Infection and Autoimmunity
Y. Shoenfeld and N.R. Rose, editors

Type I Diabetes Mellitus, Infection and Toll-Like Receptors

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1. EPIDEMIOLOGY OF TYPE-I DIABETES

During the last forty years, the incidence of type I diabetes mellitus (TIDM) has shown a significant increase in developed countries [1]. Gross geographical differences can be seen in the increase of TIDM, and Europe provides us with a good example [1]. Northern European countries have higher incidences of TIDM than southern European countries, with Finland showing the highest European (and world) incidence [2]. The genetic background is an important factor in determining the susceptibility to autoimmune disease [3], however several observations indicate that genetic differences cannot account for the uneven distribution of TIDM. First, epidemiological studies carried out in Northern Ireland [4] and England [5] have found a positive correlation between a low incidence of type I diabetes and poorer socioeconomic conditions. Second, populations migrating between countries with different values of TIDM incidence are interesting examples: children born to parents who migrated to Yorkshire from Pakistan show an incidence of TIDM indistinguishable from that seen among non-migrants in England (11.7/100,000), which is significantly higher than Pakistan's incidence of TIDM (1/100,000) [1, 6, 7]. Third, in a recent paper Hyttinen and co-workers report that after studying 22,650 twins, the concordance rate for TIDM was only 27.3% in monozygotic twins and 3.8% in dizygotic twins [2]. In summary, these data highlight the importance of environmental factors in the worldwide increase of TIDM.

2. THE HYGIENE HYPOTHESIS

The hygiene hypothesis associates the increase in the incidence of autoimmune diseases and allergy in developed countries with the effects of the environment on the immune system. It postulates that childhood infections educate the immune system on how to react to antigenic challenge. The immune system is a self-organizing system and like the brain it requires experience to learn how to behave [8]. However, as a consequence of improved hygiene, vaccination campaigns, and the use of antibiotics in industrialized countries, the "education" of the immune system has been significantly diminished, and the immune response runs out of control upon stimulation with otherwise innocuous substances.

The data obtained in the non-obese diabetic (NOD) mouse, a laboratory model of TIDM, seems to support the hygiene hypothesis. TIDM can be prevented in NOD mice by infection with bacteria, viruses or parasites [9–12]. It should be noted that TIDM protection does not need these agents to be alive: preparations of dead mycobacteria [13–15], streptococci [16] or parasites [17, 18] can also halt the process that leads to overt TIDM. Thus, microbial components supply the immune information needed to shut off autoimmune diabetes.

3. MECHANISMS OF TIDM PREVENTION

Several mutually non-exclusive mechanisms have been invoked to explain the protection from autoimmune diseases afforded by infections. Microbial epitopes can share sequence homology with regulatory self-epitopes; this "molecular mimicry"

[19] might allow infections (or more specifically, microbial molecules) to activate built-in regulatory networks. Although such a mechanism has been reported to inhibit experimental autoimmune encephalomyelitis [20] and adjuvant arthritis [21], it has not yet been reported for TIDM. In fact, molecular mimicry with microbial antigens seems to accelerate, rather than inhibit TIDM [22]. The contribution of molecular mimicry, and other mechanisms involving the adaptive immune response (antigen competition, bystander suppression or microbial superantigens) to the control of autoimmune diseases by the environment has been recently reviewed elsewhere [1].

4. TOLL-LIKE RECEPTORS

Innate immunity might also play a role in the modulation of TIDM by the environment. Toll-like receptors (TLRs) constitute a family of innate receptors recently identified in mice and humans [23–25]. TLRs were identified based on their homology with the *Drosophila melanogaster* toll receptor, which is involved in the development and the immune response of the fly [26]. Ten different TLRs have been identified thus far in mice and humans [23, 25]. However, none of the TLR knock-out mice described so far has developmental disorders, suggesting that mammalian TLRs lack a role in development or have some degree of redundancy.

Mammalian TLRs are type I transmembrane receptors that share several structural/functional features [23, 25, 27]. They present an extracellular leucine-rich (LRR) repeat whose length varies between different TLRs. LRRs are thought to mediate protein-ligand interactions; they are found in proteins with several functions not restricted to the immune response. The LRR domain of the TLRs is separated from the single transmembrane domain by a characteristic cysteine-rich domain. TLRs also share a cytoplasmic Toll/Inerleukin-1 receptor homology (TIR) domain. TIR domains are protein interaction modules shown to recruit adaptor molecules. Finally, all TLRs have at least one MyD88-dependant signaling pathway. MyD88 is a 35 kDa adaptor protein that, through its own C-terminal TIR, interacts with the TIR of activated TLRs [28, 29]. However, MyD88 is not the only adaptor molecule involved in TLR-triggered signaling, MyD88-independent signaling pathways have also been described [30–32].

Functional studies indicate that TLRs can dimerize, generating both homo and heterodimers [33–35]. Their ability to dimerize and form complexes with other surface molecules like MD2 [36] might explain the structural diversity of TLR ligands. A striking example of ligand diversity is given by TLR4, involved in the recognition of both lipopolysaccharide (LPS) [36] and chlamydial 60 kDa heat shock protein (HSP60) [37]. Although they were initially thought to recognize only pathogen-associated molecules [38], TLRs respond both to self and non-self molecules [39]. Table 1 lists some microbial and host TLR ligands.

5. TLRS AND THE IMMUNE RESPONSE

Dendritic cells (DC) are antigen-presenting cells that trigger and influence several aspects of the immune response, including the differentiation of naïve $CD4^+$ T cells into either Th1 or Th2 effector/memory cells [40, 41]. DC express several TLRs whose levels are adjusted according to the state of activation of the cell [42–46], thus by modulating DC activity, TLR ligands could potentially influence the adaptive immune response. TLR activation by self [47–49] or non-self [37, 42, 50–54] TLR ligands promotes DC maturation. Indeed, several experimental reports seem to indicate that the activation of TLRs on DC has strong effects on the immune response.

MyD88 is a pivotal molecule in the signal transduction pathway of TLRs [28, 29]. Schnare and colleagues showed that the immune response triggered by immunization with antigen in CFA, marked by Th1-type cytokines, cytotoxic activity and specific IgG2a in wild type mice, was highly impaired in MyD88-deficient mice [55]. The inability to mount a vigorous Th1 response might have been associated with a defective maturation of DCs in response to the mycobacterial component of CFA, as reported by the authors [55]. Therefore, the triggering of TLR-dependant signaling pathways are needed for the maturation of DC and the induction of a vigorous Th1 response when microbial products are used as adjuvants. Further studies have also included TLRs

Table 1. Self and non-self TLR ligands

TLR	Non-self Ligands	Self Ligands
TLR1	Mycobacterial lypoprotein Triacylated lipopeptides *B. burgdoferi* OspA	Unknown
TLR2	*P. gingivalis* LPS Zymosan Peptidoglycan (bacteria) Lipoproteins (bacteria and mycoplasma) *T. cruzi* GPI anchor *B. burgdoferi* OspA	HSP60 Surfactant protein A HSP70
TLR3	Poly (I:C) dsRNA	Unknown
TLR4	LPS Respiratory syncytial virus GroEL HSP60 Chlamydia HSP65	HSP60 HSP70 Saturated fatty acids Unsaturated fatty acids Hyaluronic acid Surfactant protein A Fibronectin
TLR5	Flagellin	Unknown
TLR6	Mycoplasma lipoproteins Lipoteichoic acid Peptidoglycan (bacteria)	Unknown
TLR7	Resiquimod Imiquimod	Unknown
TLR8	Resiquimod Imiquimod	Unknown
TLR9	CpG DNA	dsDNA
TLR10	Unknown	Unknown

in the induction of antigen specific Th2 responses [56, 57]. Overall, these results suggest that TLRs are the receptors involved in the adjuvant properties of several microbial preparations [58].

TLR ligands can also be the targets of the adaptive immune response. OspA is an outer-surface lipoprotein from *B. burgdoferi* that activates macrophages through a TLR1 and TLR2, probably complexed in a heterodimer [59]. Vaccination with OspA is being studied as a tool to fight Lyme disease [59]. Strikingly, TLR1 or TLR2-deficient mice do not mount OspA-specific immune responses upon vaccination [59]. Furthermore, humans with reduced TLR1 surface expression on $CD4^+$ cells did not mount a detectable immune response to OspA after repeated vaccination [59]. These results highlight the importance of TLRs for the design of vaccines and demonstrate that TLR ligands can simultaneously work on several components of the immune response, including $CD4^+$ T cells.

TLR signaling has been classically associated with the promotion of a Th1, pro-inflammatory, immune response. TLR activation, however, could also lead to the release of Th2 cytokines [56, 57]. Moreover, TLR signaling is needed for the induction of Th2 responses [56, 57, 60, 61] and for the maintenance of B cell memory in humans [62–64].

Thus, microbial antigens activate specific TLRs and in this way influence the immune response directed against them (and the microbe). However,

as we have already mentioned, microbial infection can also lead to the inhibition of autoimmune diabetes; is there a role for TLRs?

6. TLRS AND AUTOIMMUNITY

A healthy immune system harbors self-reactive T and B cells [65–69]. These self-reactive clones are constantly kept under the active control of regulatory cells [70–72]: the removal of the regulators leads to the onset of autoimmune disease [73]. Remarkably, regulatory T cells express several TLRs and are stimulated by TLR ligands like LPS [74]. In addition, upon activation of their own TLRs, DC can have opposite effects on regulatory cell function. DC can secrete immunomodulatory cytokines that favor induction of regulatory cells, like IL-10 [53, 60] or they can inhibit regulatory T-cells via an IL-6-mediated mechanism [75]. Accordingly, several recent papers have shown that TLRs can play a role in the modulation of TIDM by the environment.

7. IMMUNOSTIMULATORY BACTERIAL DNA MOTIFS

Bacterial DNA, like that present in CFA [76], is rich in DNA motifs that stimulate the innate immune system via TLR9-mediated mechanism [77]. These immunostimulatory DNA sequences consist of a central unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines [76]; such a sequence is referred to as a CpG motif. We have demonstrated that CpG motifs present in bacterial DNA can inhibit spontaneous diabetes of the NOD mouse [78], but not the more aggressive cyclophosphamide-accelerated diabetes [79]. Prevention of diabetes was characterized by a decrease in insulitis, and a down-regulation of the spontaneous proliferative T cell responses to HSP60 and to its 437–460 peptide (p277) which characterize NOD diabetes [78]. Moreover, we detected a concomitant increase in IgG2b antibodies to HSP60 and to p277, and not to other islet antigens (GAD or insulin) or to control antigens. The IgG2b isotype of the specific antibodies, together with the decrease in T cell proliferative responses, indicated a shift of the autoimmune process to a Th2-type in treated mice. These results suggest that immunostimulation by bacterial DNA motifs can modulate spontaneous HSP60 autoimmunity and inhibit NOD diabetes.

8. LPS

LPS from *E. Coli* stimulates innate immunity via TLR4 [23, 25]. Moreover, administration of LPS has been shown to inhibit spontaneous diabetes [80]. Furthermore, LPS-activated B cells express the Fas ligand, secrete TGFβ and can induce apoptosis of diabetogenic T cells [81]. The transfer of LPS-activated B cells could temporarily impair APC function and thereby prevented the onset of diabetes in NOD mice, and halted the pathogenic process in an adoptive transfer model of type I diabetes mellitus (T1DM) to NOD/scid mice [81]. However, the transfer of LPS-activated B cells did not promote Th2 responses to β-cell antigens. Thus, B-cell activation through TLR4 by LPS can inhibit spontaneous autoimmune NOD diabetes.

9. DOUBLE STRANDED RNA

Polyriboinosinic:polyribocytidylic acid (poly I:C) is an analogue of viral double-stranded RNA that has been shown to activate the innate immune system via TLR3 [23, 25]. Serreze and co-workers found that the repeated administration of poly I:C, alone or in combination with IL-2, completely prevented the onset of diabetes [82]. However, the therapeutic effect required continuous administration of the immunostimulants since pancreatic insulin content declined and severity of insulitis increased following cessation of treatment. T cells isolated from Poly I:C-treated mice were capable of suppressing NOD T-cell responses to alloantigens in a mixed lymphocyte culture, indicating that regulatory T cells can be induced in NOD mice by TLR3-mediated signaling pathways.

10. HSP60 AND PEPTIDE P277

Self-HSP60 is an endogenous ligand for TLR2 and TLR4 [83]. The TLR4 molecule does not seem to bind HSP60 directly, but TLR4 is required to

transduce a signal [83–85]. Macrophages exposed to soluble HSP60 secrete pro-inflammatory mediators such as TNF-α, IL-6, IL-12, and nitric oxide [47, 84, 86]. HSP60 can also induce DC maturation and Th1 responses [47, 48]. The pro-inflammatory effects of HSP60, detectable in the blood of pre-diabetic NOD mice at the peak of the autoimmune attack [87], could contribute to the onset of the disease. Thus circulating HSP60 might accelerate β-cell destruction through TLR signaling. However, the same HSP60 molecule has been shown to arrest the diabetogenic process.

HSP60 is a T and B cell antigen in human and NOD autoimmune diabetes [78, 88–90]. Vaccination of NOD mice with HSP60 [79] or its p277 peptide [91] arrested the development of diabetes and even induced remission of overt hyperglycemia [92]. Successful p277 treatment was associated with the down-regulation of spontaneous T-cell reactivity to p277 and with the induction of antibodies to p277 displaying Th2-like isotypes IgG1 and IgG2b [88].

Type I diabetes in humans was also found to be susceptible to immunomodulation by p277 therapy. A double-blind, phase II clinical trial was designed to study the effects of p277 therapy on newly diagnosed patients [93]. The administration of p277 after the onset of clinical diabetes preserved the endogenous levels of C-peptide (which fell in the placebo group) and was associated with lower requirements for exogenous insulin, compatible with the arrest of β-cell destruction. Treatment with p277 was associated with an enhanced Th2 response to HSP60 and p277. Taken together, these results suggest that treatment with HSP60 or its p277 peptide can lead to the induction of HSP60-specific regulators that can control the collective of pathogenic reactivities involved in the progression of autoimmune diabetes.

The apparent contradiction between the pro-inflammatory and the anti-diabetic effects of HSP60 may be resolved by the discovery that HSP60 can directly activate anti-inflammatory effects in T cells by way of an innate receptor. HSP60 and its fragments can regulate the physiology of inflammation itself by acting as ligands for TLR2 in T cells [94]. HSP60 activated human T-cell adhesion to fibronectin, to a degree similar to other activators: IL-2, SDF-1α and RANTES. T-cell type and state of activation was important; non-activated CD45RA+ and IL-2-activated CD45RO+ T cells responded optimally at low concentrations (0.1–1 ng/ml), but non-activated CD45RO+ T cells required higher concentrations (>1 μg/ml) of HSP60. T-cell HSP60 signaling was inhibited specifically by a mAb to TLR2, but not by a mAb to TLR4. The human T-cell response to soluble HSP60 depended on PI-3 kinase and PKC signaling, and involved the phosphorylation of Pyk-2. Soluble HSP60 also inhibited actin polymerization and T-cell chemotaxis through ECM-like gels towards the chemokines SDF-1α or ELC. Exposure to HSP60 could also down-regulate the expression of chemokine receptors CXCR4 and CCR7. Most importantly, HSP60 down-regulated the secretion of IFNγ by activated T cells (unpublished observations). These results suggest that soluble HSP60 (and its fragments), through TLR2-dependent interactions, can down-regulate T-cell behavior and control inflammation. Thus, HSP60 can have both pro- and anti-inflammatory effects on various cell types. HSP60 works as a ligand both for antigen receptors on T cells and B cells (and autoantibodies) and for innate receptors TLR4 and TLR2 on various cells types.

To further examine the contribution of innate immune signaling to autoimmune diabetes, we inserted a TLR4 mutation into NOD mice. As we mentioned above, TLR4 is needed for the activation of macrophages by HSP60 [84, 85]. Mutated TLR4 appears to markedly increase susceptibility to autoimmune type 1 diabetes (Dr. G. Nussbaum, unpublished observations). Apparently TLR4 signaling, whether by endogenous ligands such as HSP60 or foreign ligands such as LPS, can educate the immune system to avoid pathogenic autoimmunity.

11. CONCLUDING REMARKS

TLR-activation has usually been associated with the induction of pro-inflammatory immune responses. Thus the inhibition of TIDM (an inflammatory condition) by TLR-ligands is controversial. Several mechanisms might account for this paradoxical observation. First, the direct activation of regulatory cells via TLR [94]. Second, the activation of TLR-dependant anti-inflammatory responses on effector T cells [94]. Third, by inducing pro-inflamma-

tory responses, TLR ligands might trigger built-in anti-inflammatory responses. Indeed, a controlled inflammatory response has been shown to be necessary for the inhibition of NOD diabetes by CFA [95]. These mechanisms are not exclusive, and further experiments should be directed at determining the contribution of each one of them in the control of diabetes via TLR activation.

Through their interaction with microbial molecules, TLRs sense the environment. The experimental data showing that TLR-mediated activation of the immune system can inhibit the progression of NOD diabetes is in accordance with the hygiene hypothesis. The hygiene hypothesis could then be partially explained based on the "education" of the immune system by microbial TLR ligands. The role played by endogenous TLR ligands is still not clearly understood. However, the preliminary results obtained using NOD mice bearing a non-functional TLR4 might suggest that TLR activation by self ligands is involved in the control of diabetogenic T cells. Maybe the "lesson" thought by exogenous TLR ligands is continuously reinforced via the activation of TLRs by endogenous ligands, like HSP60.

Thus, the stimulation of TLRs with defined TLR ligands might allow us to translate the hygiene hypothesis into immunotherapy. New therapeutic approaches for TIDM aiming at the "re-education" of the immune system might be designed using TLR-ligands, but without the risk of infection with life-threatening pathogens. The initial success of p277 in treating TIDM might then be the first lesson to learn, in a whole new program on the treatment of autoimmne disease.

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