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TNF-α mediated modulation of T cell development and exacerbation of in vitro T1DM in fetal thymus organ culture

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Abstract

TNF-α is a pleiotropic cytokine that is constitutively expressed in the thymus. This cytokine has opposing effects on type 1 diabetes mellitus (T1DM) as non-obese diabetic (NOD) mice administered TNF-α early in life experience an acceleration in disease onset while TNF-α administered to adult NOD mice are rescued from disease entirely. Using fetal thymus organ culture (FTOC) as a model of T cell development and an associated in vitro T1DM model, we set out to reconcile the role of TNF-α in thymic development with its role in the pathogenesis of T1DM. Our data indicate that NOD derived FTOC produce a smaller percentage of double negative ($CD4^-/CD8^-$) thymocytes expressing TNF receptors compared to non-diabetic C57BL/6 (B6) derived FTOC. NOD FTOC produce more TNF-α than B6 FTOC during days 6–9 of culture, a time when negative selection of T cells is known to occur. Neutralization of this endogenous TNF-α production in NOD derived FTOC with soluble TNF receptor (sTNF R1) rescued insulin production in our in vitro T1DM model. Flow cytometric analysis of NOD FTOC treated with recombinant TNF-α (rTNF-α) or sTNF R1 demonstrated that the relative levels of TNF-α in the culture during the selection window (days 6–9) influence the ratio of immature vs. mature T cells that emerge from FTOC.

Keywords: Autoimmunity; Cytokines; Diabetes; Repertoire development; T cells

1. Introduction

Type 1 diabetes mellitus (T1DM) is a T cell-mediated, chronic autoimmune disease that is characterized by lymphocytic infiltration of the pancreatic islets of Langerhans and by the selective destruction of insulin-producing β cells in the islets. The underlying mechanism that initiates disease has been suggested to hinge upon the aberrant selection of autoreactive T lymphocytes that occurs during T cell development [1,2]. Immature T cells differentiating in the thymus interact with the major histocompatibility complex (MHC) molecules on thymic epithelium or hematopoietically derived antigen presenting cells (APC) through their T cell receptors (TCR) [3]. These interactions lead to the 'positive selection' of T cells that recognize peptides bound to self-MHC gene products of the thymus. To prevent autoimmune responses, those T cells that possess TCR specific for self-antigens are eliminated in the thymus by 'negative selection' [4,5]. The origins of T1DM are the result of the erroneous survival of autoreactive, diabetogenic T cells that have escaped negative selection.

Our laboratory has developed an in vitro fetal thymus organ culture (FTOC) model that allows the detection of developing diabetogenic T cells in only 14–21 days. This methodology involves the co-culture of mature NOD fetal thymus lobes with syngeneic fetal pancreas. Over the course of the co-culture period, mature thymocytes migrate into the adjacent pancreatic mass and mediate islet destruction in a T cell

Abbreviations: T1DM, type 1 diabetes mellitus; FTOC, fetal thymus organ culture; FPOC, fetal pancreas organ culture; TNF-α, tumor necrosis factor-alpha; rTNF-α, recombinant TNF-α; sTNF R1, soluble receptor (1) for TNF-α; NOD, non-obese diabetic; B6, C57BL/6; SP4/SP8, single positive cells expressing CD3/TCR ($CD4^+$ or $CD8^+$ as indicated); DP, double positive ($CD8^+CD4^+$); DN, double negative ($CD8^-CD4^-$); TN, triple negative ($CD8^-CD4^-CD3^-$).

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restricted fashion. This "in vitro T1DM" effect can be quantified by the subsequent measurement of insulin production by these co-cultures [6,7].

The pathogenesis of autoimmune diabetes is complex, as expected from the >23 genes found associated with the onset of disease [8], both in humans and the non-obese diabetic (NOD) mouse, the most commonly used animal model. Loci identified by genetic segregation analysis as contributing to T1DM susceptibility are provisionally referred to as *Idd* loci until specific genes are elucidated. Estimates from these analyses indicate that 50% or more of the genetic risk for T1DM is transmitted by the strain's unusual I-A^{g7} class II MHC haplotype, which was the first *Idd* gene identified (*Idd1*). There are as many as 19 non-MHC linked *Idd* genes that are also required to mediate the pathogenesis of autoimmune T1DM [9]. Some possible candidates include defects in signaling pathways, selection induced apoptotic pathways including those mediated by CD95 (Fas)/CD95L (Fas Ligand) and tumor necrosis factor-α (TNF-α) [10]. Recent work along these lines has shown that *Idd*16, an interval that maps within the MHC, contains the gene that encodes TNF-α. These authors have identified a mutation in the 5′-upstream region of this gene [11]. Mutations in this region may well contribute to the pathogenesis of T1DM through defective regulation of TNF-α expression. Indeed, NOD derived peripheral lymphocytes have shown a reduced capacity to produce TNF-α upon stimulation [12]. However, to date, no research has been done on the endogenous production of TNF-α at baseline levels, either peripherally or within the thymic microenvironment with regard to diabetic vs. non-diabetic prone animals.

TNF-α is a proinflammatory cytokine that has been shown to be a critical mediator of the inflammatory responses that characterize autoimmune diseases in general [13]. Indeed, its role in the destruction of beta islet cells, although not the focus of our particular study, has emerged as an active area of research [14,15]. The production of TNF-α has been detected by both thymocytes and stromal elements within the thymus [16] and has been shown to regulate both the proliferation, apoptosis and the maturational transition of the DN ($CD4^-/CD8^-$) subset [17].

Thus far, TNF-α has been shown to have a dual role in the progression of T1DM. Neonatal exposure to TNF-α can exacerbate T1DM onset, while adult exposure to TNF-α can avert the disease entirely [13,18]. Antibodies to TNF-α, administered over the same time periods have the ability to reverse these effects in both cases. The mitigating effects of adult administration have been attributed to TNF-α's ability to attenuate TCR signaling, thereby suppressing the autoimmune inflammatory response in animals destined to become diabetic [19]. Recent evidence suggests that the presence of TNF-α early in the life of NOD mice, destined to become diabetic, can reduce the frequency of regulatory $CD4^+CD25^+$ T cells to a degree significant enough to increase the incidence and severity of T1DM [20]. Conversely, anti-TNF-α antibodies can boost this cell population enough to avert disease entirely.

In this study we attempt to reconcile the role of TNF-α in T cell development and its apparently divergent influence on the origins and pathogenesis of T1DM using an isolated in vitro fetal thymus organ culture model. Our data indicate that the expression of TNF receptors and the endogenous production of TNF-α within the thymus are fundamentally different in diabetic (NOD) animals compared to non-diabetic controls (B6). Furthermore, we demonstrate that the addition of recombinant TNF-α (rTNF-α) or the neutralization of endogenous TNF-α via administration of soluble TNF R1 (sTNF R1) to FTOC can significantly impact the development of T cells as they emerge from the thymus such that they can no longer cause T1DM.

2. Materials and methods

2.1. Mice

Breeding pairs of NOD/Lt mice were obtained as a gift from the laboratory of Dr. Edward Leiter at the Jackson Laboratory (Bar Harbor, ME). Our colony was maintained in a specific pathogen free vivarium at the University of Arizona Central Animal Facility and propagated by brother–sister mating in accordance with protocols reviewed and approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). Mice were allowed free access to standard breeder chow (S-2335 irradiated breeder chow; Harlan Teklad, Madison, WI) and autoclaved drinking water. The incidence of T1DM in NOD/Lt females in our colony at the University of Arizona is 80–90% by 40 weeks of age. NOD/Lt mice were then bred to produce timed-pregnant females. Timed pregnant C57BL/6 mice were also purchased from National Cancer Institute (Frederick, MD). The fetuses were removed from pregnant females at the indicated time points (plug date = 0 days of gestation (dg)). We consistently found that our animals were variable with regard to their stage of development even though they had been vaginally plugged on the same day. Therefore developmental assessment of these mice was based on their characteristics as given in "The Mouse, its Reproduction and Development" [21].

2.2. Fetal thymus organ culture (FTOC)

The organ culture methods used have been described in detail by our laboratory and others [22–24]. Briefly, at least 6 thymus lobes, dissected from 13–16 dg fetal mice were placed on the surface of Millipore (25 μm thick, 0.45 μm pore size; Millipore, San Francisco, CA) filters, which were supported on blocks of surgical Gelfoam (Upjohn Co, Kalamazoo, MI) in 10 × 35 mm plastic Petri dishes with 3 mL of medium. Organ culture media consisted of Dulbecco's modified Eagle's medium (4.5 g/L D-glucose; JRH, Lenexa, KS) supplemented with 20% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), streptomycin (100 μg/mL), penicillin (250 mg/mL), gentamycin (10 μg/mL), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (2×10^{-5} M), and 3.4 g/L sodium bicarbonate. The cultures were grown in a humidified incubator in 5% CO_2 at 37 °C. Cells were harvested as previously described [6,23]. The thymus lobes were placed into a solution of Accutase (ISC Bio Express, Kaysville, UT). The tissue was incubated at 37°C for 15 min. The lobes were then dispersed into a single cell