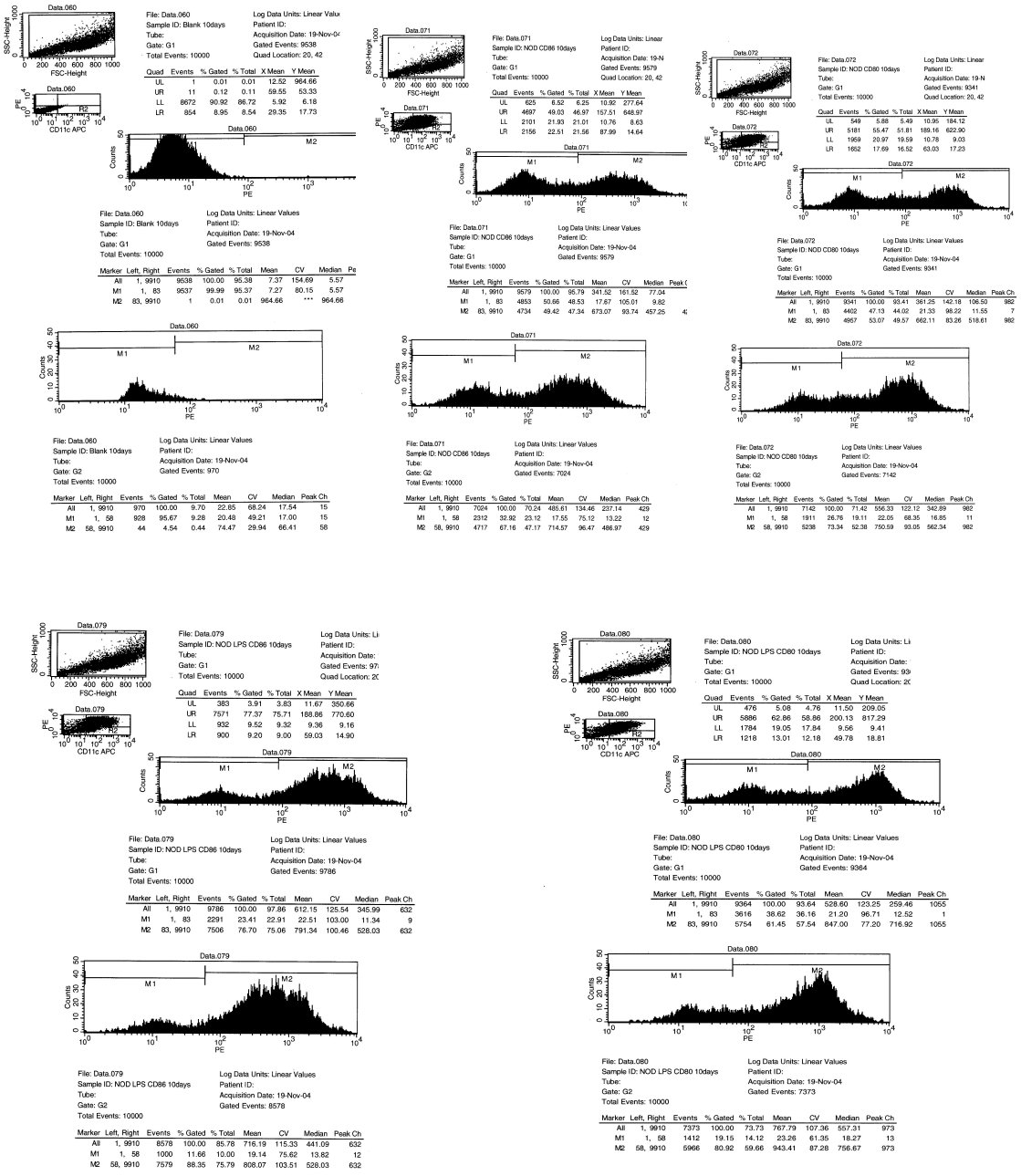


Fig. 3.8 Co-stimulatory molecule expression in dendritic cells from bone marrow cultures with and without LPS stimulation

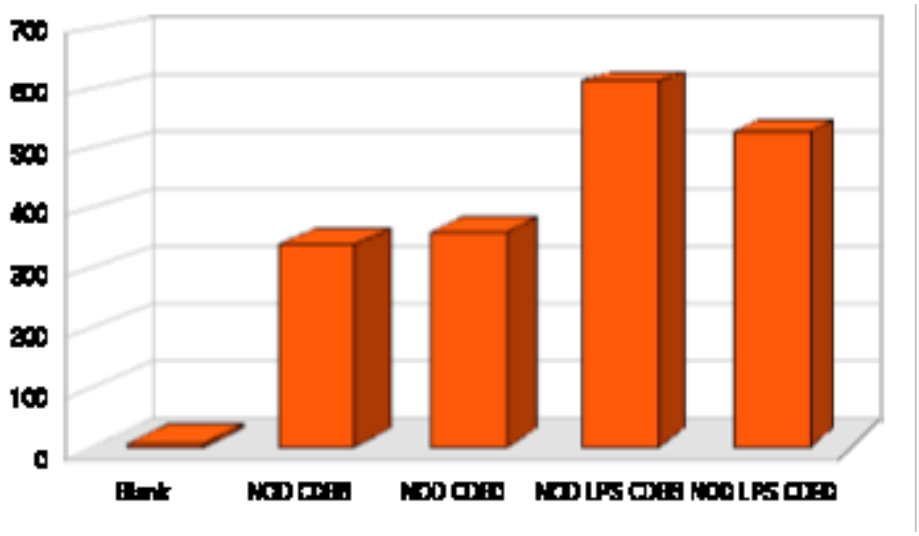


Fig. 3.9 Mean Fluorence Intensity graph of CD80 and CD86 in dendritic cell cultures from the bone marrow of NOD mice with and without LPS treatment

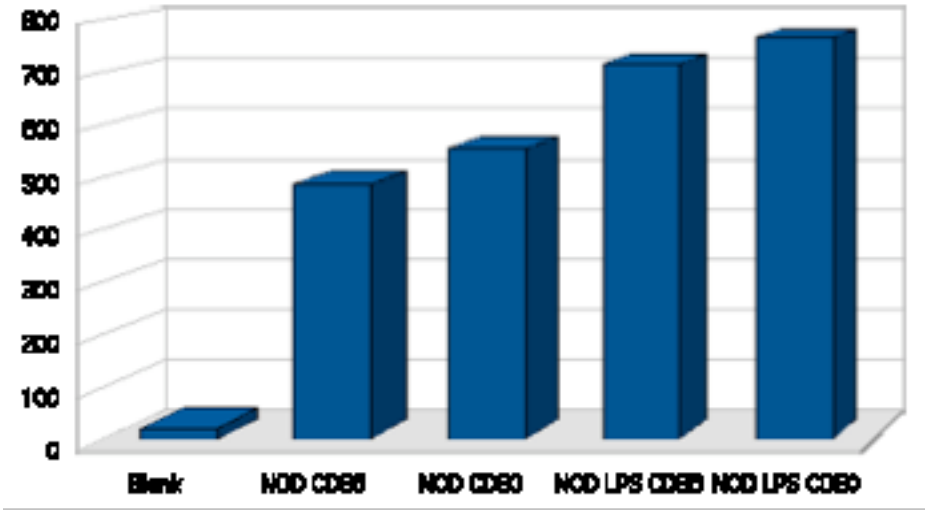
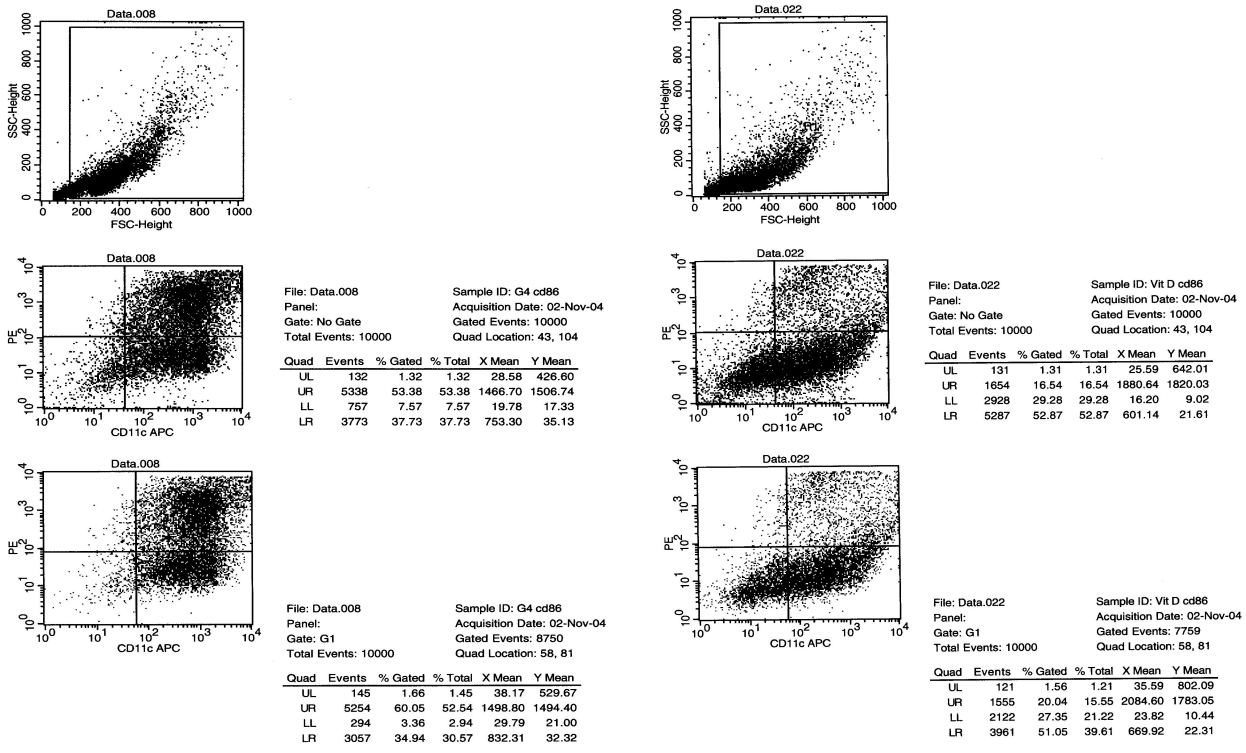


Fig. 3.10 Mean Fluorence Intensity graph of CD80 and CD86 in dendritic cell cultures from the bone marrow of NOD mice with and without LPS treatment (gated for CD11c +cells)

As figures 3.9 and 3.10 show, we established a protocol to produce dendritic cells from bone marrow expressing good levels of the costimulatory molecules, CD86 and CD80. In accordance with expected results, costimulatory molecule levels increase when cells are stimulated using LPS. Some cultures produce a lower percentage of CD11c+ cells, however this protocol does produce a significant percentage of CD11c+ cells for our study.

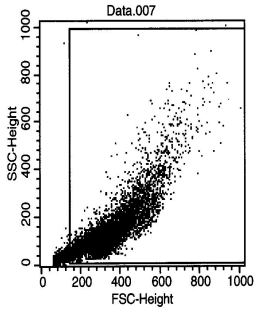
Cells cultured at different times can give slightly different levels of expression of both cd11c and costimulatory molecules. In other words, there can be slight variability between different batches of cultures. However, this batch difference is insignificant as each molecule behaves in each culture in exactly the same way.

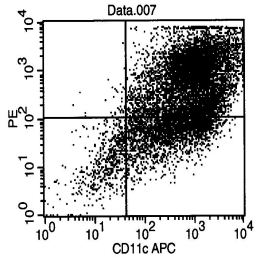
In order to manipulate the expression of costimulatory molecules and consequently the behavior of dendritic cells, we treated cells cultures with chemicals having immunomodulatory action and we observed their effects on these cells.



A **B**

Fig. 3.11 CD86 expression in DCs from untreated (A) BM cultures and vit. D treated (B) cultures.





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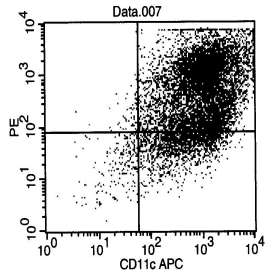
LR	2329	23.29	23.29	677.58	55.68



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Gate: G1 Gated Events: 9110
Total Events: 10000 Quad Location: 58, 81

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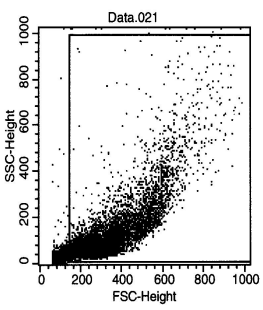
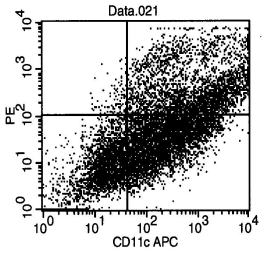
UL	65	0.71	0.65	42.64	333.99
UR	7336	80.53	73.36	1652.66	1210.96
LL	213	2.34	2.13	31.50	27.99
LR	1496	16.42	14.96	690.78	48.03





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Total Events: 10000 Quad Location: 43, 104

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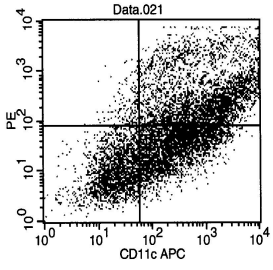
UL	105	1.05	1.05	27.24	322.92
UR	2946	29.46	29.46	2040.07	826.79
LL	2473	24.73	24.73	17.83	12.41
LR	4476	44.76	44.76	449.53	37.29



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Gate: G1 Gated Events: 8028
Total Events: 10000 Quad Location: 58, 81

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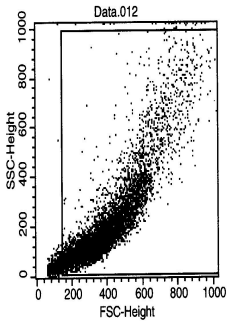
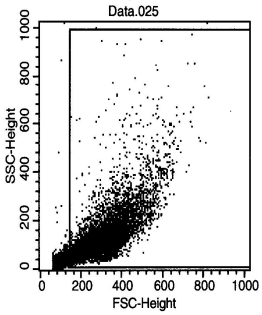
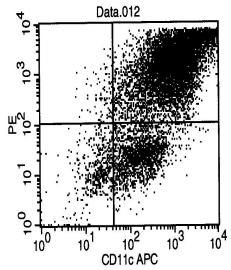
UL	164	2.04	1.64	38.34	294.40
UR	3113	38.78	31.13	2003.71	747.79
LL	1795	22.36	17.95	24.94	14.26
LR	2956	36.82	29.56	480.64	35.95

A B

Fig. 3.12 CD80 expression in DCs from untreated (A) bone marrow cultures and vit. D treated (B) cultures







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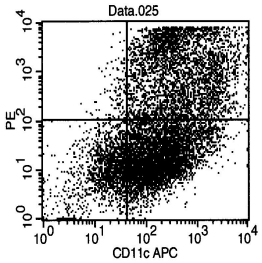
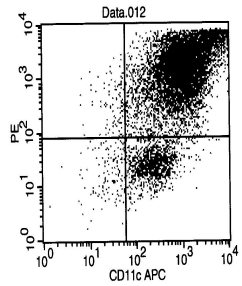
UL	96	0.96	0.96	27.18	555.37
UR	7651	76.51	76.51	1681.57	2223.71
LL	497	4.97	4.97	21.35	18.77
LR	1756	17.56	17.56	288.75	30.95



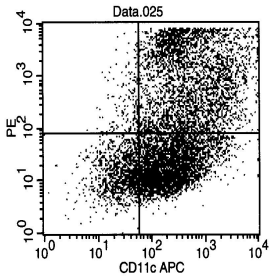
File: Data.025 Sample ID: Vit D lps cd86
Panel: Acquisition Date: 02-Nov-04
Gate: No Gate Gated Events: 10000
Total Events: 10000 Quad Location: 43, 104

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	121	1.21	1.21	28.11	746.83
UR	3282	32.82	32.82	1132.21	2095.09
LL	1536	15.36	15.36	21.63	15.76
LR	5061	50.61	50.61	353.42	24.75



File: Data.012 Sample ID: G4 lps cd86
Panel: Acquisition Date: 02-Nov-04
Gate: G1 Gated Events: 8753
Total Events: 10000 Quad Location: 58, 81

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	112	1.28	1.12	36.54	559.16
UR	7343	83.89	73.43	1715.95	2253.83
LL	141	1.61	1.41	30.75	26.71
LR	1157	13.22	11.57	328.67	29.49

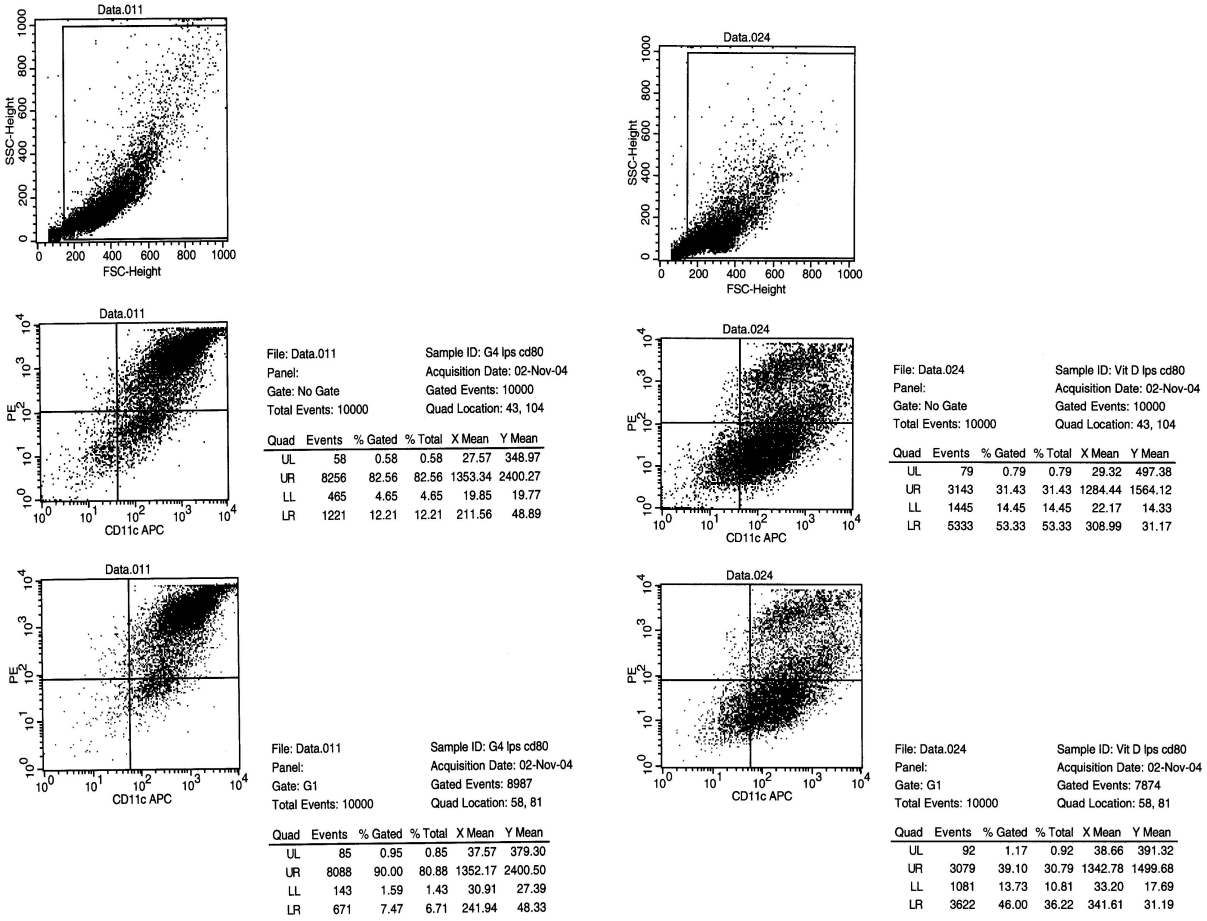


File: Data.025 Sample ID: Vit D lps cd86
Panel: Acquisition Date: 02-Nov-04
Gate: G1 Gated Events: 8201
Total Events: 10000 Quad Location: 58, 81

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	191	2.33	1.91	39.03	700.20
UR	3216	39.21	32.16	1194.11	2031.51
LL	1181	14.40	11.81	31.41	17.79
LR	3613	44.06	36.13	401.99	24.35

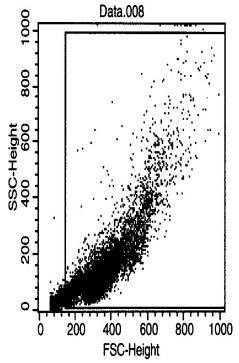
A B

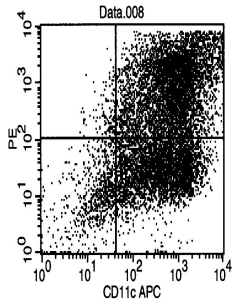
Fig. 3.13 CD86 expression of DCs from untreated (A) bone marrow cultures and vit. D treated (B) cultures LPS stimulated



A B

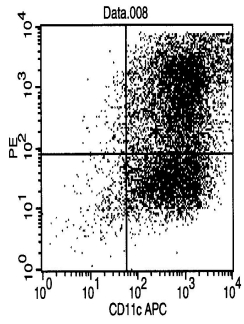
Fig. 3.14 CD80 expression of DCs from untreated (A) bone marrow cultures and vit D treated (B) cultures LPS stimulated





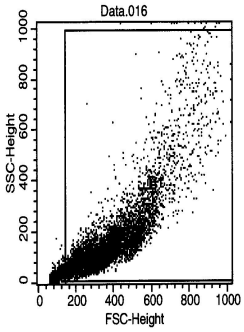
File: Data.008 Sample ID: G4 cd86
Panel: Acquisition Date: 02-Nov-04
Gate: No Gate Gated Events: 10000
Total Events: 10000 Quad Location: 43, 104

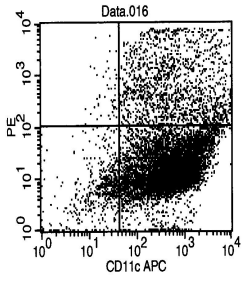
Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	132	1.32	1.32	28.58	426.60
UR	5338	53.38	53.38	1466.70	1506.74
LL	757	7.57	7.57	19.78	17.33
LR	3773	37.73	37.73	753.30	35.13



File: Data.008 Sample ID: G4 cd86
Panel: Acquisition Date: 02-Nov-04
Gate: G1 Gated Events: 8750
Total Events: 10000 Quad Location: 58, 81

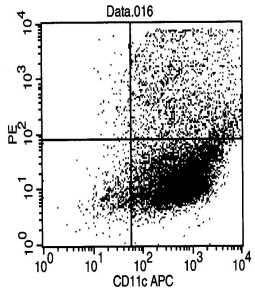
Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	145	1.66	1.45	38.17	529.67
UR	5254	60.05	52.54	1498.80	1494.40
LL	294	3.36	2.94	29.79	21.00
LR	3057	34.94	30.57	832.31	32.32





File: Data.016 Sample ID: ASP cd86
Panel: Acquisition Date: 02-Nov-04
Gate: No Gate Gated Events: 10000
Total Events: 10000 Quad Location: 43, 104

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	54	0.54	0.54	26.73	784.67
UR	1492	14.92	14.92	1736.71	1409.60
LL	564	5.64	5.64	21.70	10.91
LR	7890	78.90	78.90	838.38	20.02



File: Data.016 Sample ID: ASP cd86
Panel: Acquisition Date: 02-Nov-04
Gate: G1 Gated Events: 9246
Total Events: 10000 Quad Location: 58, 81

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	83	0.90	0.83	37.78	819.30
UR	1561	16.88	15.61	1817.66	1313.55
LL	439	4.75	4.39	32.73	11.61
LR	7163	77.47	71.63	849.64	19.20

A B

Fig. 3.15 CD86 expression of DCs from untreated (A) bone marrow cultures and aspirin treated (B) cultures

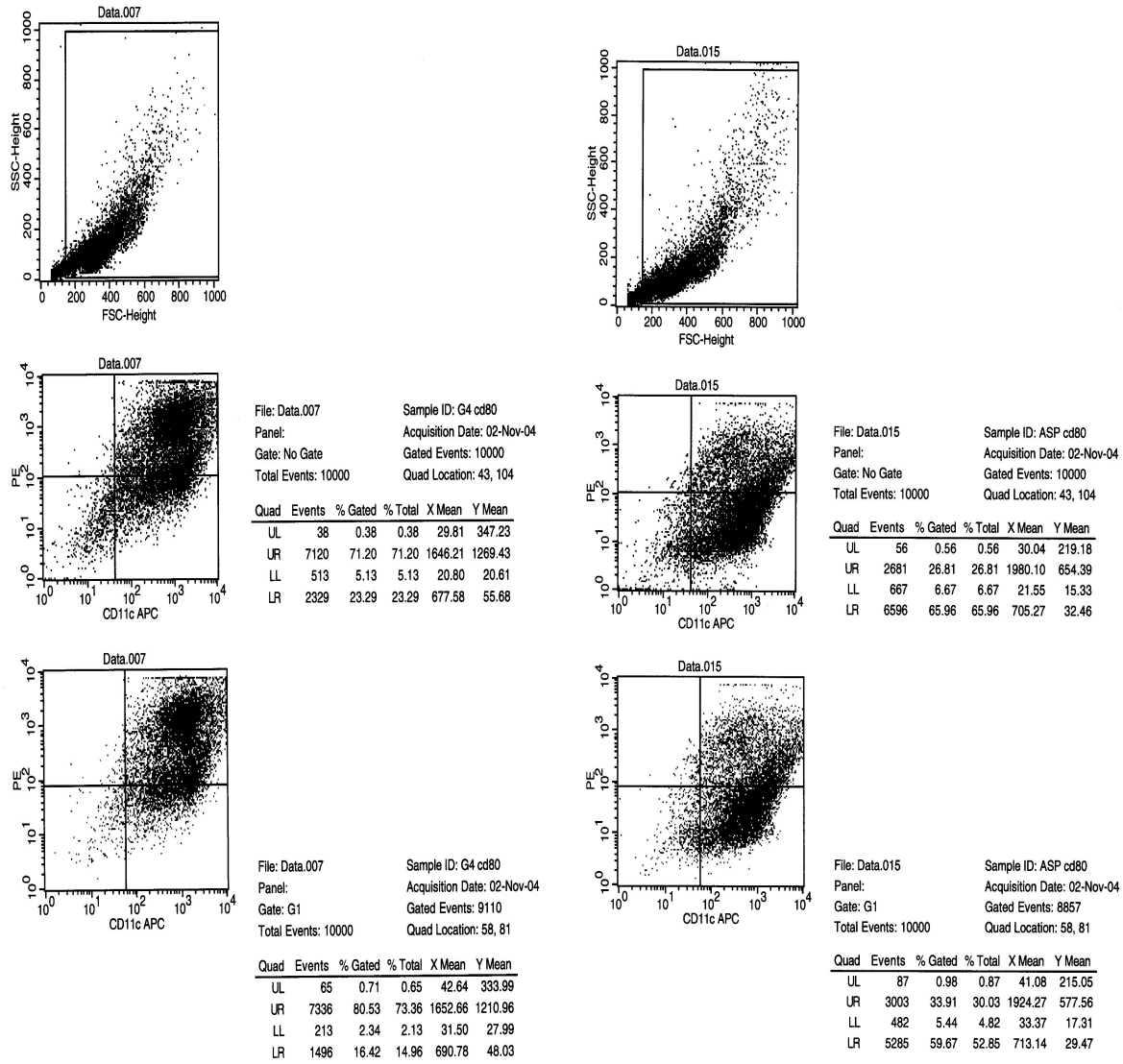
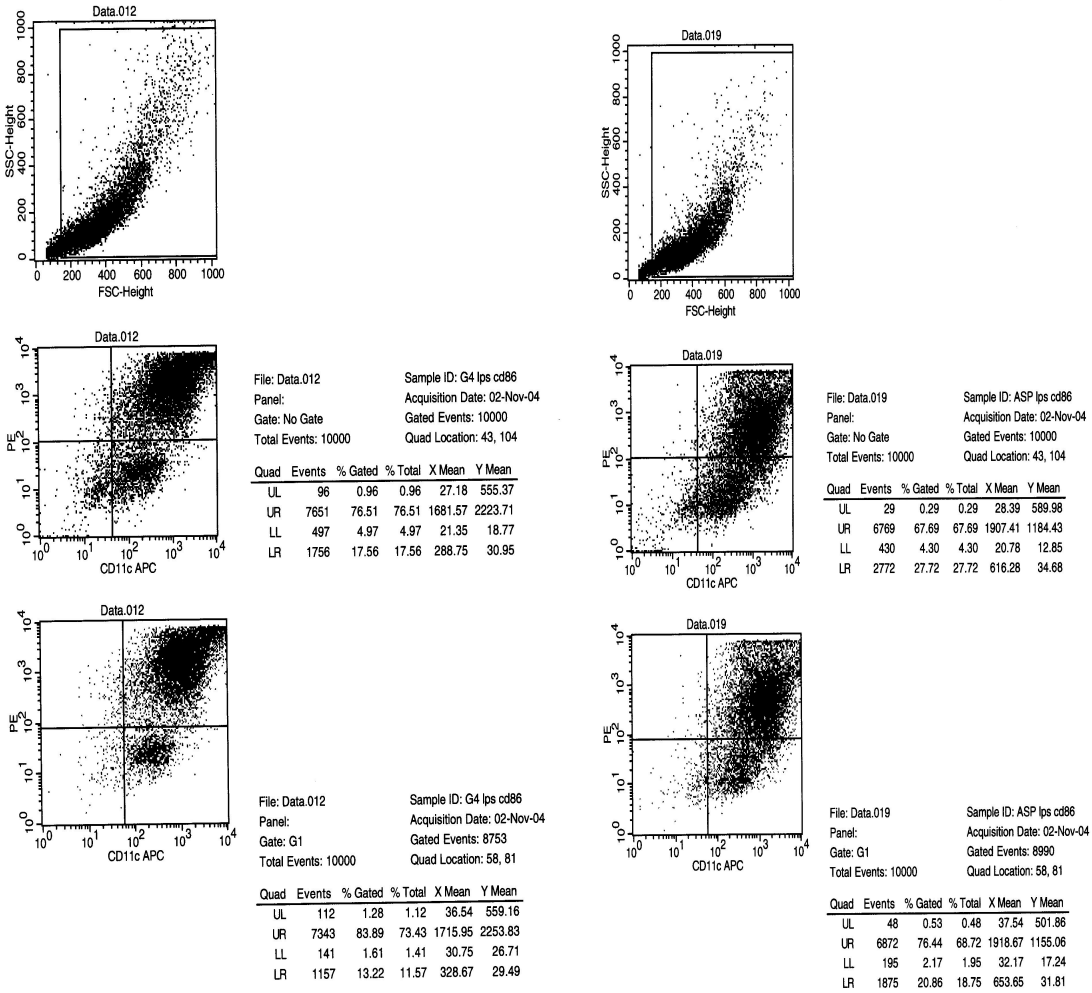
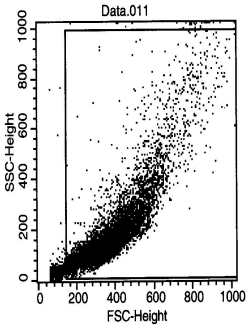


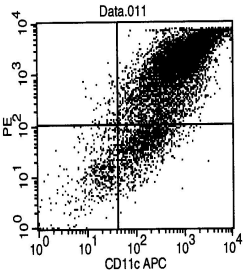
Fig. 3.16 CD80 expression of DCs from untreated (A) bone marrow cultures and aspirin treated (B) cultures



A **B**

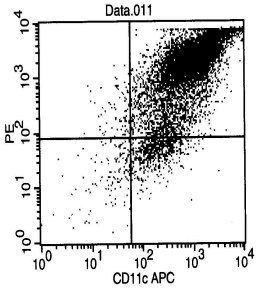
Fig. 3.17 CD86 expression of Ccs untreated (A) bone marrow cultures and aspirin treated (B) cultures LPS stimulated





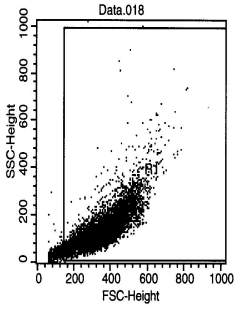
File: Data.011 Sample ID: G4 lps cd80
Panel: Acquisition Date: 02-Nov-04
Gate: No Gate Gated Events: 10000
Total Events: 10000 Quad Location: 43, 104

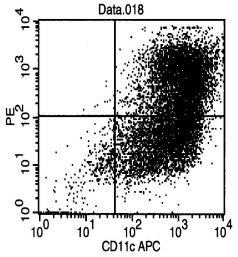
Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	58	0.58	0.58	27.57	348.97
UR	8256	82.56	82.56	1353.34	2400.27
LL	465	4.65	4.65	19.85	19.77
LR	1221	12.21	12.21	211.56	48.89



File: Data.011 Sample ID: G4 lps cd80
Panel: Acquisition Date: 02-Nov-04
Gate: G1 Gated Events: 8987
Total Events: 10000 Quad Location: 58, 81

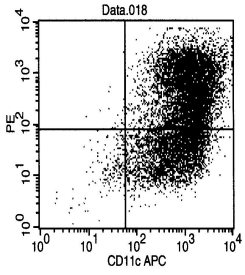
Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	85	0.95	0.85	37.57	379.30
UR	8088	90.00	80.88	1352.17	2400.50
LL	143	1.59	1.43	30.91	27.39
LR	671	7.47	6.71	241.94	48.33





File: Data.018 Sample ID: ASP lps cd80
Panel: Acquisition Date: 02-Nov-04
Gate: No Gate Gated Events: 10000
Total Events: 10000 Quad Location: 43, 104

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	13	0.13	0.13	28.81	226.83
UR	5739	57.39	57.39	1752.66	965.80
LL	354	3.54	3.54	19.75	13.40
LR	3894	38.94	38.94	879.89	38.75



File: Data.018 Sample ID: ASP lps cd80
Panel: Acquisition Date: 02-Nov-04
Gate: G1 Gated Events: 9266
Total Events: 10000 Quad Location: 58, 81

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	21	0.23	0.21	40.20	287.73
UR	6046	65.25	60.46	1740.93	913.54
LL	187	2.02	1.87	33.42	20.93
LR	3012	32.51	30.12	940.85	34.50

A B

Fig. 3.18 CD80 expression of DCs from untreated (A) bone marrow cultures and aspirin treated (B) cultures LPS stimulated

The following graph shows the Mean Fluoresce Intensity for CD86 in BM cultures treated with vitamin D, aspirin, IL10, with and without LPS stimulated vs untreated cells (G4 cells).

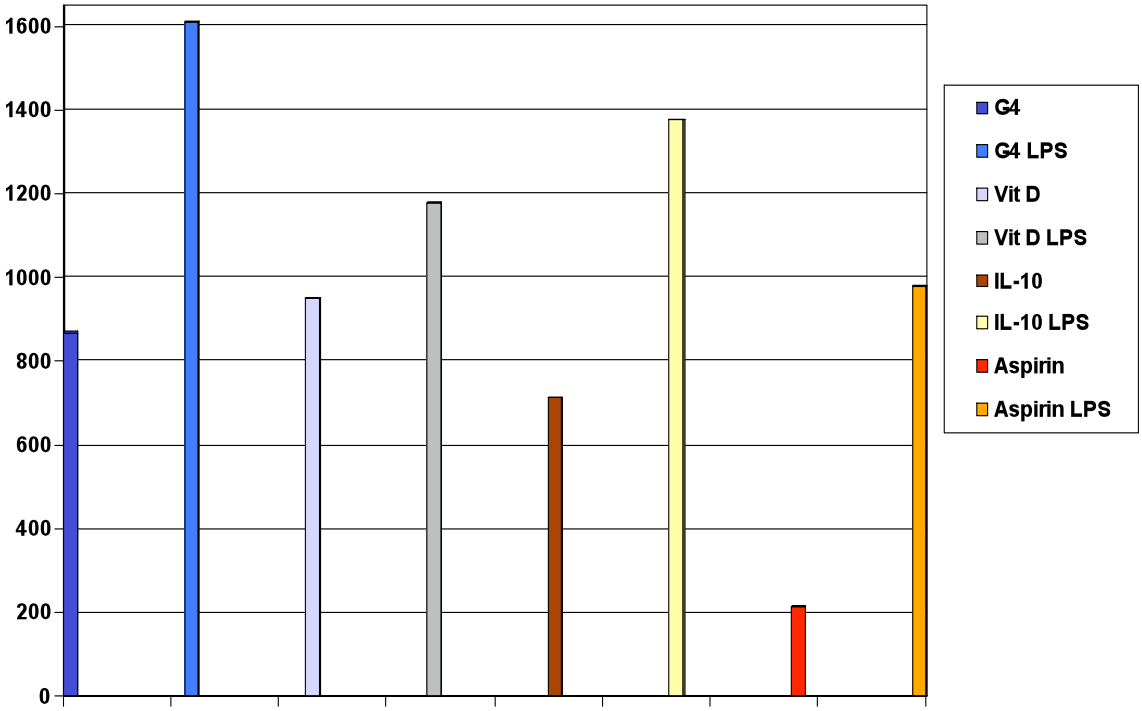


Fig. 3.19 MFI for CD86 on Dcs from treated vs untreated BM coltures with/without LPS

Mature dendritic cells are able to active T lymphocytes. In Type 1 Diabetes, T cell activation leads to beta cell destruction. Immature dendritic cells are not able to present antigen to lymphocytes and to active T cells. Dendritic cells unable to active T cells are tolerogenic dendritic cells (dendritic cells able to induce immune tolerance). Type 1 Diabetes is a complex disease but inducing tolerance can be a strategy to start fighting autoimmune diabetes.

Antigen presentation is an important mechanism of defense against many enemies, like viruses, bacteria, etc., so the gold standard to achieve would be to induce a specific tolerance able to stop the autoimmune process. However, in order to be able to induce a

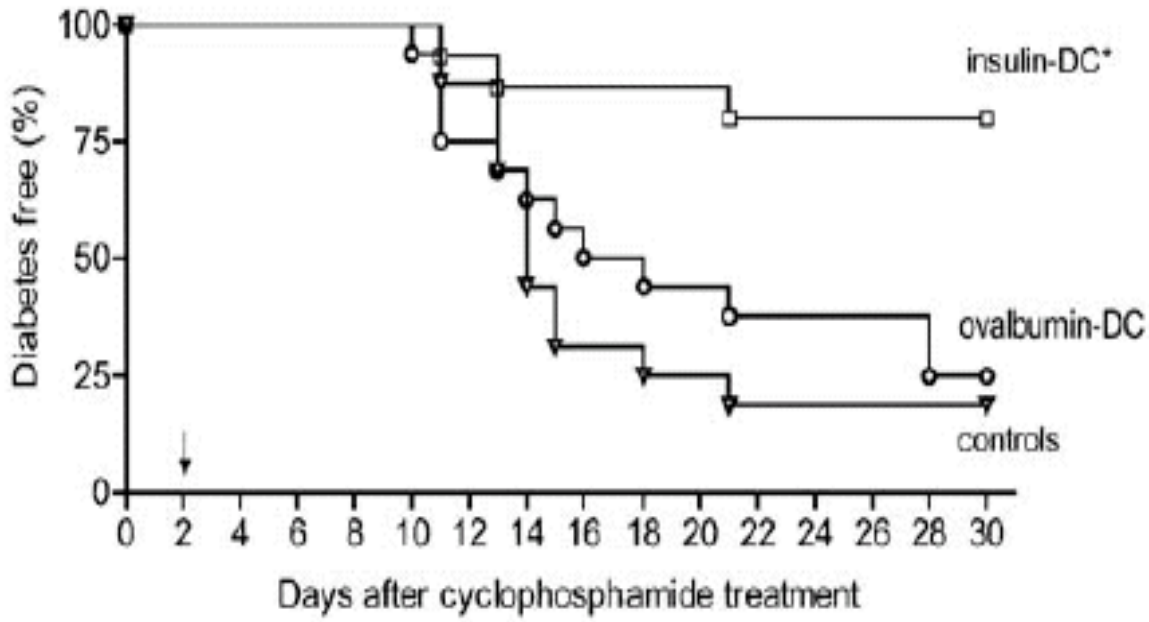
specific tolerance, we should know how to modulate dendritic cell phenotype and behavior. We studied dendritic cells and dendritic cell modulation characterized by changes in the surface molecules.

We tested drugs on our bone marrow cultures checking DCs maturation and surface molecule modulation by analyzing treated cells versus untreated cells. We observed that treatment with chemicals, especially aspirin and vitamin D, lower the costimulatory molecule expression which may be helpful in transitioning dendritic cells towards tolerogenic behavior.

CY in Vivo Experiments

In the paper Diabetologia (2003) 46: 1357-1365, authors stimulated with insulin or OVA the generation of an NOD DC1 cell line with /without IL10 treatement and then they injected the cell lines into mice two days after CY treatment. They showed mice treated with the insulin treated DC cell line were protected from diabetes.

Fig. 3.20 Mice treated with the insulin treated DC cell line are protected from diabetes (Diabetologia 2003, 46: 1357-1365)



We wanted to apply this model to our experimentation. So, we pulsed two groups (treated or not treated with aspirin) of our BMDCs with 400ug/ml insulin for 6 h, and we injected them in mice two days after CY injection.

Unfortunately it didn't work, so we repeated the experiment without insulin pulsation. Shortly, we realized the CY model wasn't a good model for our experiment. The CY model gave us 60% diabetes incidence. Even if this percentage corresponds to the literature, our study was in need of a diabetes model giving a better diabetes incidence.

Although the CY model was not optimal for our study, we still wanted to pursue the CY mechanism of diabetes induction. We induced diabetes by CY in 3 NOD mice. We isolated T cells from the diabetic mice and we transferred 5 x 10^6 total T cells into 5 male and 1 female NOD.SCID recipients. Transferring T cells from non CY-induced diabetic mice to NOD.SCID recipients induces diabetes. We wanted to study if the transfer of the T cells from CY-induced diabetic mice would also induce diabetes in the NOD.SCID recipients. Sixty days after the T cell transfer, both males and female were healthy. The experiment demonstrated that CY is able to directly induce diabetes in NOD mice, while T cells from CY-induced diabetic NOD does not. We concluded CY induces diabetes through a non-T cell mediated mechanism.

Co-Transfer experiments

To study the tolerance induction ability of aspirin treated dendritic cells from bone marrow, we cultured bone marrow from 10 week old NOD mice, we treated them with aspirin (see methods section) and we transferred the cells into NOD.SCID recipient mice along with T cells from diabetic mice. The control group was represented by NOD.SCID mice who received the same treatment using untreated dendritic cells from bone marrow instead of aspirin treated DCs. On both treated and untreated dendritic cells from the same experiment, we performed flow cytometry studies to analyze surface molecule expression (fig. 3.21- 3.22 – 3.23).

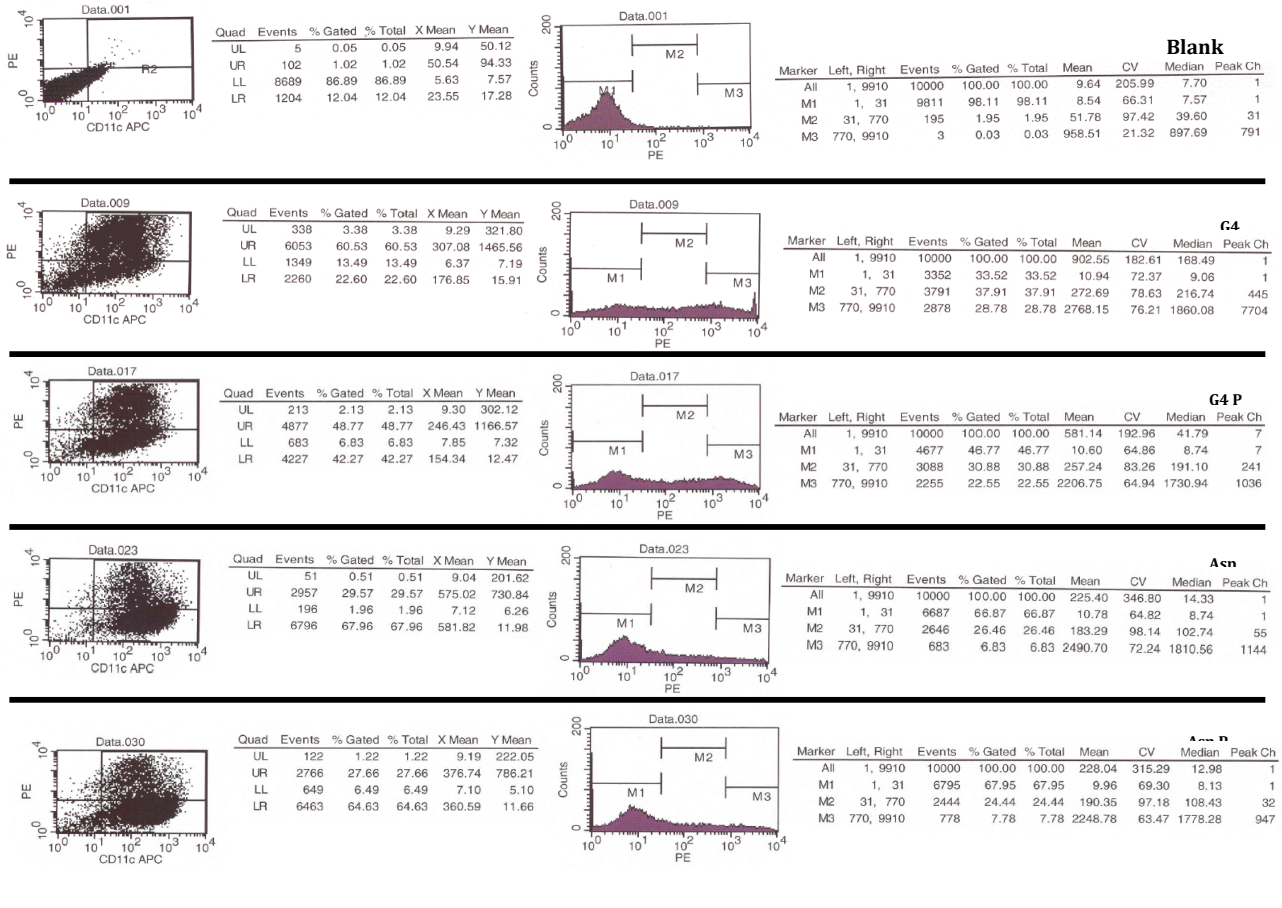


Fig. 3.21 CD86 expression on treated/untreated BMDCs

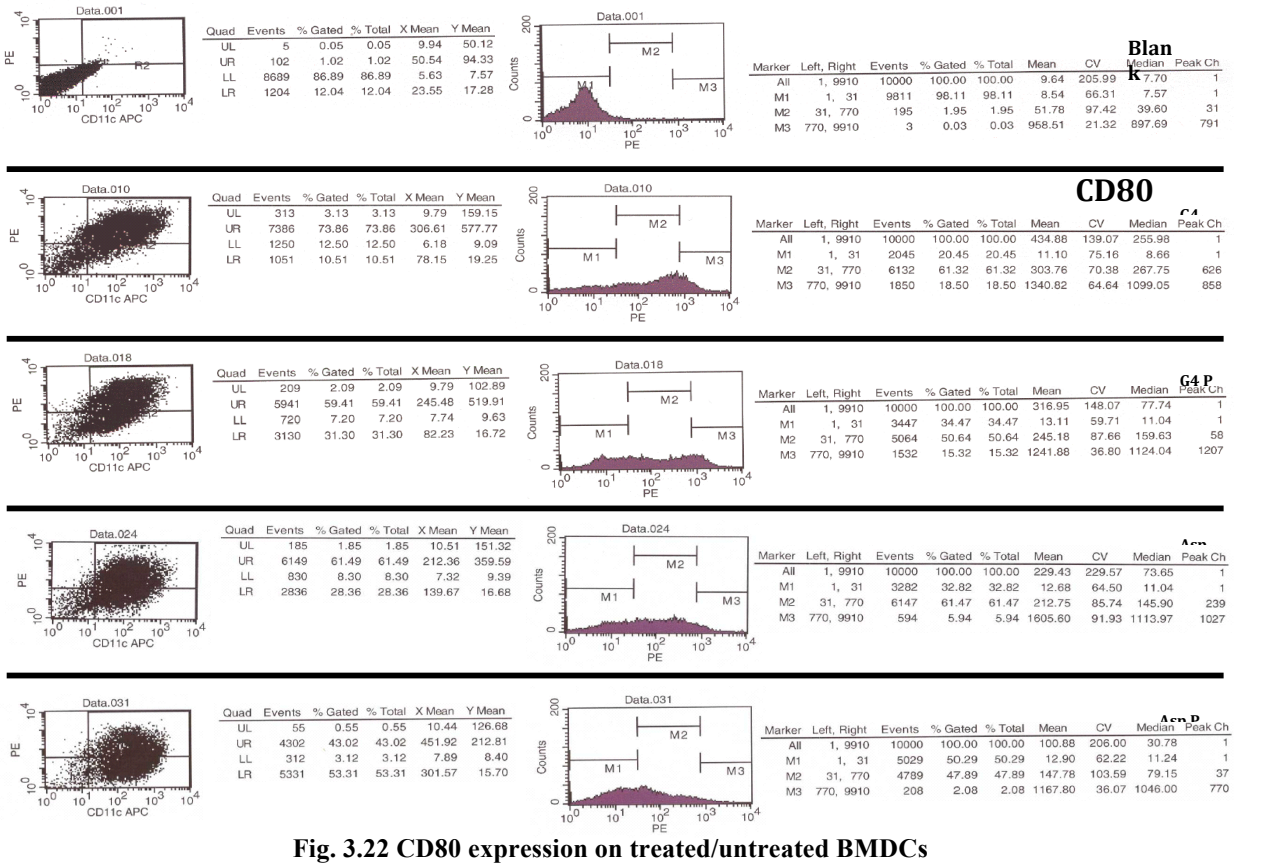
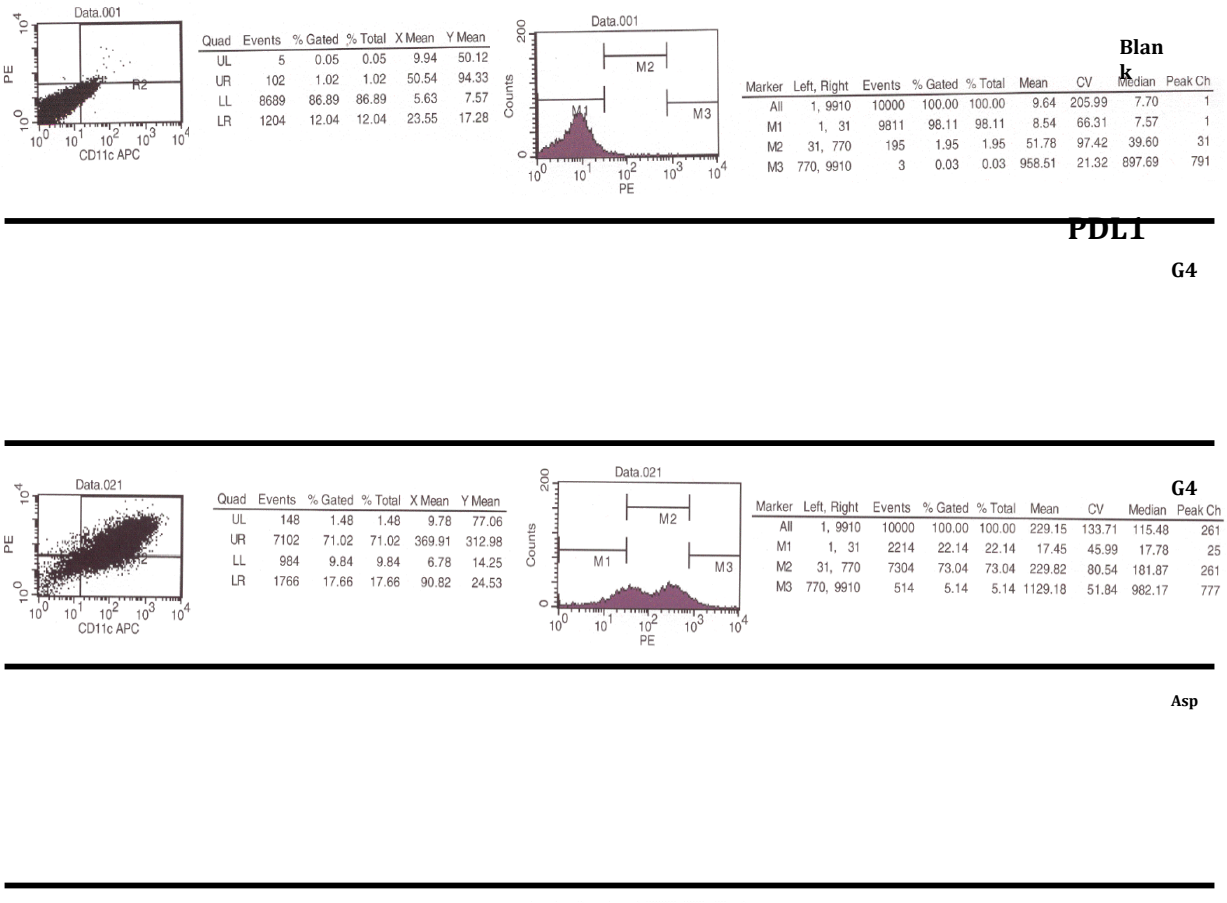


Fig. 3.22 CD80 expression on treated/untreated BMDCs



Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	5	0.05	0.05	9.94	50.12
UR	102	1.02	1.02	50.54	94.33
LL	8689	86.89	86.89	5.63	7.57
LR	1204	12.04	12.04	23.55	17.28

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	9.64	205.99	7.70	1
M1	1, 31	9811	98.11	98.11	8.54	66.31	7.57	1
M2	31, 770	195	1.95	1.95	51.78	97.42	39.60	31
M3	770, 9910	3	0.03	0.03	958.51	21.32	897.69	791

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	148	1.48	1.48	9.78	77.06
UR	7102	71.02	71.02	369.91	312.98
LL	984	9.84	9.84	6.78	14.25
LR	1766	17.66	17.66	90.82	24.53

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	229.15	133.71	115.48	261
M1	1, 31	2214	22.14	22.14	17.45	45.99	17.78	25
M2	31, 770	7304	73.04	73.04	229.82	80.54	181.87	261
M3	770, 9910	514	5.14	5.14	1129.18	51.84	982.17	777

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	81	0.81	0.81	10.20	94.01
UR	8855	88.55	88.55	545.25	340.73
LL	407	4.07	4.07	7.37	13.07
LR	657	6.57	6.57	76.16	25.00

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	304.65	91.95	259.46	299
M1	1, 31	866	8.66	8.66	17.20	51.72	18.27	29
M2	31, 770	8616	86.16	86.16	283.00	57.96	266.55	299
M3	770, 9910	540	5.40	5.40	1114.93	43.34	1000.00	805

Fig. 3.23 PDL1 expression on treated/untreated BMDCs

In the co-transfer model, 2 weeks after the co-transfer, a second shot of dendritic cells (without T cells from diabetic mice) is necessary. So, once again we cultured bone marrow from 10 week old NOD mice, we treated them with aspirin (see methods section) and we transferred the cells into NOD.SCID recipient mice. The control group was represented by NOD.SCID mice who received untreated dendritic cells from bone marrow. On both treated and untreated cells from the same experiment we performed flow cytometry studies to analyze surface molecule expression

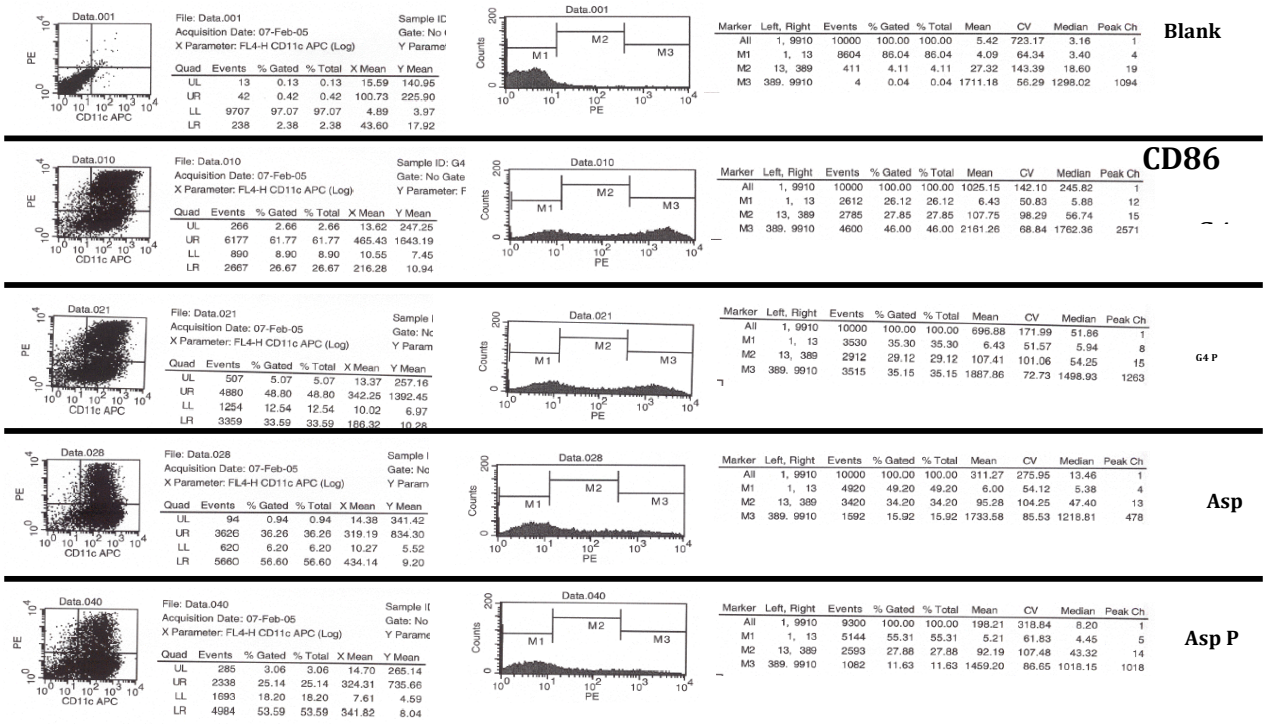
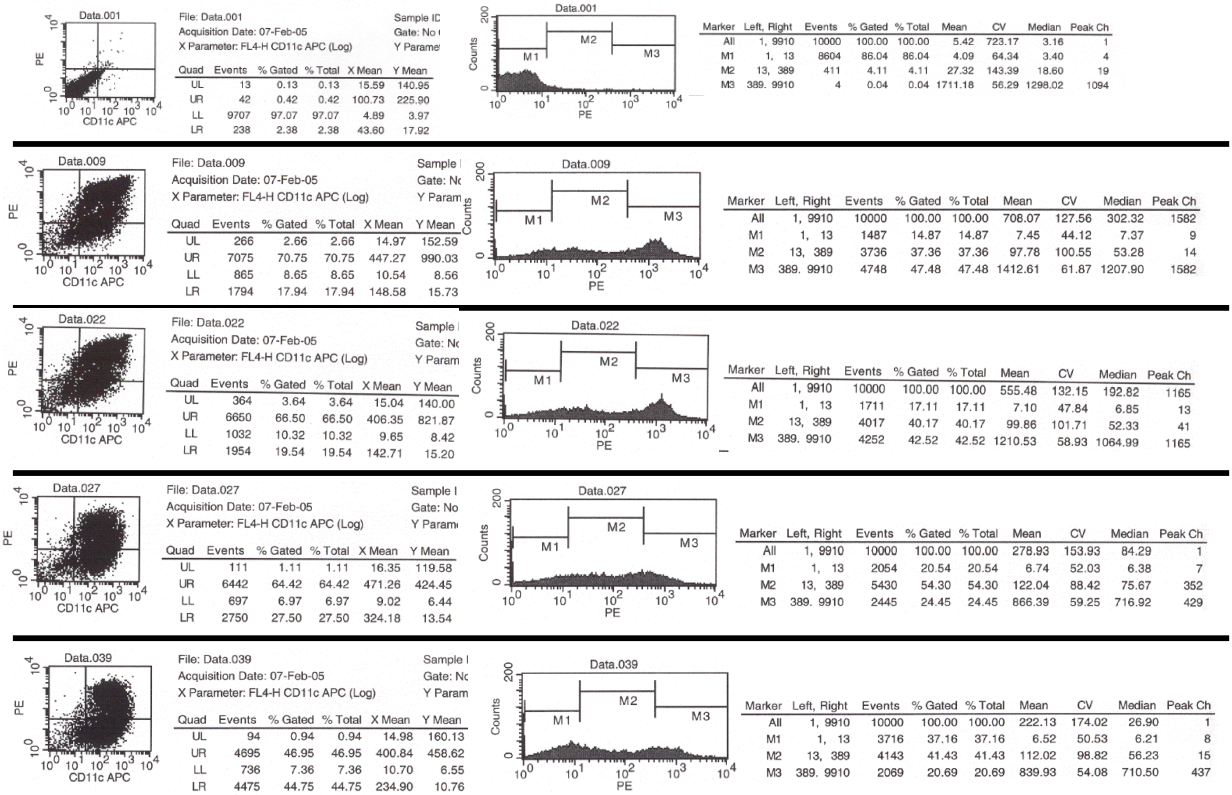


Fig. 3.24 CD86 expression on treated/untreated BMDCs

Fig. 3.25 CD80 expression on treated/untreated BMDCs



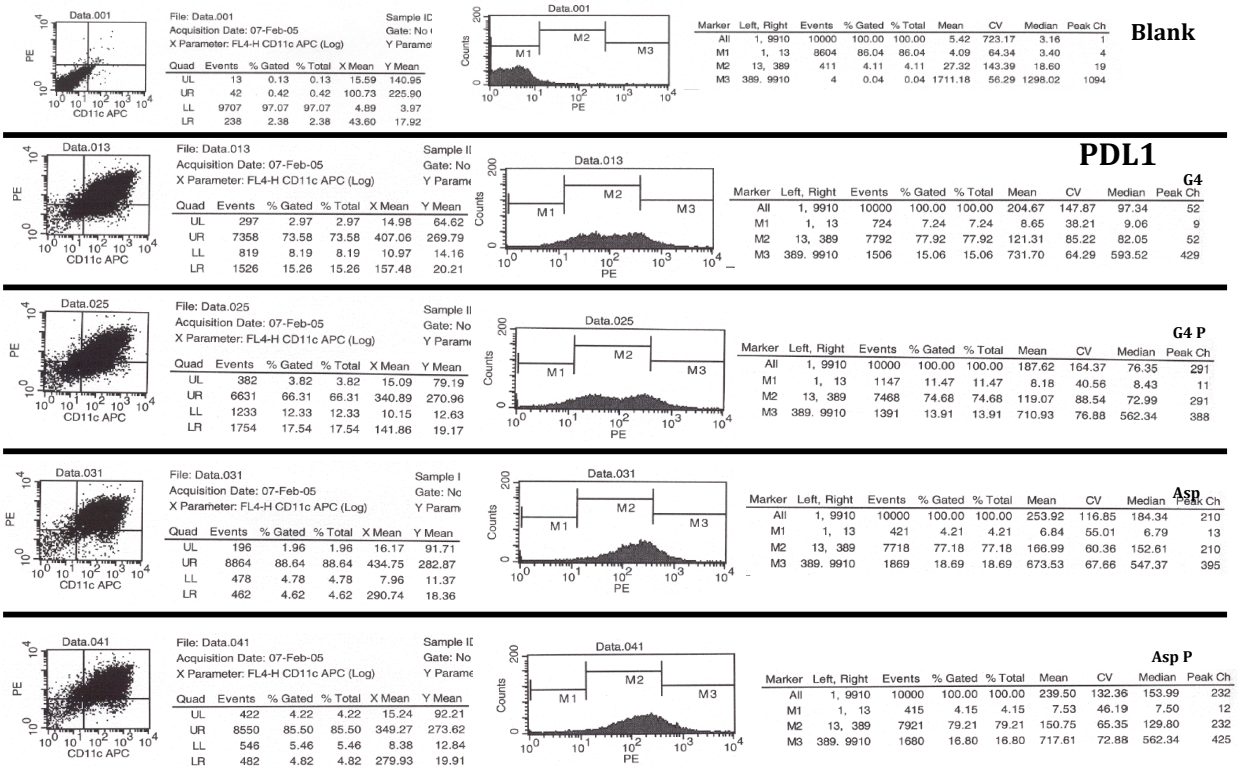
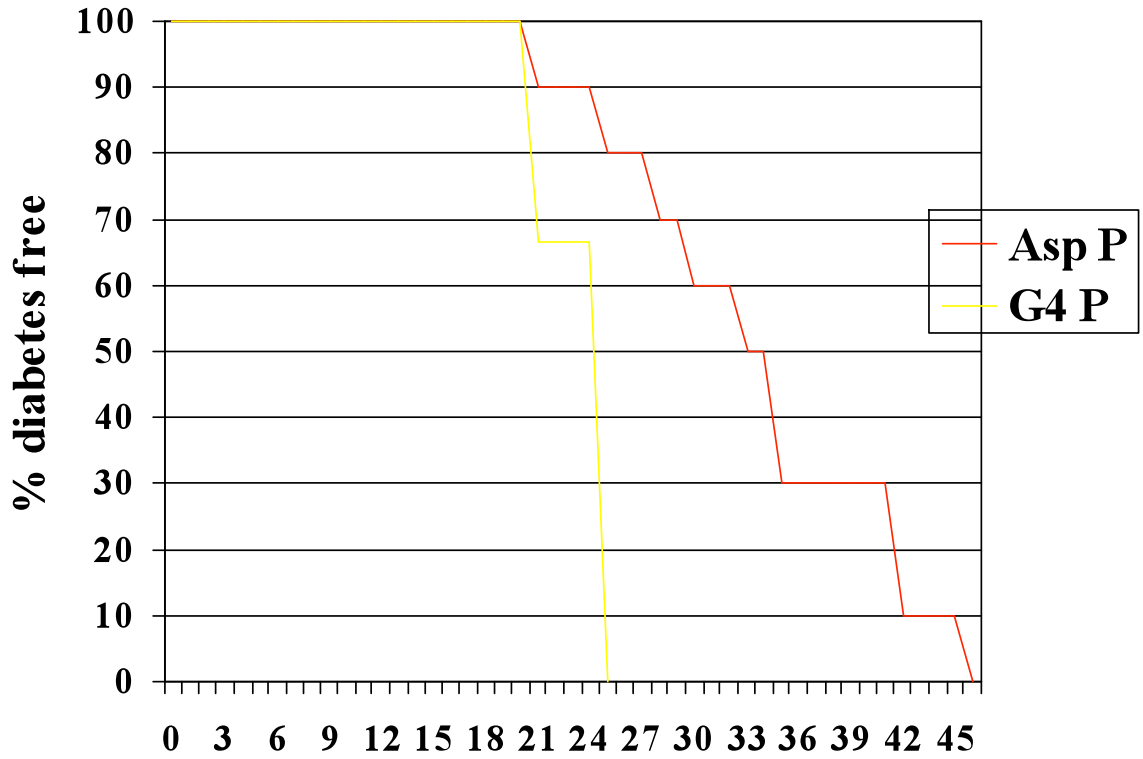


Fig. 3.26 PDL1 expression on treated/untreated BMDCs

The following is the graph of the co-transfer result:

Fig. 3.27 Delay on diabetes onset in mice transferred with Aspirin treated insulin pulsed BMDCs



The diabetes onset in mice treated with Aspirin treated, insulin peptide pulsed BMDCs was delayed compared to mice treated with untreated insulin pulsed BMDCs. The experiment shows that aspirin modulation of bone marrow dendritic cells may be useful in the prevention of diabetes onset in mice.

We repeated the experiment hoping to increase the difference in the time of diabetes onset in the two groups. Again we analyzed BMDCs by flow cytometry.

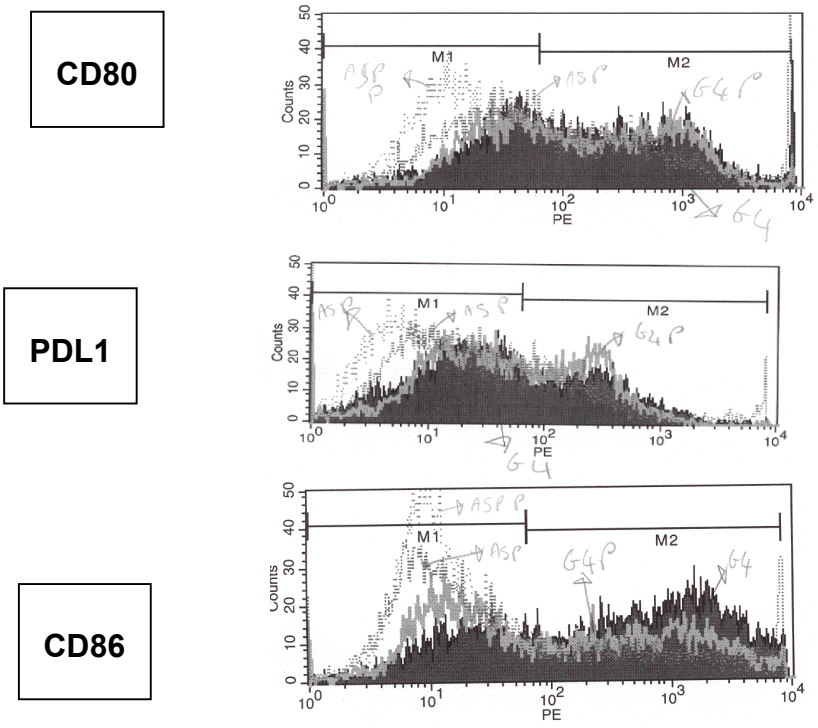
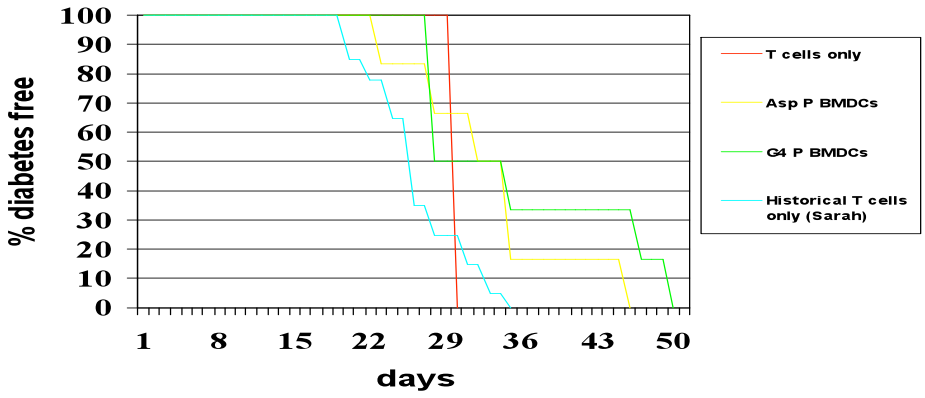


Fig. 3.28 MFI expression for CD80, CD86 and PDL1 on treated and untreated BMDCs

However, we didn't have the expected results.

Fig. 3.29 Graph showing diabetes onset in the transferred mice



Analyzing data from the first and the second transfer together, the graph shows no difference between the two treatment groups.

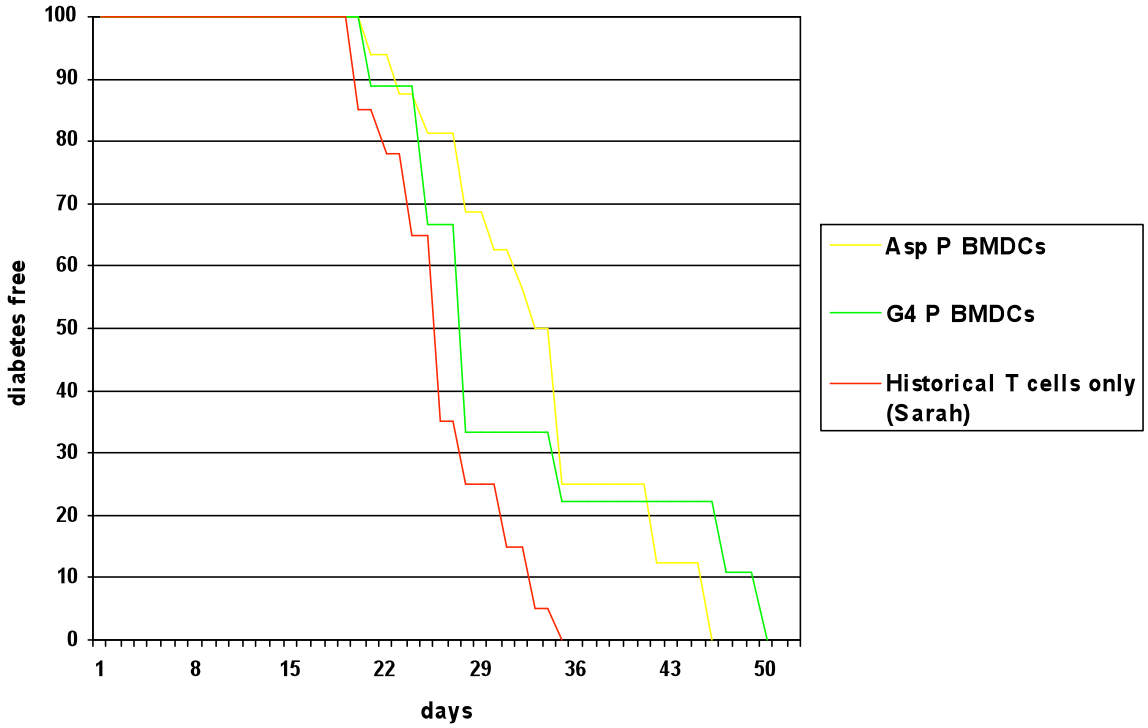


Fig. 3.30 Graph showing diabetes onset in mice from both the co-trasnfer experiments

A marker for dendritic cell maturation, CD209, was checked by RT-PCR (Fig. 3.31) on samples from BMDCs cultures (some samples are from the cultures used for the co-transfer experiments, some samples are from other cultures). The presence of a band where the arrow in the picture is pointing should be an indicator of immature dendritic cells, the absence of a band should be a sign of mature dendritic cells.

Fig. 3.31 RT-PCR on BMDCs. The wells 5, 7, 12, 13 are samples from the co-transfer experiments. 7= aspirin treated cells used for the first co-transfer experiment (immature) 5=untreated cells used for the second co-transfer experiment (mature) 12, 13=aspirin treated used for the second co-transfer (mature) all the samples are beta-actin normalyzed

The samples that are interesting to us are the samples 5, 7, 12 and 13. While samples 5 and 7 look as we expected, samples 12 and 13 are different. Something was different with these cells. We thought we were injecting immature cells into our recipient mice while we were acutally injecting mature cells. This was probably caused by an operator mistake or a failure in the aspirin mechanism to give us the expected results.

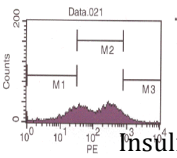
We tried the same experimental model using vitamin D treated BMDCs, however the study showed that there was no difference at all between mice receiving untreated BMDCs and mice receiving vitamin D treated BMDCs.

In order to understand why we did not obtain the expected results, we re-analyzed all the data. (fig. 3.32) By analyzing the expression of PDL1, we noticed that in the first aspirin co-transfer experiment, PDL1 expression is higher in aspirin treated BMDCs than in untreated BMDCs in both the initial and the booster transfers. While in the second aspirin co-transfer experiment, PDL1 expression was good in the initial transfer but was lower than the untreated BMDCs in the booster transfer.

Analyzing the flow cytometry results of the vitamin D co-transfer experiment, we noticed that vitamin D doess not change the expression of PDL1 on BMDCs, which means that the PDL1 expression on vitamin D treated BMDCs is the same as on untreated BMDCs. The consequence of this observation is that may be PDL1 a key molecule in Type 1 Diabetes.

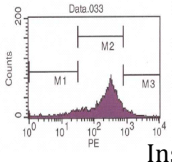
FIG.3.32 a-PDL1 expression in the first aspirin co- transfer experiment

Insulin peptide pulsed untreated BMDCs first injection first co- transfer



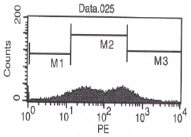
Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	229.15	133.71	115.48	261
M1	1, 31	2214	22.14	22.14	17.45	45.99	17.78	25
M2	31, 770	7304	73.04	73.04	229.82	80.54	181.87	261
M3	770, 9910	514	5.14	5.14	1129.18	51.84	982.17	777

Insulin peptide pulsed aspirin treated BMDCs first injection first co- transfer



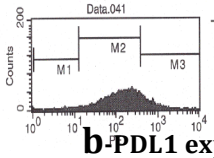
Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	304.65	91.95	259.46	299
M1	1, 31	866	8.66	8.66	17.20	51.72	18.27	29
M2	31, 770	8616	86.16	86.16	283.00	57.96	266.55	299
M3	770, 9910	540	5.40	5.40	1114.93	43.34	1000.00	805

Insulin peptide pulsed untreated BMDCs second injection first co-transfer



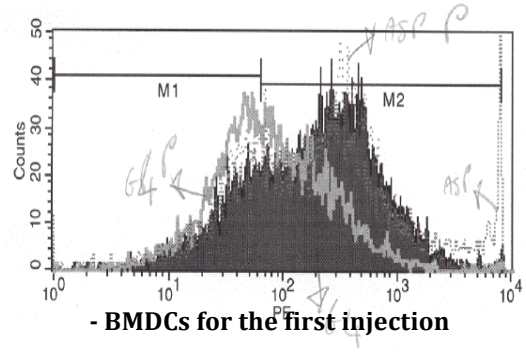
Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	187.62	164.37	76.35	291
M1	1, 13	1147	11.47	11.47	8.18	40.56	8.43	11
M2	13, 389	7468	74.68	74.68	119.07	88.54	72.99	291
M3	389, 9910	1391	13.91	13.91	710.93	76.88	562.34	388

Insulin peptide pulsed aspirin treated BMDCs second injection first co-transfer



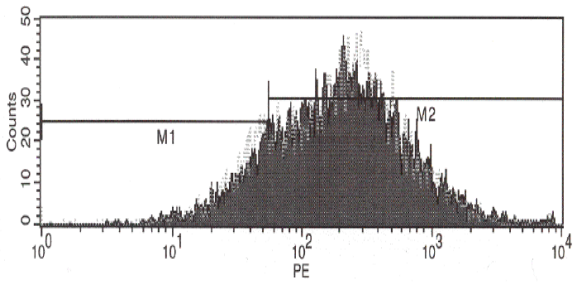
Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	239.50	132.36	153.99	232
M1	1, 13	415	4.15	4.15	7.53	46.19	7.50	12
M2	13, 389	7921	79.21	79.21	150.75	65.35	129.80	232
M3	389, 9910	1680	16.80	16.80	717.61	72.88	562.34	425

b-PDL1 expression in the second aspirin co- transfer experiment



- BMDCs for the first injection

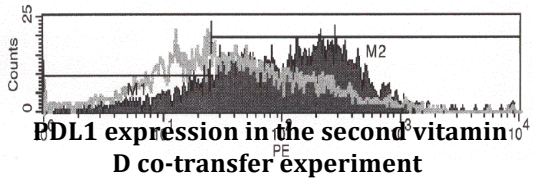
PDL1 G4 P vs Asp P



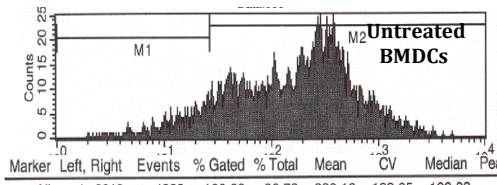
- BMDCs for the second injection

C- PDL1 expression in the first vitamin D co-transfer experiment

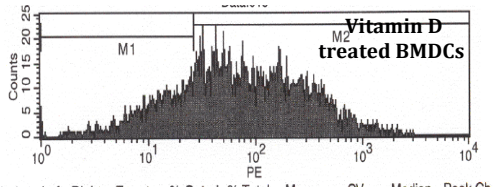
Black line=untreated BMDCs
White line=vitamin D treated BMDCs



PDL1 expression in the second vitamin D co-transfer experiment



Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	4339	100.00	86.78	290.16	122.85	192.82	375
M1	1, 27	427	9.84	8.54	15.72	43.97	16.25	25
M2	27, 9910	3915	90.23	78.30	319.90	113.48	226.71	375



Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	4186	100.00	83.72	152.52	154.44	64.36	31
M1	1, 27	1017	24.30	20.34	14.41	47.52	14.20	24
M2	27, 9910	3180	75.97	63.60	196.25	129.98	104.60	31

G4= untreated BMDCs
G4 P=insulin peptide pulsed untreated BMDCs
Asp=aspirin treated BMDCs
Asp P=insulin peptide aspirin treated BMDCs

Other Treatments

We started to treat our BMDCs cultures using different chemicals in order to modulate PDL1. The following graphs (Fig. 3.33) show a synopsis of these studies.

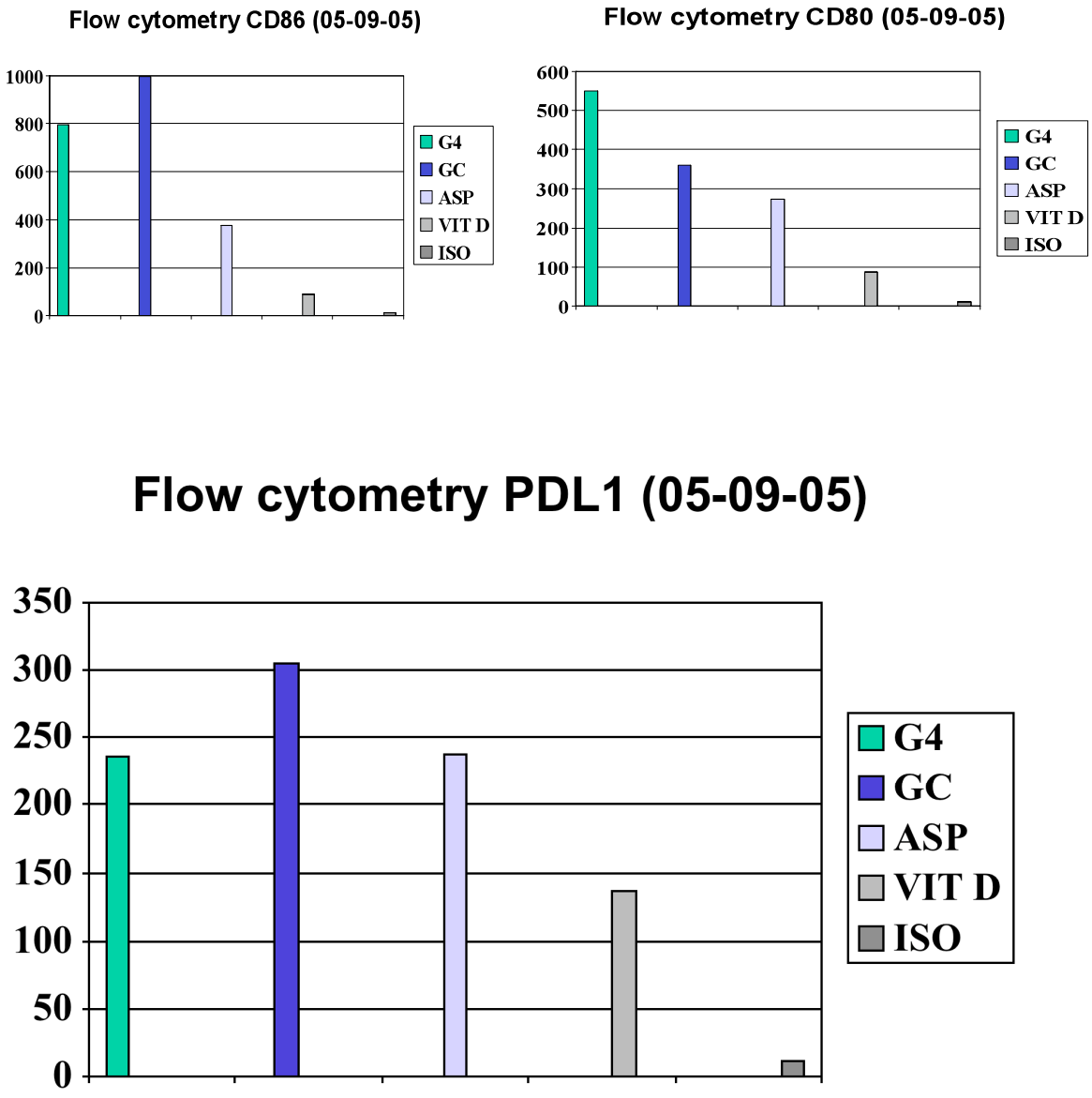


Fig. 3.33 CD80, CD86 and PDL1 expression on Untreated (G4) vs. GCSF treated (GC), Aspirin treated and vitamin D treated BMDCs

ISO=isotype control

We tried an experiment where we compared untreated cells (G4) versus vitamin D treated cells, versus vitamin D and G-CSF treated cells (Fig. 3.34).

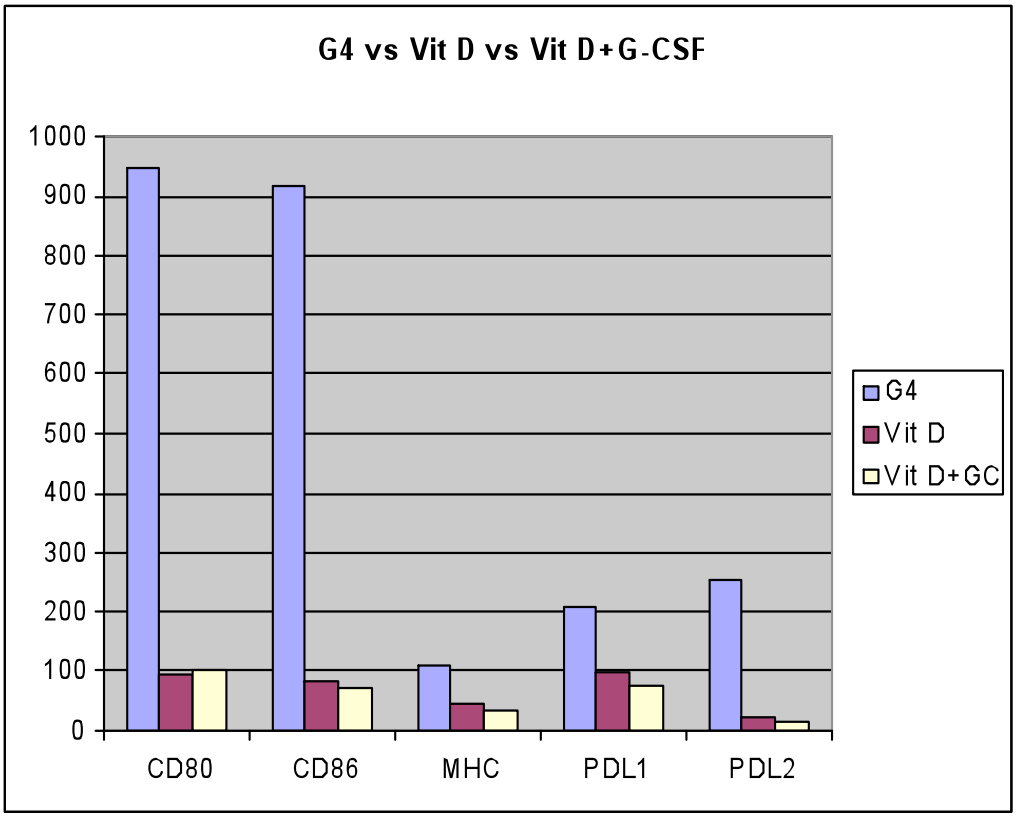


Fig. 3.34 Co-stimulatory molecule expression on untreated (G4) vs Vitamin D (Vit D) or Vitamin D and G-CSF (Vit D+GC) treated BMDCs

These chemicals are good in lowering CD80 and CD86, however, these treatements did not increase the PDL1 expression, the key of this study.

Many chemicals were tested until a natural chemical, aloe vera, caught our attention. We focused some experiments on aloe vera and we tried to use different concentrations (1 ml and 3 ml) of the chemical with and without aspirin and vitamin D (Fig. 3.35).

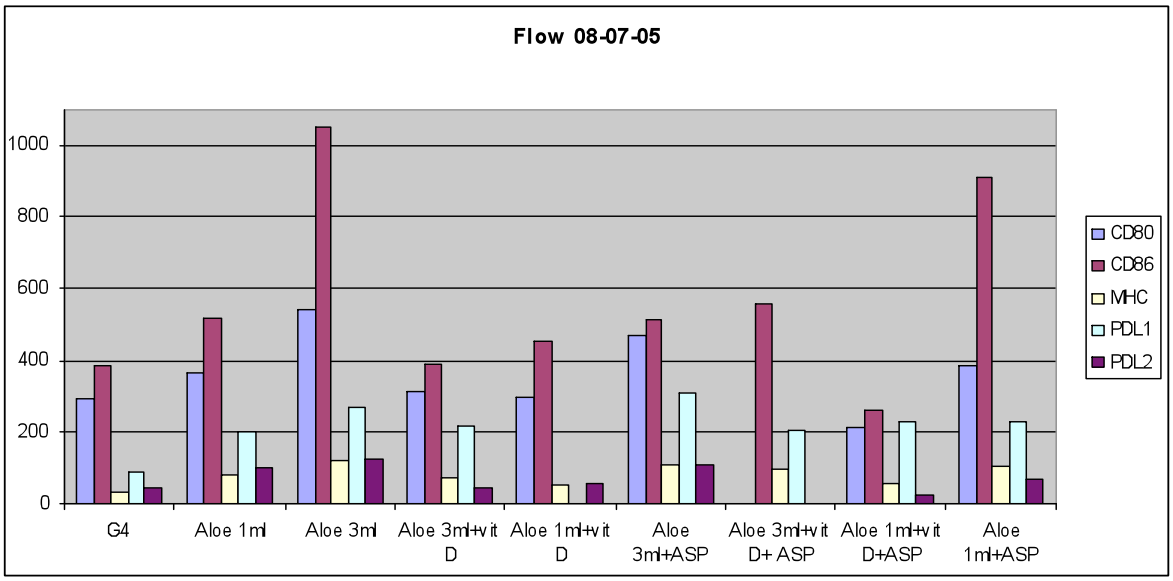
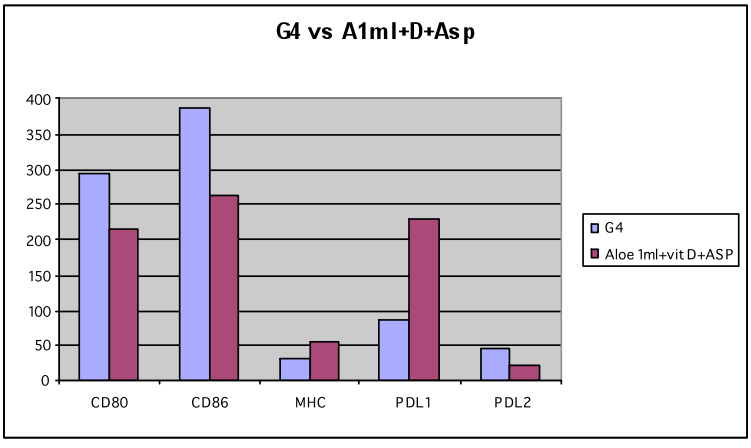


Fig. 3.35 Molecule expression on untreated (G4) BMDCs vs Aloe Vera (Aloe) or Aloe Vera plus Vitamin D and/or Asp treated BMDCs at various concentration of Aloe Vera

One of these combinations caught our attention. Analysing data from the set of cells treated with a low dose (1ml) of Aloe Vera plus vitamin D and aspirin, we observed an increase of PDL1 and a simultaneous decrease of the costimulatory molecules, CD80 and CD86 (Fig. 3.36).

Fig. 3.36 CD80, CD86, PDL1 expression on untreated (G4) vs Aloe plus Vit D and Aspirin treated



BMDCs

At this point we wanted to test the activity of these BMDCs and their potential tolerogenic power. We performed MLR using treated and untreated BMDCs from different treatements. Following there is a graph showing results for the MLR of aspirin treated and vitamin D treated BMDCs versus untreated (G4) cells (fig. 3.37).

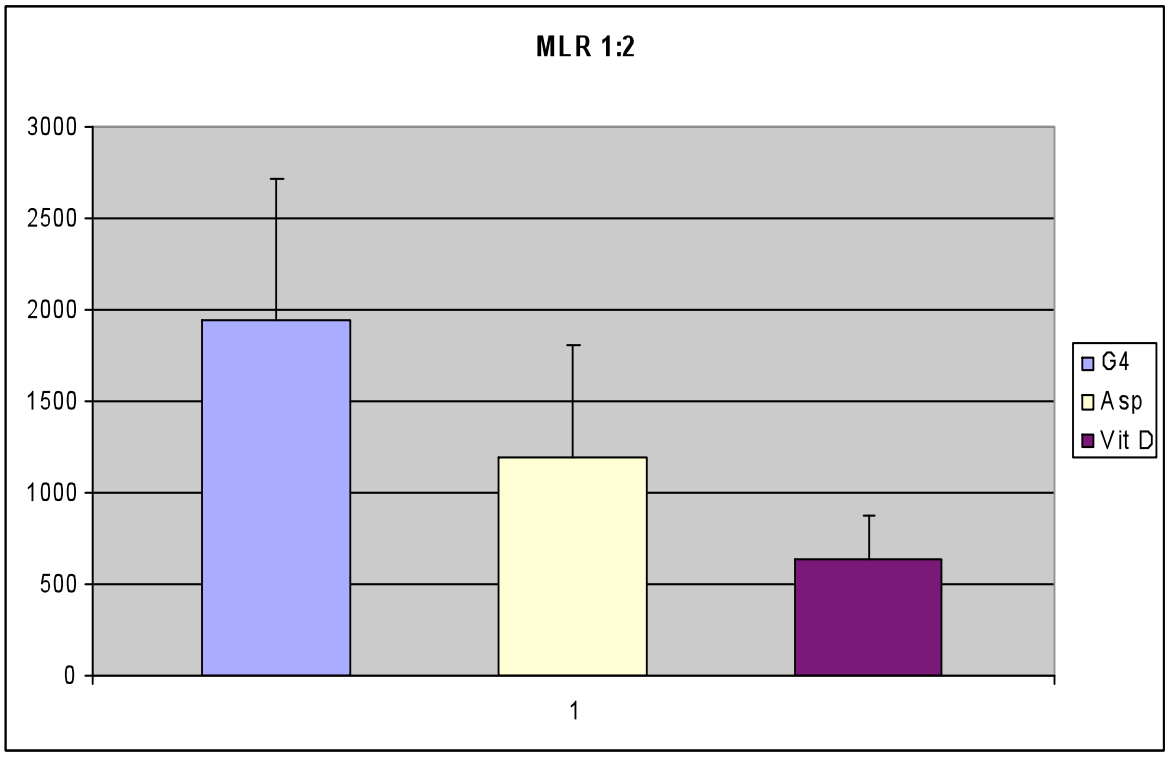


Fig. 3.37 MLR of untreated (G4) vs aspirin and vitamin D treated BMDCs

Aspirin treated and vitamin D treated BMDCs have less power to active T cells, so the two chemicals are useful in producing tolerogenic BMDCs in vitro. We then analyzed MLR data for all the treatments we tested earlier by flow cytometry (Fig. 3.38).

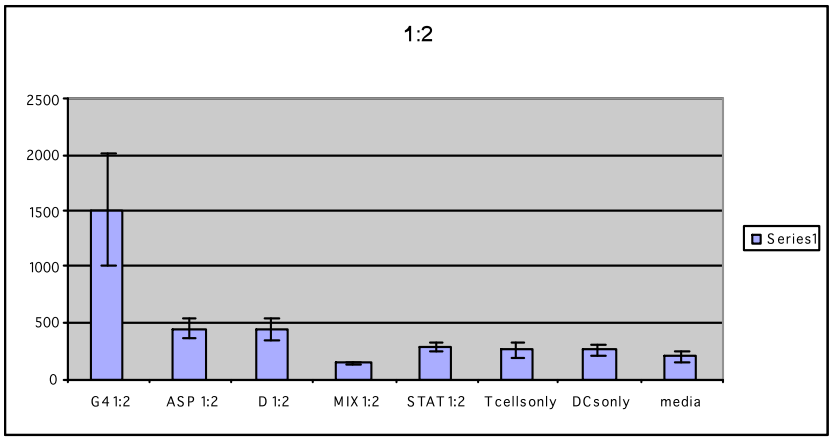


Fig. 3.38 MLR (1:2) of untreated (G4) vs aspirin treated (ASP), vitamin D (D) treated, Aloe plus vitamin D and aspirin treated, statins treated BMDCs

In order to test the role of PDL1 in the interaction of DCs and T cells, we cultured BMDCs and we treated them with aspirin. We used a PDL1 antibody to block the activity of PDL1 in a plate of the cultured cells. We then performed the MLR in aspirin treated, untreated and aspirin treated, PDL1 blocked BMDCs (Fig. 3.39). The following graph shows that aspirin treated BMDCs, where PDL1 expression is higher than untreated BMDCs, have lower T cell proliferation than in the MLR using untreated BMDCs. However, in the MLR where the aspirin treated cells were treated with the PDL1 antibody, the proliferation was higher, even higher than in the MLR using untreated cells. This shows that PDL1 plays a role in the interaction between DCs and T cells and may have a key role in Type 1 Diabetes.

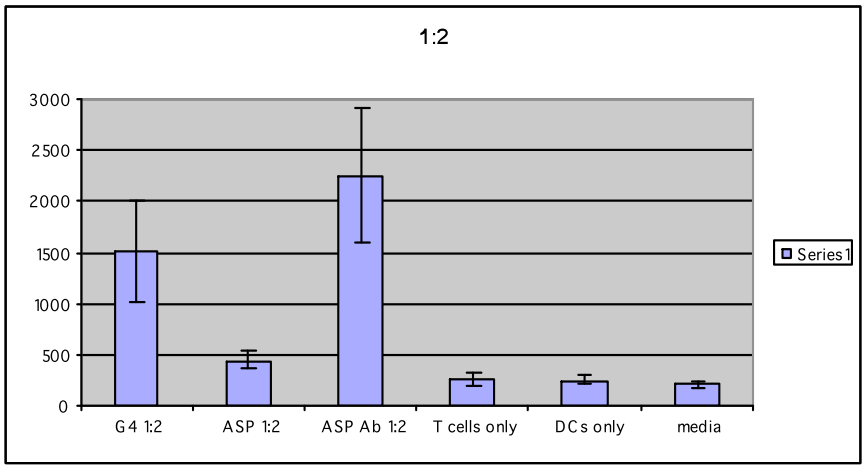


Fig. 3.39 MLR of untreated (G4) vs aspirin treated (ASP) and aspirin treated PDL1 Ab blocked (ASP Ab) BMDCs

MLR using the aloe+vitamin D +aspirin treated BMDCs was performed. The combo treatment lowered the T cell proliferation compared to untreated cells, however the PDL1 Ab was not able to restore the T cell proliferation. At the moment we do not have an explanation for this result, but we hypothesize that aspirin and the combo treatment have different mechanisms of action.

Wanting to explore the role of PDL1, we cultured the NOD DC1 cell lines and used a lentivirus vector to overexpress PDL1 in these cells (3.40).

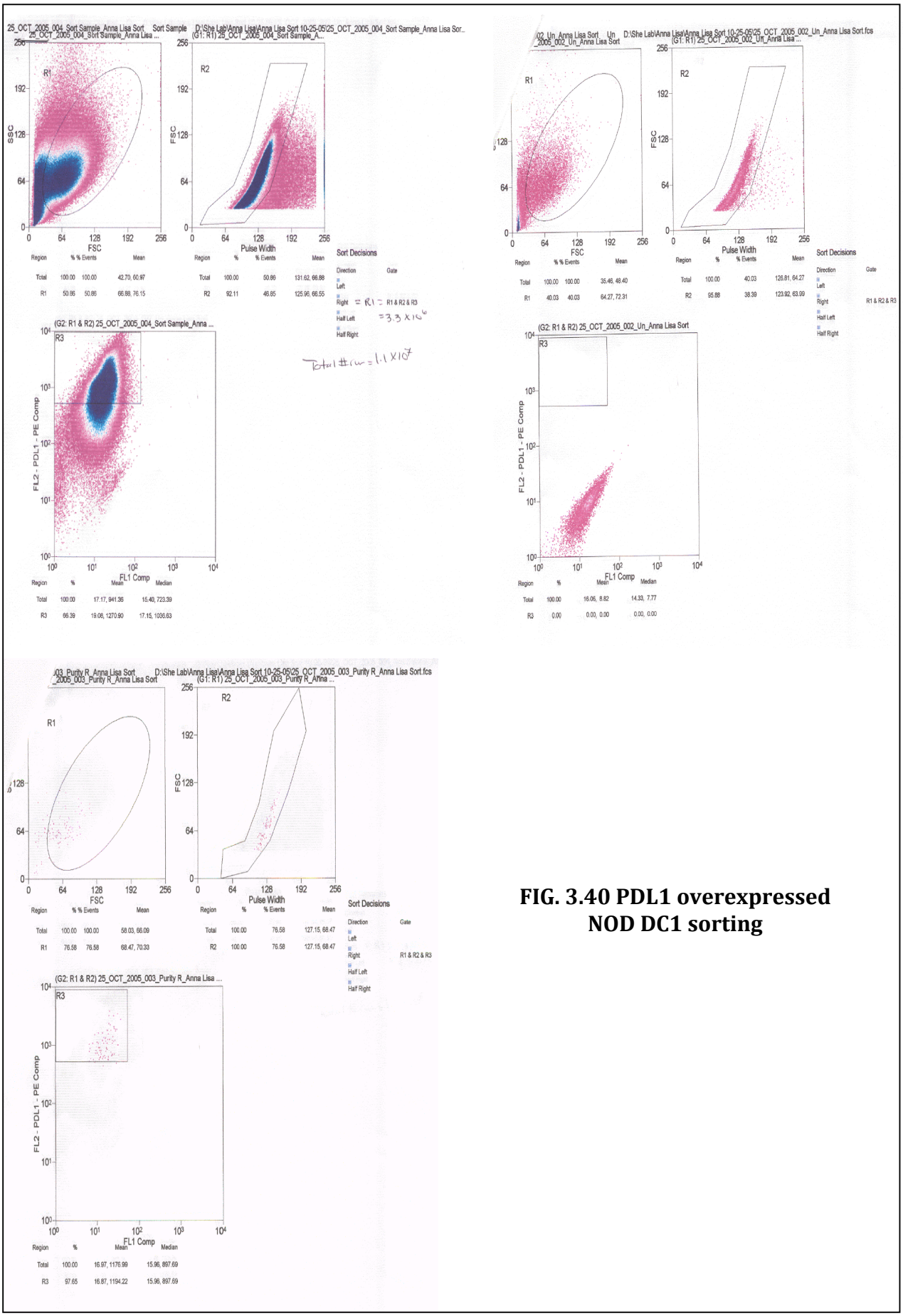


FIG. 3.40 PDL1 overexpressed NOD DC1 sorting

At this point, we performed an MLR (Fig. 3.41) using the PDL1 sorted NOD DC1 cell lines and the experiment showed these cells are more tolerogenic than untreated NOD DC1 cell lines. Because the only difference among the two groups of cells is the PDL1 overexpression, it is clear that PDL1 may be a key molecule in inducing tolerogenicity.

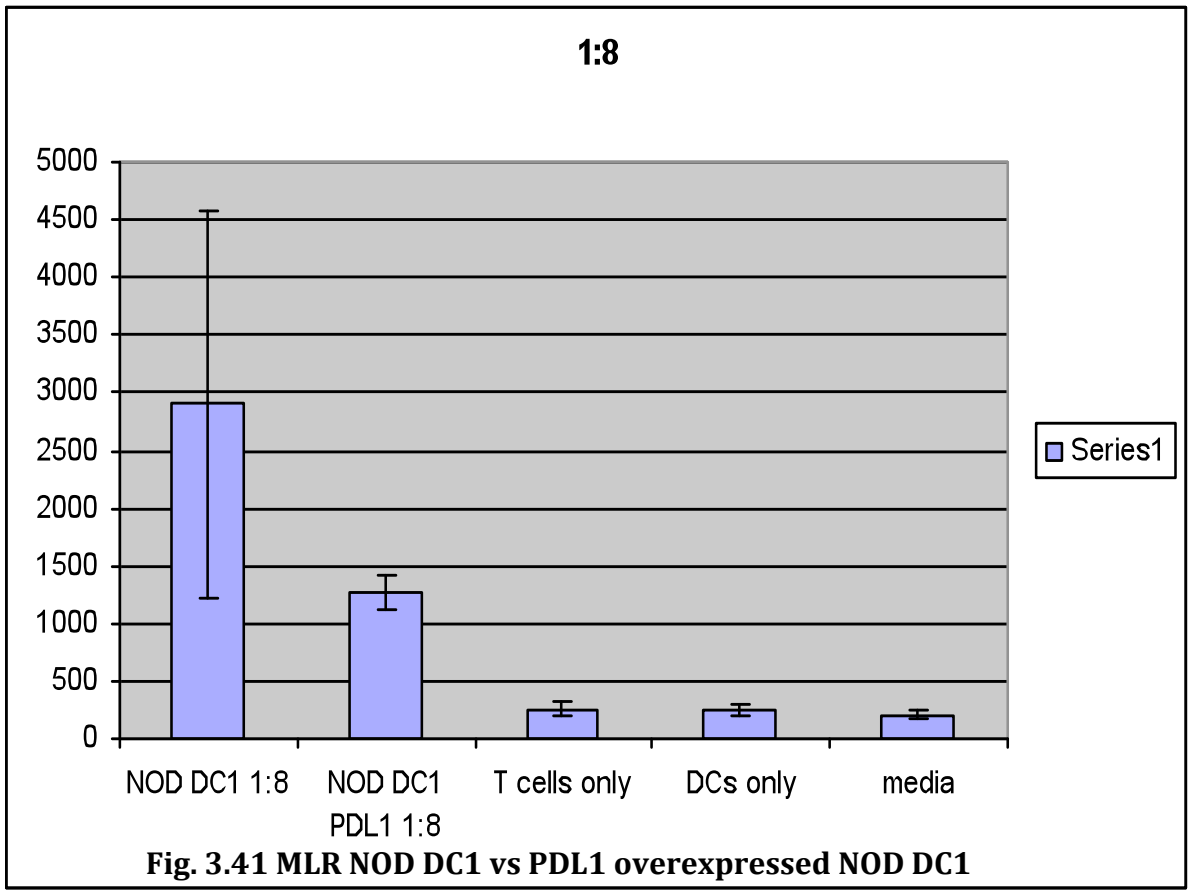


Fig. 3.41 MLR NOD DC1 vs PDL1 overexpressed NOD DC1

The immune system plays a pivotal role in Type 1 Diabetes. Dendritic cells, T cells and their interaction are a critical point in diabetogenesis. Inducing tolerogenicity can delay and hopefully stop the diabetic process. The aim of this study was to explore the method of inducing tolerogenicity and identifying functionally important dendritic cell markers to, eventually, generate engineered dendritic cells for the prevention of Type 1 Diabetes. Many studies have shown immature DCs are more tolerogenic. We wanted to use safe-to-use-in-human chemicals and drugs to induce tolerogenicity and to identify key molecules for tolerogenicity in Type 1 Diabetes. We thought looking for safe-to-use-in-human chemicals could give us the chance to better deduce a therapy that could be used in humans in the future.

We have shown drugs such as vitamin D, aspirin and aloe vera are able to modulate dendritic cell surface molecules and DC maturation, thereby inducing tolerance. The modulation of some molecules such as CD86 and CD80 are able to induce tolerance in vitro but not in vivo explaining the lack of success of all the in vivo protocols for Type 1 Diabetes worldwide. This means that other molecules should have more specific action in tolerogenicity in vivo.

We identified PDL1 as one possible key molecule for in vivo tolerogenicity in Type 1 Diabetes. Our experiments demonstrated a strong correlation between PDL1 expression and tolerogenicity in vitro and in vivo and the involvement of different molecules between in vitro and in vivo experiments. We demonstrated that engineering DCs towards tolerogenicity is possible. More studies are necessary, but we strongly believe PDL1 plays a pivotal role in tolerogenic mechanisms in Type 1 Diabetes. In addition, we believe it is possible to engineer DCs (high-expressing-PDL1-DCs) to induce tolerance in Type 1 Diabetes using safe-to-use-in-human chemicals and drugs. Finally, we strongly believe we opened new prospectives for further studies in immunology and diabetology and new prospectives on the prevention and hopefully a cure for Type 1 Diabetes.

In Vivo Prevention Protocol

The aim of this protocol is to prevent and/or reverse type 1 diabetes in NOD mice by using a therapy safe to humans and young children. We hypothesize that using combo therapy with the long-term use of tolerogenic and beta-cell protection/regeneration drugs following a short-term immunosuppression is the best strategy.

Type 1 Diabetes is an autoimmune disease, involving genetic, environmental and immunological factors. Its immunological features include the involvement of T and dendritic cells along with other cells and cytokines. The disease results from immunological damage to the pancreas and loss of beta cells with a lack of insulin production and increased blood sugar.

Several tissue parts in the body, just like the skin or the liver, have the ability to regenerate after damage. It appears that the potential of regeneration is very limited for pancreatic beta cells, although it is not clear if the lack in regeneration is due to the beta cell itself or to the autoimmune attack.

The dream of the endocrinologists and researchers is to be able to replace the beta cell loss with new cells and to cure diabetes. Many in vitro and in vivo studies have been performed but new problems continuously arise and the reversal of Type 1 Diabetes is still a goal to be achieved. Studies have shown that some neogenesis is possible by the process of transdifferentiation and stem cell activation by GLP1 or gastrin stimuli. In the clinical field, beta cell transplantation has been considered. Although this technique opens a window of disease- free time, the autoimmune cascade remains a big concern.

Type 1 Diabetes is a multifactorial disease and it is now clear that acting on a single piece of the puzzle does not allow us to destroy the disorder. Only, the simultaneous attack to all the elements involved in the process would allow us to prevent and to cure Type 1 Diabetes. In line with this, we analyzed the cascade of events involved in the pathology and the instruments already available in modern medicine to establish a new strategy for therapy. The aim was to prevent and/or reverse Type 1 Diabetes using a therapy that is safe to humans and young children.

The key to pathogenesis is the autoimmune attack, which results in the loss of pancreatic beta cells. Theorically, the block of the autoimmune events and a sufficient amount of islet mass should be able to allow the appropriate insulin production and the reversal of the disease. Therapeutic strategy should be directed to all the components involved in the process; the autoimmune cascade, the protection of the beta cells and the regeneration of the islets. The therapeutic attack against the autoimmune events involves two phases: the blocking of the active elements by a short and strong immunosuppression and the modulation of precursors by the induction of tolerance. The first step should allow us to block the active phase of the pathological cascade while the second step should allow us to maintain the result.

Type 1 Diabetes appears when the majority of the islets are already lost, however, a few of them can still be active even if not sufficiently productive. It is of vital importance to protect these islets from damage. So, a third step of the therapeutic strategy is the administration of agents able to protect the beta cells. However, the blocking of autoimmune events and the protection of the beta cell are still not sufficient to achieve the appropriate insulin production. That is why our strategy includes another step: the regeneration of islets.

Summarizing, the blocking of the autoimmune cascade, the induction of tolerance, the protection and the regeneration of the pancreatic beta cells should, theorically, allow us to achieve the dream of all endocrinologists: the reversal of Type 1 Diabetes.

We considered all the agents available for each one of our goals. The aim of this protocol was to prevent and/or reverse Type 1 Diabetes in NOD mice using a therapy safe to humans and young children. To achieve this aim, we used a combo therapy involving long-term use of tolerogenic and beta-cell protection/regeneration drugs following short-term immunosuppression. Many agents were considered like cyclosporine, tacrolimus, mycophenolate, glucocorticoids, azathioprine, sirolimus, OKT3, basiliximab, daclizumab, proteosome inhibitors (Velcade), FTY720, anti-integrins, PDL-1 Ig, deoxyspergualin (NFk-b inhibitor), CTLA4-Ig, statins, TGF-β, TNF-a, CCR5, CXCR3, DC treatment, insulin peptide, IGRP peptides, DIAPEP277, proinsulin peptide, vitamin D, G-CSF, nicotinamide, vitamin E, hepatocyte growth factor (HGF), GLP-1, Epidermal Growth Factor (EGF) and Gastrin. However, the

criteria of human safety and, unfortunately, economical concerns, influenced our choices. With these restrictions in mind, we proposed the best cocktail of drugs possible.

We used 12 week old NOD female mice from Jackson Lab. Mice were housed pathogen-free and all the animal experiments were performed according to the IACUC guidelines and approved by the Animal Use Committee at Medical College of Georgia. We gave them 1mg/Kg of rapamycin i.p. (in 500ml deionized water containing 1g sodiumcarboxymethylcellulose and 1.25 g Tween 80), 5 mg/Kg i.p. of vitamin D (in peanut oil), 0.045 mg per mouse of vitamin E by oral route, 500 mg/kg i.p. of nicotinamide (in sterile PBS) every other day for 2 weeks. At this point, mice were 14 weeks old. We stopped the treatment. We waited 2 weeks and then immunized mice using insulin peptides (16 weeks old mice). We immunized the mice with human recombinant insulin B chain, amino acids 9-23 (B9-23) diluted in isophane insulin diluent and mixed with incomplete Freund's adjuvant (IFA). Immunizations were subcutaneously administered in the inguinal, axillary, and dorsal neck regions. The control group received veichles without drugs. So, basically, i.p. injection of deionized water containing sodiumcarboxymethylcellulose and Tween 80 without rapamycin, i.p. injection of peanut oil without vitamin D, oral peanut oil without vitamin E, i.p. injection of sterile PBS without nicotinamide and subcoutaneous injections of incomplete Freund's adjuvant without insulin peptides.

We bought rapamycin from CALBIOCHEM cat n. 553210, the sodiumcarboxymethylcellulose from CALBIOCHEM cat. 217277, vitamin D from CALBIOCHEM cat n. 679101, peanut oil from SIGMA cat n. P2144, vitamin E from SIGMA cat n. T1539, nicotinamide from SIGMA cat n N0636. B9-23 insulin peptide SO#00895554 (Invitrogen).

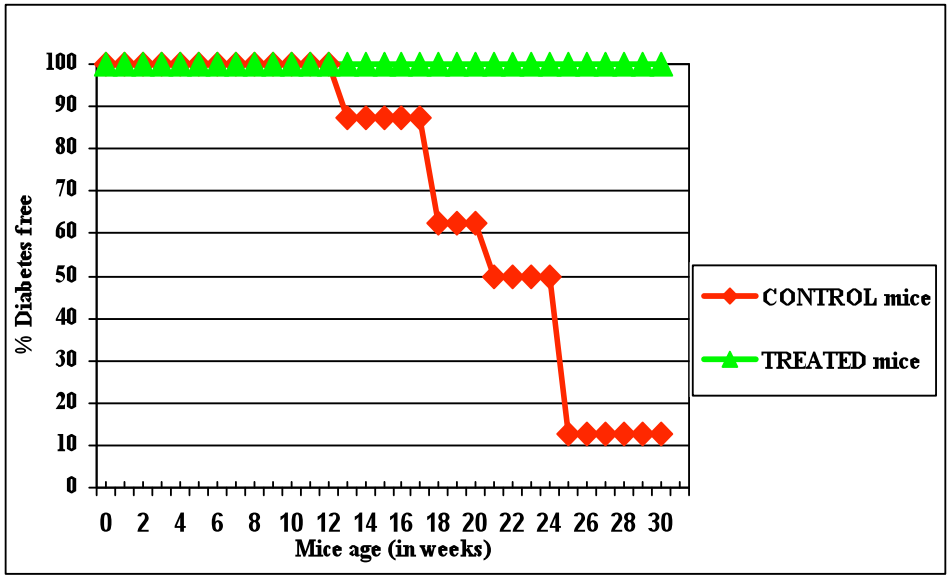


Fig. 3.42 Graph - Prevention experiment in NOD female mice

We used 8 NOD female mice per each group. Mice were treated simultaneously and the handling of animals and materials was the same for both groups. The diabetes incidence in the colony is 90%. Mice were monitored for diabetes before starting the experiment and twice a week after, until the age of 30 weeks old.

The graph (Fig. 3.42) represents the result of the experiment. On the x axis there is the age of the mice while on the y axis is the percentage of diabetes-free mice. The red line represents the control mice who received just vehicle without drugs while the green line represents the treated group. At 30 weeks old, 100% of treated mice were diabetes free while in the control group only 12.5 % of mice were diabetes free.

Type 1 Diabetes is a multifactorial disease where immunological events cause beta cell loss and a lack of insulin production. Three big events lead to the high blood glucose levels: the immunological attack of the beta cells, the islet loss and the lack in beta cell regeneration. Therefore, it is not possible to use a single therapeutic strategy to cure diabetes. In fact, it is impossible to stop the islet loss without stopping the immunological attack and it is impossible to improve the cells regeneration just stopping the immunological events. A more complex strategy of therapy is necessary.

In this study we analyzed a combo therapy that is safe for humans and young children using long-term tolerogenic and beta-cell protective/regenerative drugs followed by short-term immunosuppression to prevent/reverse Type 1 Diabetes.

We applied the protocol to 12 week old female mice. We gave them rapamycin, vitamin D and nicotinamide i.p. and vitamin E by oral route every other day for 2 weeks. We stopped the treatment and after 2 weeks we immunized them using insulin peptides. Using this strategy the three steps involved in disease manifestation; immunological attack, beta cels loss and islet regeneration, were all targeted by some component of the therapy. Rapamycin was used to stop the immune events, insulin peptides and vitamin D to promote a shift of dendritic cells toward tolerogenic DCs while vitamin E and nicotinamide were given to improve islet regeneration. One hundred percent of the treated mice remained diabetes free at 30 weeks of age, while only 12.5 % of the control mice were healthy at the same age.

Observing these results we are able to suggest that a combo therapy would be useful in diabetes therapy and would be the right future approach for diabetes treatment in humans. Single strategies of therapy have been tried in mice and humans in many different protocols but none have led us to complete prevention/reversal of the disease. While some treatments, like insulin peptide immunization, gave good results in mice when administered at a young age (4 weeks), the same results were not maintained while transferring these protocols to humans. Especially when taking into account the difficulty of applying the therapy before the onset of overt diabetes. Those single treatments, which worked well in young mice, no longer had any beneficial affects when applied to older mice. Our protocol appears to work in older mice just prior to diabetes onset. In fact, only a few protocols currently published are able to achieve this result. While we know there is a need to confirm these results with other experiments using a larger number of mice, our protocol is one of the few promising protocols for Type 1 Diabetes prevention, worldwide. The safety of the drugs utilized and the short time of therapy are, also, promising for a transferring this therapeutic approach to humans.

To test the limits of our combo therapy, we tried to apply it to a sick mouse (diabetic mouse). We checked the mouse and on the initial day of diabetes diagnosis, we started the therapy. We followed the mouse checking it for diabetes twice a week until 30

weeks of age and a high glucose reading was not observed in the animal. While we know it is not possible to draw conclusions from a single mouse experiment, we believe this is encouraging for future experiments. Unfortunately, economical concerns did not allow us to repeat both the prevention and the reversal experiments. However, we believe we are authorized to consider this combo therapy one of the most promising protocols for the prevention and reversal of Type 1 Diabetes in mice and, hopefully, in humans, worldwide.

Conclusions

-Type 1 Diabetes is a complex disease. Genetic, immunological and environmental factors play a role in the pathogenesis of the disease.

-Dendritic cells are antigen-presenting cells. The interaction of dendritics cells and T cells plays a pivotal role in T cell proliferation.

-Type 1 Diabetes is an autoimmune disease where beta cells of the pancreas are destroyed by immune cells, especially T cells.

-Modulating dendritic cells may be a key to modulate T cell proliferation and the autoimmune events in Type 1 Diabetes.

-Many chemicals have immunological properties. Some of these chemicals are safe for use in humans and have already been approved for use in other medical conditions.

-In this study we modulated dendritic cells from bone marrow cultures using different chemicals.

-Many of these chemicals are able to modulate costimulatory molecules on our dendritic cells and are effective in T cell suppression in vitro. Among these we found aspirin, statins, and vitamin D to be the moste effective.

-However, dendritic cell modulation is not always enough to modulate T cell proliferation in vivo.

-We hypothesed that PDL1 plays a pivotal role in the DC/T cell interactions, especially in vivo.

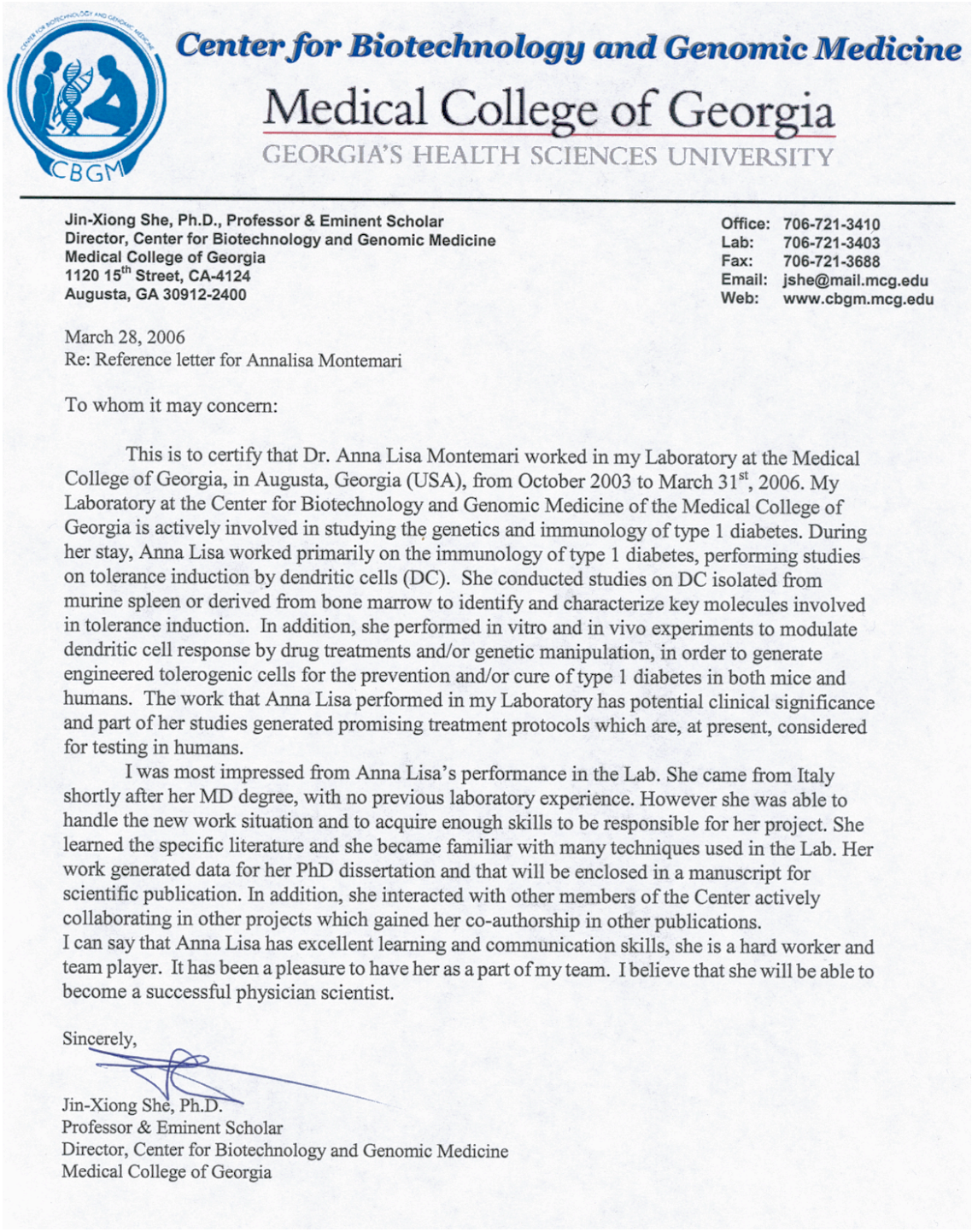
In fact, the in vivo experiment (aspirin cotransfer experiment) with high PDL1 expressing DCs showed protective effects in Type 1 Diabetes onset while the cotransfer experiment (vitamin D cotrasfer) using low expressing PDL1 cells showed no protection.

-We have reason to believe PDL1 plays a pivotal role in T cell proliferation and our hypotesis is supported by in vitro experiments (MLRs) demonstrating lower T cell proliferation using dendritic cells treated with chemicals (aspirin, aloe+aspirin+vitamin D) modulating PDL1 expression toward an increase of the expression itself or using dendritic cells overexpressing PDL1 by a vector.

-Further studies need to be performed to study the PDL1 role in Type 1 Diabetes.

As interesting as it is to study the immunological aspects of Type 1 Diabetes, the more complicated and more important aim is to identify a strategy of therapy. Type 1 Diabetes is a complex disease. Many cells and many events are involved in the mechanism of the disease. There is a genetic predisposition to the disease. Many environmental triggers are supposed to have a role in the pathogenesis. When the disease is triggered, the immune system will destroy beta-cells. The target of the prevention and cure of Type 1 Diabetes is basically a three-step strategy: 1) preserve the residual pancreatic mass 2) stop the autoimmune process 3) regenerate the beta cell mass. To develop a cure for Type 1 Diabetes, all three steps need to be addressed.

We developed a protocol using a three-step strategy that was successful in preventing Type 1 Diabetes in NOD mice prior to the onset of diabetes (10-12 weeks old). To our knowledge this is one the few protocols that has been successful in preventing diabetes in NOD mice so late in the course of the disease. The chemicals used in this protocol are absolutely safe for humans and have already been approved by the FDA for other medical conditions. Further studies should be performed to corroborate the findings of this study, however transitioning this three step strategy from mice to humans should be considered at this time.



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March 28, 2006
Re: Reference letter for Annalisa Montemari

To whom it may concern:

This is to certify that Dr. Anna Lisa Montemari worked in my Laboratory at the Medical College of Georgia, in Augusta, Georgia (USA), from October 2003 to March 31st, 2006. My Laboratory at the Center for Biotechnology and Genomic Medicine of the Medical College of Georgia is actively involved in studying the genetics and immunology of type 1 diabetes. During her stay, Anna Lisa worked primarily on the immunology of type 1 diabetes, performing studies on tolerance induction by dendritic cells (DC). She conducted studies on DC isolated from murine spleen or derived from bone marrow to identify and characterize key molecules involved in tolerance induction. In addition, she performed in vitro and in vivo experiments to modulate dendritic cell response by drug treatments and/or genetic manipulation, in order to generate engineered tolerogenic cells for the prevention and/or cure of type 1 diabetes in both mice and humans. The work that Anna Lisa performed in my Laboratory has potential clinical significance and part of her studies generated promising treatment protocols which are, at present, considered for testing in humans.

I was most impressed from Anna Lisa's performance in the Lab. She came from Italy shortly after her MD degree, with no previous laboratory experience. However she was able to handle the new work situation and to acquire enough skills to be responsible for her project. She learned the specific literature and she became familiar with many techniques used in the Lab. Her work generated data for her PhD dissertation and that will be enclosed in a manuscript for scientific publication. In addition, she interacted with other members of the Center actively collaborating in other projects which gained her co-authorship in other publications.
I can say that Anna Lisa has excellent learning and communication skills, she is a hard worker and team player. It has been a pleasure to have her as a part of my team. I believe that she will be able to become a successful physician scientist.

Sincerely,

Jin-Xiong She, Ph.D.
Professor & Eminent Scholar
Director, Center for Biotechnology and Genomic Medicine
Medical College of Georgia

School of Medicine
Department of Pediatrics
Section of Endocrinology & Diabetes

MCG
Children's Medical Center

April 8, 2006

Dear Review Committee Member:

It is my great pleasure to recommend Anna Lisa Montemari for a position in your residency program. I am a Professor of Pediatrics at the Medical College of Georgia and the Chief of Pediatric Endocrinology. As the clinical director of the diabetes research program, I worked with Anna Lisa throughout her time in Dr. She's laboratory. Anna Lisa proved to be a very thoughtful and diligent scientist. She maintained an excellent clinical focus on her research project that examined the role of vitamin D and a number of anti-inflammatory agents on the development of diabetes in NOD mice. Her presentations at weekly laboratory meetings demonstrated her ability to analyze data effectively and devise new approaches when technical problems or unexpected results arose.

Anna Lisa was very popular member of the laboratory group who was eager to help others. She was a tireless worker who showed dedication both to her own project and to the goals of the entire laboratory group. I think her intellect, perseverance and outgoing personality will help her become an excellent clinician. I highly recommend her for a residency position.

Yours sincerely,

Andrew Muir, MD

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Augusta, March 30, 2006

To whom it concerns:

It is with great pleasure that I write this letter of recommendation for Dr.Anna Lisa Montemari, MD who is applying for a residence traineeship in Italy.

Dr. Montemari has joined Dr. Jin-Xiong She laboratory, located in the Center for Biotechnology and Genomic Medicine, at Medical College of Georgia in October 2003 as a student from European Endocrinology and Metabolism PhD Program. At that time, she has already obtained her MD degree in Italy and had achieved an exceptional expertise in the different gene expression assays. She has also contributed to the studies focused on the risk factors associated with different HLA genotypes of susceptibility to Type 1 Diabetes, which have been recently published.

Since her arrival to the Center, Dr Montemari has been working on the project focused on the function of the environmental factors in type 1 diabetes pathogenesis and the function of the vitamin D3 as an autoimmune regulator. In particular, she has been studying how vitamin D3, aspirin and IL-10 treatments of dendritic cells may help to generate tolerogenic dendritic cells, which might prevent type 1 diabetes. Using this strategy she has found that these treatments upregulate PDL1 expression and reduce CD80 and CD86 expression on bone marrow derived dendritic cells. These phenotypic changes are compatible with phenotype of tolerogenic dendritic cells, which can be adoptively transferred to diabetic animals to evaluate their therapeutic potential. The manuscript presenting these results has been currently in preparation.

Dr. Montemari is highly intelligent and critically thinking young researcher. Moreover, she is a very pleasant person to work with. Her professional experience gained during time he spent in the Center for Biotechnology and Genomic Medicine should be a valuable asset for any program in biomedical sciences, especially in the fields related to tolerance and T cell immunology.

If you need additional information, please feel free to contact me.

Sincerely,

Leszek Ignatowicz Ph.D.

Certificate of Postdoctoral Training

Presented to

Anna Lisa Montemari, M.D.

The Medical College of Georgia
hereby awards this certificate for completion of
Postdoctoral Training
October 2003-March 2006

Faculty Mentor

MEDICAL COLLEGE OF GEORGIA · GEORGIA HEALTH SCIENCES UNIVERSITY · 1828

Frank Traiber
VP for Research

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