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Review

Is Lack of Peripheral Tolerance Induction a Cause for Diabetes in the Non-Obese Diabetic Mouse?

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Abstract. The non-obese diabetic (NOD) mouse is a spontaneous animal model for type 1 diabetes characterized by a selective destruction of the insulin producing β cells in the pancreas. As in humans, the disease is controlled by several susceptibility genes, some of which map to the major histocompatibility complex on chromosome 17. However, environmental factors also contribute to the development of the disease in the NOD mouse, presumably through controlling the balance between the Th1 and Th2 response in the animal. Recent observations have shown that the NOD mouse has abnormalities in the development of bone marrow-derived antigen-presenting cells. These include the most potent activators of naive T cells, the dendritic cells, which exist in at least two different sub-populations; DC1 cells, responsible for activation of Th1 cells, and DC2 cells, which produce Th2 cells. In addition to activating naïve T cells, the dendritic cells are also involved in generating central and peripheral tolerance to self molecules. In this process DC2 cells appear to be more important for the development of peripheral tolerance than DC1 cells. Besides abnormalities in the development of bone marrow-derived antigen-presenting cells, the NOD mouse also has a defect in the thymic selection of T cells, leading to a higher concentration of autoreactive T cells. We speculate that the NOD mouse may develop an imbalance in the two subsets of dendritic cells with a skewing towards DC1 cells, thus having a reduced ability to generate peripheral tolerance to a number of autoantigens.

Key words: peripheral tolerance; non-obese diabetic mouse; antigen-presenting cells.

Induction of the Immune Response

The immune system has evolved to protect the host from invading pathogens. Two general systems of immunity to infectious agents have been selected during evolution: innate or natural immunity, and acquired (adaptive) or specific immunity. Innate immunity refers to the species's basic resistance to infections. It is characterized by a limited ability to distinguish one pathogen from another and it uses families of receptors

Abbreviations used: AIDC – activation-induced cell death, APC – antigen-presenting cells, CSF – colony-stimulating factor, CTL – cytotoxic T lymphocytes, CTS – cataract-prone Shionogi, DC – dendritic cells, GM-CSF – granulocyte macrophage stimulating factor, G0 – agalacto-IgG, IFN – interferon, LCMV – lymphocytic choriomeningitis virus, LPS – lipopolysaccharide, M-CSF – macrophage stimulating factor, MHC – major histocompatibility complex, NOD – non-obese diabetic, PAMP – pathogen-associated molecular pattern, PRR – pattern-recognizing receptor, SDS/PAGE – sodium dodecyl sulfate/polyacrylamid gel electrophoresis, TCR – T cell receptor.

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encoded in the germ line to identify potentially noxious substances. These receptors are referred to as pattern--recognizing receptors (PRRs), which are able to recognise conserved pathogen-associated molecular patterns (PAMPs) associated with microorganisms[29, 30]. The acquired immunity is characterized by its display of specificity, diversity, memory and self/non-self discrimination. The specificity of the system is a result of its ability to generate a tremendous diversity in its recognition molecules allowing the system to specifically recognize billions of uniquely different structures on foreign antigens. The acquired immune system generates its vast number of antigen-recognizing receptors through the process of somatic gene rearrangement and various means of diversification, which creates random specificities. However, induction of an immune response is only appropriate if the antigen recognized is derived from, or belongs to, a pathogen. Activation of lymphocytes specific for self-antigens, or benign persistent environmental antigens, may result in autoimmune disorders and deleterious hypersensitive reactions, respectively, which is why the imune system's ability to distinguish self from non-self is crucial[29, 30]. Evidence is now accumulating that the cellular and soluble components of innate immunity provide instructions that enables the acquired immune response to select appropriate antigens and the strategies for their elimination[9]. Thus, innate and acquired immunity are closely interlinked, and acquired immunity does not occur independently of innate immunity.

Immunological tolerance, or unresponsiveness, to self-antigens is one of the cornerstones of the aquired immune system, which is why extensive mechanisms have evolved to ensure that effector cells with high specificity for self molecules are eliminated. Tolerance to self is induced by the encounter of immature lymphocytes with self-antigens in the primary lymphoid tissues: the bone marrow in the case of pre-B cells, and the thymus in the case of thymocytes (central tolerance). Once the effector cells are released from the primary lymphoid tissues both the B and T cells may undergo a further selection in the periphery to establish tolerance to antigens not present or not present in sufficient quantities in these primary lymphoid tissues (peripheral tolerance)[32]. In the present review we will restrict ourselves to a discussion of T cell immunity/ /tolerance, as insulin-dependent diabetes is believed to be a T cell-mediated autoimmune disease.

Activation of naive T cells requires two seperate signals in order to induce proliferation. Signal one is received through the antigen receptor (TCR), which recognizes small peptides derived from the proteolytic degradation of polypeptides, presented in complex with major histocompatibility complex (MHC) class I molecules (to $CD8^+$ T cells) or MHC class II molecules (to $CD4^+$ T cells). The second signal is delivered through the co-receptors CD28 (for positive stimulation of the T cells) and CTLA-4 (for down-regulation of the T cell response), which interacts with the co-stimulators CD80 (B7-1) or CD86 (B7-2) expressed on the antigen--presenting cells. A third signal can also be provided via stimulation by various cytokines. If the T cell receives only signal one, it does not become fully activated and may either die through programmed cell death, or apoptosis, or be rendered unresponsive or anergic[58].

The co-stimulators CD80 and CD86 are expressed on specialized antigen-presenting cells (APC), such as dendritic cells (DC), macrophages and B cells, which also express MHC class II molecules for activation of $CD4^+$ T cells, Dendritic cells are considered the most potent professional antigen-presenting cells, and although B cells and macrophages might be able to stimulate T cells that have already experienced antigen, the current belief is that only interdigitating DC are able to activate naive $CD4^+$ T[1, 54]. Dendritic cells are, as such, of key importance in ensuring a careful regulation of the $CD4^+$ T cell activation.

Dendritic cells are derived from bone marrow stem cells. They populate the different tissues as immature DC, which are unable to stimulate T cells. The immature DC are characterized by high synthesis but low cell surface expression of MHC class II molecules and low expression of the requisite accessory co-stimulators[62, 63]. They are, though, extremely efficient in capturing proteins, which are processed by proteases and loaded onto either MHC class I or MHC class II antigens, depending on the processing and presenting pathway. When DC have captured an antigen and the PRRs expressed on the immature DC interact with a PAMP, a series of events induce the immature DC to differentiate into mature DC[29, 30]. This maturation is crucial for the initiation of adaptive immunity. A profound change in MHC class II distribution, where the cell surface expression is increased but the synthesis of MHC class II is stopped, characterizes the mature DC. Also, a strong up-regulation of the co-stimulator molecules, induction of integrins and co-receptors, such as LFA-1, and production of various cytokines and chemokines is observed with maturation[36, 62]. Mature DC are no longer able of capturing and processing antigen, but are very efficient in presenting antigen. During maturation, the DC migrate into the secondary lymphoid tissue, where they can activate naive T cells expressing T cell recep-

tors specific for antigenic peptides complexed with the MHC class II molecules on the surface of the DC. Many of the antigenic peptides presented by the MHC class II molecules will have originated from self-proteins sampled in the periphery, but the T cells with specificity for these peptides have, in many cases, already either been deleted or have been rendered ignorant by the central tolerance mechanisms.

The T cell response can be characterized as a Th1 or Th2 response, depending on the types of cytokines predominantly synthesized in the activated T cells. Th1 cells secrete IFN-γ, TNF-α and IL-12, while Th2 cells produce IL-4, IL-5 and IL-10[21] (Fig. 1). The functional significance of the Th1 and Th2 cell subsets is that their distinct patterns of cytokine secretion lead to strikingly different T cell actions. Th1 cells and their cytokines are the mediators in cellular immune responses, which are characterized by high levels of cytotoxic T lymphocyte (CTL), macrophage and natural killer (NK) cell activation. These cells are thus specialized to eliminate pathogens invading host cells. The Th2 cells are effective stimulators of humoral immune responses, and thus elicit an antibody-mediated reaction that provides protection against extracellular pathogens, such as helminths[42]. The mechanisms determining the differentiation into the two subsets are not fully understood at present, but it seems to rely on signals received very early in the activation process. It is known that the cytokine microenvironment plays an important role in Th0 cell differentiation towards the Th1 or Th2 cell type during immune responses. As DC act as the priming APC for most T cell responses, their role in the differentiation of T cells has now begun to be studied. RISSOAN et al.[46] have recently proposed that the DC not only provide the signals for activation of the T cells, but also the selective signals resulting in either Th1 or Th2 immunity. By sophisticated phenotyping, they characterized two subpopulations of DC from human peripheral blood, which they designated as DC1 and DC2. The DC1 subset was designated as myeloid DCs, and the DC2 subset had a few lymphoid features and did not differentiate into macrophages in response to

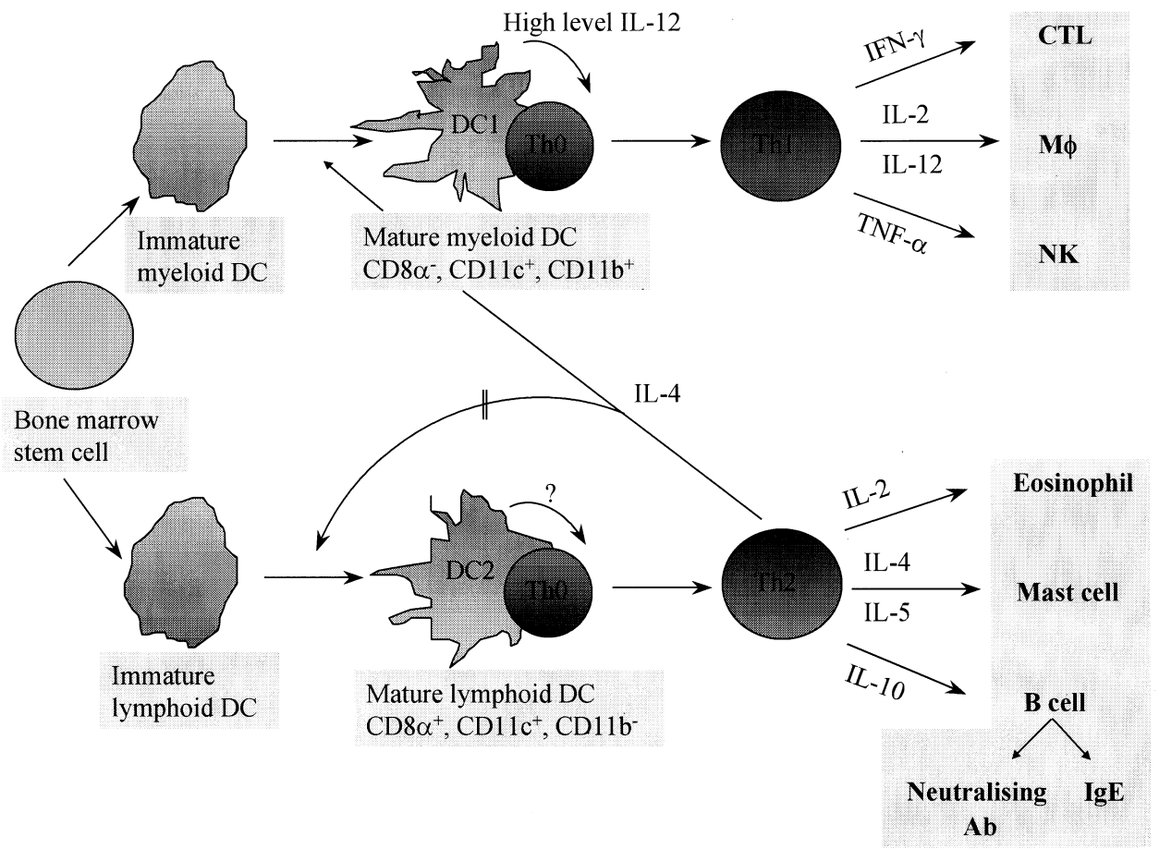

Fig. 1. DC1 and DC2 subpopulations provide selective signals to Th0 cells resulting in either Th1 or Th2 immune responses. It is as yet not clear whether the DC subpopulations are derived from different immature DC precursors or immature DC can polarize into mature DC1 or DC2 cells depending on different extracellular signals

GM-CSF and M-CSF. Activation of DC1 resulted in the production of a high level of IL-12 and promoted Th1 activation whereas DC2 cells induced Th2 differentiation (Fig. 1). Moreover, it was found that the Th2 cytokine IL-4 enhanced DC1 cell maturation and killed DC2 cells, an effect which was blocked by IFN-γ. Thus, a negative feedback loop from the mature Th cells may selectively inhibit prolonged Th1 or Th2 responses by regulating survival of the appropriate dendritic cell subset. The DC's involvement in the initial commitment of naïve Th cells into the Th1 or Th2 subset has also been explored by KAPSENBERG and collegues[19], who have suggested that the migrating DC carry and additional "signal 3" which biases the naïve Th cell towards either a Th1 or Th2 phenotype.

If an animal is to have a highly specific, yet not self-reacting, immune system, it must in some way be able to tolerate its own antigens. Thus, careful surveillance of the activities of immune cells is necessary to prevent destruction of self tissue. Most individuals do have an immune system able to tolerate or ignore self throughout life. There are, however, some situations where the immune system fails to prevent self-reactions and this can have serious health consequences. Many aspects of autoimmunity and autoimmune diseases have been investigated, but the exact mechanism behind the generation of these complex diseases, i.e. the breakdown of tolerance to self, is still not fully understood. Invading pathogens may share antigenic sequences with peptides derived from self-molecules (antigenic mimicry). If a high T cell response to the foreign peptide is induced, it may break the immunological tolerance or ignorance to self and induce a persistent autoreactive response. Other situations may be related to the way cells in the body are dying. Normally, cells are eliminated in the body through programmed cell death or apoptosis. Mechanisms to eliminate apoptotic cells without causing harm to the individuals have been developed, as, for example, the large number of apoptotic thymocytes in the thymus are cleared out by resident macrophages. However, if cells are particularly stressed or in another way affected, they may die abnormally by necrosis. This releases a range of intracellular molecules, notably molecules such as RNA and DNA, heat shock proteins and other stress molecules[10]. These molecules are not normally available to the immune system and, because there is an extensive sequence conservation of these molecules from pathogens to animals and because they are potent activators of dendritic cells, their release may elicit an autoimmune response. Many autoimmune diseases are T cell-mediated, and several studies indicate that a functional imbalance between the two Th cell subsets is a key determinant in establishing autoimmune pathology[21, 42].

Type 1 (insulin-dependent) diabetes mellitus is an example of a chronic organ-specific autoimmune disease attacking the insulin-producing cells in the pancreas, resulting in insulin deficiency. As a relatively common disease, type 1 diabetes mellitus has been extensively studied. Even so the triggering of the breakdown in tolerance, or the mechanism through which, this occurs, is still not understood, and much less information is available on the etiology of the disease than on the pathogenesis. We will here use a spontaneous mouse model for type 1 diabetes, the non-obese diabetic (NOD) mouse, to argue that one of the contributing factors for the disease progression is the inability to induce normal or sufficient peripheral tolerance.

Pathogenicity of Diabetes in the NOD Mouse

The NOD mouse is a spontaneous model for human type 1 (insulin-dependent) diabetes mellitus. The NOD mouse was derived from a cataract mouse found among outbred Jcl: ICR mice in 1966. During inbreeding and selection of the cataract-prone phenotype resulting in the cataract-prone Shionogi (CTS) strain, a female mouse with diabetic signs as observed and used to establish the NOD strain in 1980[27] (Fig. 2). As in human type 1 diabetics, the disease in the NOD mouse appears to be a chronic autoimmune disease characterized by a lymphocytic infiltration of the islets of pancreas, production of a number of autoantibodies with islet-reactivity and, finally, a selective destruction of the insulin-producing β cells in the pancreas. The lymphocytic infiltration starts as early as 3–6 weeks of age[11, 20] with an initial influx of DC and macrophages, which are later followed by T and B cells[17]. The lymphocytic inflammation consists initially of peri-islet located lymphocytes, which are not activated and remain benign for a long time. However, at 10 to 14 weeks of age, β cell-specific $CD4^+$ and $CD8^+$ T cells become activated and, within 3 weeks, they destroy a sufficient number of insulin-producing β cells to cause clinical diabetes. Depending on environmental factors, the incidence of diabetes in a typical high-incidence colony is between 80–90% in females and 40–50% in males by 30 weeks of age. The switch from benign to malignant autoimmunity in the NOD mice appears to be central to the disease progression in the NOD mouse. This, as yet unexplainable, switch from benign to malignant infiltration of the islets has also been observed in the

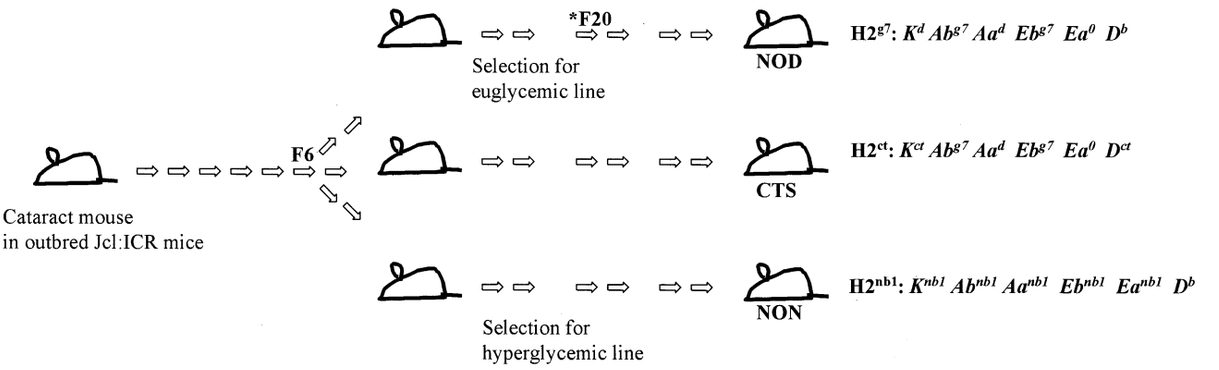

Fig. 2. The genotype of the NOD mouse and its related lines. The NOD mouse was derived from a cataract mouse identified among Jc1: ICR outbred mice, which was used to develop an inbred cataract-prone strain, the CTS mouse (cataract Shionogi): During the breeding, two sublines were separated at the 6th generation selected for development of euglycemic and hyperglycemic phenotype. At the 20th generation (*20) a diabetic female mouse of the euglycemic line was identified and used to establish the NOD mouse[27]

BDC2.5 T cell receptor transgenic NOD mouse[20], which expresses on most of its T cells the rearranged T cell receptor genes isolated from a diabetogenic CTL line. Although a massive intra-islet inflammation of T cells is seen from 3 weeks of age in the transgenic NOD mice, they do not develop diabetes until 10–25 weeks of age, much like the pattern of diabetes onset in the control NOD mice.

The T cells play a crucial role in the development of diabetes in the NOD mouse. Many experiments have shown that T cells isolated from diabetic females can accelerate the onset of diabetes if injected into irradiated non-diabetic NOD mice[60]. Th1 cells are believed to be the primary $CD4^+$ T cells mediating type 1 diabetes, and, indeed, β cell-specific T cell clones that exhibit a Th1 phenotype have been found to efficiently transfer diabetes in NOD[57]. However, such transfer studies have also shown that both $CD4^+$ and $CD8^+$ cells are required for the development of diabetes in the recipient mice. Moreover, it has been shown that depleting young animals of DC and macrophages prevents insulitis and diabetes[13], demonstrating an important role for these cells in the onset of the autoimmune disease in the NOD mouse.

Autoantibodies to many of the same islet-derived antigens observed in humans have also been found in the NOD mouse[37, 64]. We have compared insulin-specific autoantibodies in non-diabetic and diabetic NOD mice with control mice and found no difference in the isotype or concentration of the insulin-specific Ig. Where we did find a difference was in the concentration of insulin-specific agalacto-IgGs (G0s), which sharply increased from 12 weeks and onwards in the NOD mice compared with control mice (MALLING et al. unpublished observations). Agalacto-IgG does not have terminal galactose in the N-linked oligosaccharide chain in the Fc region. Although this glycosylation modification is naturally occurring, the relative level of G0 antibodies in systemic autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is higher than seen in age- and sex-matched normal controls (recently reviewed by DELVES[8]). Using a collagen-induced arthritis model, RADEMACHER et al. [43] showed that *in vitro* produced G0-antibodies specific for collagen can be pathogenic. It was interesting to note that the increase in the G0 of the insulin-autoantibodies in the NOD mice coincided in time with the shift from benign to malignant autoimmunity. Any link between these two events has yet to be established, though.

The role of autoantibodies in the development of diabetes has not been fully explored. There is no evidence that these autoantibodies are pathogenic, and the disease cannot be transferred by serum from affected subjects[49]. However, the absence of B cells in the NOD mice prevents the development or at least significantly delays the onset of diabetes. This has been demonstrated by crossing the $Ig\mu^{0/0}$ mutation onto the NOD background to create congenic NOD.$Ig\mu^{0/0}$ mice that do not produce B cells due to a lack of B cell receptors[48]. These mice do not become diabetic at the normal incidence rate. The protective effect of $Ig\mu^{0/0}$ has been interpreted as if the B cells function as antigen-presenting cells for naïve T cells, because injection of antibodies from diabetic NOD mice into NOD.$Ig\mu^{0/0}$ mice does not induce diabetes in the recipient mice[49]. It cannot be excluded, however, that autoantibodies have more than a pedestrian function in the development of diabetes in mice and humans.

Genetic Predisposition

As in humans, the disease in the NOD mouse is determined by several genes (*Idd1-19*) (Table 1). At least three of these genes have been linked to the MHC region, whilst the non-MHC genes map to other chromosomes[52, 61]. The non-MHC genes are collectively responsible for the autoimmune-susceptible genotype of the animals, whereas the H2 alleles determine the predominant feature of the autoimmune disease. This can be demonstrated by the fact that the congenic NOD.H2^{b} (K^b A^b E^b D^b) mouse does not develop diabetes but is a model for the autoimmune Sjögren' disease, and NOD.H2^{h4} (K^k A^k E^k D^k) mouse spontaneously develops thyroditis but not diabetes[59]. The MHC class II molecules especially are a key components in the development of diabetes, and the homozygosity of the class II genes have been shown necessary for disease susceptibility.

The MHC-linked diabetogenic genes collectively contribute 42% to the familial clustering of diabetes in the NOD mouse[56]. The NOD mouse has a unique MHC haplotype: K^d Ab^{g7} Aa^d Eb^{g7} Ea^0 and D^b (Fig. 2). The only MHC class II restriction element expressed in the mouse, I-A, has an unusual structure, with a histidine and a serine in positions Aβ56 and Aβ57, where most other Aβ subunits encode a proline and an aspartic acid, respectively. Construction of NOD mice containing transgenes encoding mutated and different MHC alleles have shown that the unusual structure of the ab^{g7} [25, 41] and the lack of I-E expression[25, 33] are critical for the diabetogenic phenotype and consistent with the involvement of CD4$^+$ cells in the autoimmune phenotype. The mutated Ab^{g7} and the Ea^d transgenes appear to protect the NOD from developing diabetes through different mechanisms. Thus, peri-islet infiltration of T cells, but no switch to malignant infiltration, was observed in the NOD.Ab^{g7}-Pro56 and NOD.Ab^{g7}-Asp[57] transgenic mice, while restoring the expression of I-e with a functional Ea^d gene completely abrogated all signs of autoimmunity[41]. In addition, either the structure or the expression of MHC class I alleles may also be important for the diabetogenic phenotype, because introducing *H2-L*^{d} as a transgene back-crossed into the NOD protects against development of diabetes[31]. This protection could, though, also be derived from the genomic region flanking the transgene co-introduced into the NOD mice through the back-crossing.

At least four additional MHC-linked diabetogenic genes exist in the NOD mouse besides the MHC class I and class II alleles. Analysis of congenic intra-MHC recombinant NOD mice, where a >6.6 cM region centromeric to *Lpm*2 in the NOD mouse was replaced with the similar region derived from a B10.A(R209) mouse, showed that this region protects the animals from developing both insulitis and diabetes[12], even though these mice have the rest of the MHC region of the NOD mouse. More refined dissection of the region suggests that at least 3 diabetogenic genes map to this region. Furthermore, construction of congenic NOD.H2^{ct} containing the MHC region of the CTS mouse (Fig. 2), which shares the MHC class II alleles with the NOD mouse, has demonstrated that there exists at least one additional diabetogenic gene (*Idd16*)[14], possibly located in the MHC class III region[26]. The phenotype of these four MHC-linked diabetogenic genes has yet to be established.

Table 1. Chromosomal localization of the *Idd* loci

Locus	Adjacent gene(s)	Chromosome	cM position
Idd1	MHC	17	19.5
Idd2	*Ncam*	9	22.0
Idd3	*Fgf2*	3	19.2
Idd4	*Nf1*	4	44.0
Idd5	*Fn1/Pax3*	5	40.0
Idd6		7	syntenic
Idd7	*Sle*	7	4.0
Idd8	*Tcra*	14	3.5
Idd9	*Elp1*	4	82.0
Idd10	*Tshb/Amy1*	3	48.5
Idd11	*Ssdh1*	11	64.6
Idd12	*Glud/Tcra*	14	12.0
Idd13	*Il1*	2	71.0
Idd14		5	syntenic
Idd16	MHC, *Tnfa*	17	18.0
Idd17	*Glrb, Fgg*	3	39.0
Idd18	*Amy1, Egf*	3	56.3
Idd19	*Raf1*	6	57.5

Further information can be found at
http://www.informatics.jax.org//searches/marker from.shtml

Environmental Factors

The disease in type 1 diabetes mellitus is genetically complex, and several susceptibility and protective genes have been identified in both human diabetes and in animal models of the disease. However, the concordance rate in monozygotic twins is only about 50%, and the large differences in diabetes incidence between colonies of NOD mice indicates that the determination of type 1 diabetes is multifactorial, with a number of as yet unidentified environmental factors contributing to the disease. Some of the environmental factors known to influence the development of diabetes in NOD mice

include diet, viral infection and exposure to microbial agents and temperature (see[42] for review). Studies have shown that NOD mice kept on an essential fatty acid--deficient diet or a diet where chloroform-soluble components have been removed have a significantly reduced cumulative incidence of diabetes without affecting the insulitis process[2, 5]. A viral origin of diabetes was one of the first etiological hypotheses, but, intriguingly, it has been shown that NOD animals raised in a pathogen-free environment have an increased incidence (>90%) of disease, suggesting that certain pathogenic infections can reduce or prevent the onset of the disease. Direct infections with a number of viruses can also prevent diabetes in the NOD mice (see[42] for review). In addition, immunisation protocols with adjuvants containing microbacterial extracts will induce protection from disease in the NOD mice[42, 53]. Finally, induction of stress has also been shown to induce protection against development of diabetes in the animals[47]. It thus seems that a general activation of the immune system and up-regulation of APC function will protect the susceptible animals from developing type 1 diabetes.

The mechanisms responsible for the effects the environmental factors have on the onset of diabetes in the NOD mouse have yet to be determined. However, there are strong indications that their influence on the disease may be caused by a shift in the balance between a Th1 and Th2 response. If the autoreactive immune response in autoimmune-prone individuals is initiated as a Th1 type, it will progress to a full-blown disease, whereas a general shift in the cytokine balance towards a Th2 response prevents the autoimmune disease. The onset of diabetes in NOD mice, which are deficient in IL-4, can, for example, be prevented by IL-4 injections[44], strongly suggesting that a Th2 response protects against development of diabetes.

Autoreactivity

Several studies have shown that the NOD mouse has a defect in the thymic selection of T cells, leading to a higher concentration of autoreactive T cells released into the periphery[7, 18]. RIDGWAY et al.[45] likewise proposed that the disease susceptibility in the NOD mouse could be explained by an inefficient negative selection of T cells in the thymus and that this was caused by the I-A^{g7} molecules. They immunised NOD mice, congenic NOD.$H2^{h4}$ mice (H2 haplotype: K^k A^k E^k D^k), NOD-Ab^k transgenic mice (homozygous for I-A^{g7} but also expressing I-$A^{k/d}$ molecule) and congenic B10.$H2^{g7}$ (containing the MHC region of the NOD mouse on a C56BL/10 background) with different self peptides mixed with complete Freund's adjuvant. They then examined the lymph nodes for the presence of autoreactive $CD4^+$ cells, i. e., $CD4^+$ T cells able to proliferate in response to APC expressing the appropriate self-antigens. They found that NOD mice homozygous for I-A^{g7} did elicit proliferation of autoreactive T cells irrespective of whether the I-A^k transgene was expressed or not, but that (NOD×NOD.$H2^{h4}$) F1 mice heterozygous for I-A^{g7} failed to induce autoreactive $CD4^+$ cells. Neither the congenic NOD.$H2^{h4}$ nor B10.$H2^{g7}$ mice were able to elicit detectable levels of autoreactive $CD4^+$ cells, demonstrating that homozygous I-A^{g7} is necessary but not sufficient for inducing proliferation of autoreactive T cell clones.

In an attempt to understand the lack of tolerance induction in the NOD mouse, we made a study on DC derived from bone marrow of NOD, NOD.$H2^{h4}$ and control CBA mice. We found that both the NOD and the NOD.$H2^{h4}$ dendritic cells expressed significantly less MHC class II on their surface as well as both of the co-stimulator molecules CD80 and CD86. The number of cells expressing MHC class II and CD80/86 as well as the number of molecules per cell were greatly decreased compared with those on dendritic cells from non-autoimmune CBA mice. These findings correlated with the failure of these dendritic cells to induce T cell proliferation in an antigen-dependent manner (STRID et al., submitted for publication).

The crystal structures of MHC class II molecules have shown that the relatively well-conserved aspartic acid residue at position Ab57 forms a salt bridge with residue Arg76 on the α-helix of Aa to stabilize the peptide-binding groove. The serine at position 57 of the Ab^{g7} prevents formation of this salt bridge, giving I-A^{g7} an intrinsic instability characterized by the formation of a high fraction of unstable molecules in SDS/PAGE analysis. This makes the MHC class II molecules poor binders of peptides with weak affinities for and fast dissociation rates of the peptides[3]. In addition, the I-A^{g7} molecule has a short half-life on APC[3].

According to the avidity model of the thymic selection of T cells, it is not the intrinsic affinity of the TCR for its ligand alone which determines the faith of the thymocytes, but the avidity of the interaction between the thymocyte and the APC defined as the combination of the intrinsic TCR affinity, the density of the TCR on the thymocytes, the density of the peptide: MHC ligand on the thymic DC and the potency of the costimulatory signalling. The fact that I-A^{g7} is a poor peptide binder and may be expressed at a lower concentration on the

APC in the thymus would thus have a far-reaching effect on thymic selection by dramatically decreasing the effective dose of I-A^{g7}: self-peptides on the selecting DC surface. The net result of a low dose of MHC: peptide complex would be an increase in TCR affinity to achieve an equivalent avidity threshold for positive and negative selection. This would result in the selection of a population of high-affinity, self-reactive and potentially autoreactive T cells.

Defects in Bone Marrow-Derived Cells

There is increasing evidence for abnormalities in bone marrow-derived cells in diabetics. LANGMUIR et al.[24] showed that NOD mice are somewhat deficient in their *in vitro* response to IL-3GM-CSF and IL-5. They found that fewer progeny cells were produced *in vitro* from bone marrow cell suspensions in response to increasing concentrations of the growth factors compared with bone marrow cells derived from the non-diabetic BALB/c mice. Looking at the cytokines produced by the APCs, we (STRID and LUND, unpublished results) and others[50] have not found any difference in TNF-α production between LPS-stimulated peritoneal macrophages from NOD and diabetes-resistant control strains, which contrasts with the findings of JACOBS et al. [15, 16]. Nor did we find any differences in the cytokine profiles between LPS-stimulated bone marrow-derived dendritic cells from NOD and control mice (STRID and LUND, submitted for publication). However, LPS-stimulated macrophages from the NOD mouse are known to be poor IL-1 producers[15, 16, 50], suggesting that at least some macrophages in the NOD mouse are not fully differentiated. Indeed, SERREZE et al.[50, 51] found a defect in the development of macrophages from NOD bone marrow cell suspensions in response to CSF-1 *in vitro*. These NOD-derived CSF-1-generated macrophages showed a functional deficiency, where the MHC class I expression was aberrantly down-regulated in response to IFN-γ, while MHC class I expression on macrophages derived similarly from C57BL/KsJ mice remained on the same level after IFN-γ treatment[51]. In addition, the bone marrow-derived NOD macrophages required the presence of both CSF-1 and IFN-γ to induce expression of significant levels of the receptor for CSF-1, the *c-fms* proto-oncogen. In contrast, macrophages from diabetes-resistant control mice expressed high levels of *c-fgm* in response to CSF-1 only, and macrophages from the non-obese non-diabetic NON mice down-regulated the expression of *c-fgm* when incubated with both CSF-1 and IFN-γ. The NOD macrophages also had an abnormal regulation of the IFN-γ receptor, *Ifgr*[50]. The difference between NOD and control mice was observed as differences in the mRNA levels, which may be caused by a reduced activity of protein kinase C in the NOD macrophages[50]. The developmental abnormalities in the cytokine-stimulated macrophages in the NOD mouse are controlled by non-MHC linked diabetogenic genes[51].

It is now widely accepted that the dendritic cells are the most important and potent professional APC. Even so not much work has been published on dendritic cells in the NOD mouse. We have, however, recently described that the development of DC, as that of macrophages, in the NOD mice is abnormal compared to that of DC derived form diabetes-resistant control strains. As mentioned previously, we found that myeloid DC generated by cultivating bone marrow stem cells in the presence of GM-CSF expressed very low levels of MHC class II molecules and the co-receptors CD80 and CD86 on the cell surface even following activation with both LPS and IFN-γ. In addition, we could not induce IL-2 production in T cells in an antigen-specific manner (STRID et al., submitted for publication). Bone marrow-derived DC from the congenic NOD.H2^{h4} (K^k, A^k, E^k, D^k) mouse also failed to express MHC class II, CD80/CD86 and to stimulate T cell proliferation, suggesting that, like the defective development of macrophages, the differentiation abnormalities of dendritic cells in the NOD mice are not controlled by genes located in the MHC region (STRID et al., submitted for publication).

An abnormal development in the production of dendritic cells appears to be associated with diabetes also in the other spontaneous model for type 1 diabetes; the BB-DP rat. A lower number of dendritic cells could be isolated from the BB-DP rat compared with non-diabetic Wistar rats. The DC had the same immature phenotype as we have described for the NOD mouse, with low MHC class II and co-receptor expression and an inability to elicit an allo-MLR response *in vitro*[6]. Even human diabetics appear to have a defect in the development of DC. TAKAHASHI et al.[55] obtained a lower yield of DC in response to GM-CSF and IL-4 from adherent peripheral blood cells from diabetic patients compared with age-, sex- and MHC class II-matched non-diabetic controls. In addition the *in vitro*-derived DC from the diabetic patients showed a reduced expression of the co-receptors CD80 and CD86 as well as a lower stimulation of autologous $CD4^+$ T cells compared with dendritic cells derived from the non-diabetic controls.

The abnormalities in the development of myeloid

DC in type 1 diabetic humans and in the spontaneous animal models for type 1 diabetes do not seem to render them unable to elicit an immune response. There are no indications that type 1 diabetics are immunologically incompetent. The apparent immaturity of diabetic DC may, however, suggest that there is a linkage between the abnormal production of certain bone marrow-derived hematopoietic cells and the ability to induce self-tolerance.

Is There an Imbalance in the Development of DC in Diabetics and Can this Explain the Lack of Self-Tolerance in these Patients?

Peripheral tolerance is the mechanism which maintains unresponsiveness to antigens that are present only in peripheral tissues and not in the generative lymphoid organs. Peripheral mechanisms may also inactivate or kill lymphocytes that are specific for ubiquitous self-antigens but which escaped central tolerance, for any reason. Tolerance in $CD4^+$ T lymphocytes may be induced either by a process of functional inactivation (anergy) or by apoptotic cell death as a result of antigen stimulation (activation-induced cell death, AICD). It is also possible that the functions of $CD4^+$ T cells are suppressed by other suppressive lymphocytes. The consequence of antigen recognition, i.e. activation or tolerance, depends mainly on two factors: how the antigen is presented to lymphocytes (the nature of the APC, concentration of the antigen, tissue location and persistence of the antigen), and how the responses of specific lymphocytes to that antigen are regulated. Recognition of self antigen in peripheral lymphoid tissues is a normal phenomenon, but pathological autoimmunity should be prevented by controlling what happens to lymphocytes after they respond to self-peptides. Since DC are likely to be the activators of most T cell responses, it does not seem unreasonable to believe that DC play an important role in the induction of peripheral tolerance as well as central tolerance.

The autoimmune response in the NOD mouse appears to consist of two seperate events. First, there is a benign infiltration into important endocrine organs, such as the islet of Langerhans, which starts very early in life. Even if the animal expresses predominantly β cell-reactive $CD8^+$ T cells, no tissue damage is seen during this phase. However, at 10 weeks or later, an autoreactive immune response is initiated in the majority of the female and, depending on the environment, also in a fraction of the male mice, which very rapidly leads to destruction of the insulin-producing cells. It has yet to be established what precipitates this malignant autoimmune response. But why do not all the animals become diabetics, and why does an induction of Th2 responses in the animals appear to protect against development of type I diabetes? We hypothesize that the spontaneous development of autoimmune disease(s) in the NOD mouse relates to a failure of peripheral tolerance induction. This is supported by the experiments of CLARE-SALZLER et al.[4], who reported that transfer of DC derived from the pancreatic lymph node of NOD mice, but not from any other lymph nodes, protected against development of diabetes, suggesting that an increase in the capacity to induce peripheral tolerance will protect against development of the autoimmune disease. The insufficient induction of peripheral tolerance, as hypothesized, in unrelated to the MHC alleles of the NOD mouse, since NOD.$H2^{h4}$ congenic mice spontaneously develop autoimmune thyroditis if fed iodine in their drinking water.

PULENDRAN et al.[38] demonstrated that two distinct DC subtypes could be differentially expanded in mice *in vivo*. Treatment of the animals with GM-CSF *in vivo* preferentially expanded a DC subset with features characteristic of DC2 cells, which have the ability to activate naïve $CD4^+$ T cells into a Th2 type. In contrast, mice injected with the cytokine Flt-3 ligand[28, 38] dramatically expanded a different subset of DC with high expression of IL-12 and IFN-γ[39]. The Flt-3 ligand-treated mice produced a high level of Th1 cells and, if immunised with ovalbumin, they predominantly produced IgG2a antibodies, whereas GM-CSF-treated mice immunised with ovalbumin predominately elicited an IgG1 antibody response. PULENDRAN and colleagues[40] have previously shown that mice treated with Flt-3 ligand had a dramatically enhanced sensitivity of antigen-specific B and T cell responses and that the Flt-3-treated mice could elicit a productive immune response to otherwise tolerogenic protocols.

Human diabetics and the two spontaneous animal models for type 1 diabetes, the BB-DP rat and the NOD mouse, share a defect in the development of DC. Fewer monocytes cells are produced from primary (bone marrow) and secondary (spleen) lymphoid cell suspensions or from peripheral blood cells in response to various growth factors (GM-CSF, M-CSF, IL-3, IL-5) relative to appropriate non-diabetic controls[6, 24, 55]. In addition, at least in the case of the animal models, the GM-CSF-generated dendritic cells appear to be developmentally arrested at a precursor state, with little or no MHC class II and CD80/CD86 cell surface expression (STRID et al., submitted)[6]. The GM-CSF-generated DC from the NOD mouse also proved to produce a very limited amount of

IL-12 in response to LPS and IFN-γ compared with non-autoimmune controls, indicating that they may be of the DC2 subset (STRID et al., submitted). This abnormal developmental response to GM-CSF could affect the balance of the DC1/DC2 subset in the autoimmune-prone animals or individuals both in the primary and secondary lymphoid tissues as well as in the periphery. A deficient maturation of the DC2s in the autoimmune--susceptible animals or individuals could polarize the DC population towards a predominant DC1 subset. If the observation of PULENDRAN et al. [38, 39], that the DC1s are less able to induce central and peripheral tolerance in the host, is correct, the individuals with a predominant DC1 population may be more prone to developing autoimmune diseases. In addition, a skewing towards a DC1 response to self-antigens would shift the T cell response from a non-destructive Th2 to the destructive Th1 response prevailing in the animals or individuals that develop diabetes.

Although a lack of centrally and peripherally induced tolerance/ignorance to self proteins contributes to the development of the disease in the NOD mouse, it cannot explain the shift from benign to malign autoimmunity in the mice. A number of transgenic models have been explored to search for explanations for insulin-dependent diabetes. In these mice, foreign transgenes, LCMB gp120[34, 35] or membrane-attached ovalbumin[22, 23], were put under the control of the rat insulin promoter (RIP) and microinjected into C56BL/6 mice in order to direct the expression to the insulin-producing cells in the pancreas. One of the transgenic lines also expressed the transgene in the thymus[35], whereas no presence of transgenic mRNA could be detected in the thymus of the two other transgenic lines[23, 34]. Despite this, central tolerance against the transgene could be observed, as the ovalbumin transgenic mice, when crossed with transgenic mice expressing an ovalbumin--specific H2-K^b-restricted T cell receptor, showed a significant deletion of the antigen-specific T cells[23]. The lessons from these 3 transgenic mouse lines were that, although the transgene was predominantly expressed in the insulin-producing β cells, the whole T cell population appeared ignorant towards the antigen, i.e., the induction of peripheral tolerance was adequate to regulate the T cell population. If the animals were infected with LCMV, however, β cell destruction was observed within weeks, and the autoimmune response could be transferred with $CD4^+$ and $CD8^+$ T cells to non-infected transgenic mice[34, 35]. Autoimmune destruction was observed to require a threshold level of antigen-specific T cells[22, 23] and, although $CD8^+$ T cells alone would be able to induce autoimmune disease in the animals upon transfer, the threshold level was much higher than in the presence of antigen-specific $CD4^+$ T cells[22].

Extending this discussion to the NOD mouse, which spontaneously develops diabetes, one may presume that, around week 12, a number of β cells will suddenly be destroyed and numerous intra-cellular proteins released and captured by DC. The dendritic cells will migrate to the draining lymph node, where they will present the self antigen to T cells and elicit an autoimmune response. Because of the unfavourable balance in DC1/DC2 cells, the threshold level of activated β cell--specific T cells required for mounting a full-blown autoimmune response is much lower than in non-diabetic control animals.

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