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Mechanisms of autoimmunity in the non-obese diabetic mouse: effector/regulatory cell equilibrium during peak inflammation

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Summary

Immune imbalance in autoimmune disorders such as type 1 diabetes may originate from aberrant activities of effector cells or dysfunction of suppressor cells. All possible defective mechanisms have been proposed for diabetes-prone species: (i) quantitative dominance of diabetogenic cells and decreased numbers of regulatory T cells, (ii) excessive aggression of effectors and defective function of suppressors, (iii) perturbed interaction between effector and suppressor cells, and (iv) variations in sensitivity to negative regulation. The experimental evidence available to date presents conflicting information on these mechanisms, with identification of perturbed equilibrium on the one hand and negation of critical role of each mechanism in propagation of diabetic autoimmunity on the other hand. In our analysis, there is no evidence that inherent abnormalities in numbers and function of effector and suppressor T cells are responsible for the immune imbalance responsible for propagation of type 1 diabetes as a chronic inflammatory process. Possibly, the experimental tools for investigation of these features of immune activity are still underdeveloped and lack sufficient resolution, in the presence of the extensive biological viability and functional versatility of effector and suppressor elements.

Keywords: autoimmunity; diabetogenic cells; effector T cells; regulatory T cells; type 1 diabetes.

Introduction

The perturbed functional balance between effector and suppressor immune mechanisms has been extensively investigated in the context of the pathogenesis of type 1 diabetes (T1D). The dispute over the cause of quantitative and qualitative equilibrium between effector and regulatory cells in non-obese diabetic (NOD) mice and humans suggests that all possible mechanisms are involved to a certain extent in inflammatory insulitis. In essence, immune dysfunction has been attributed to: (i) increased numbers of diabetogenic cells and reciprocal decreased numbers of suppressor cells, (ii) aggressive effector cell function and reciprocal decreased suppressor cell activity, (iii) decreased sensitivity of effector cells to apoptosis and negative regulation, and (iv) increased susceptibility of suppressor cells to apoptosis. However, most proposed mechanisms have been confounded by studies that could not reproduce the experimental observations and/or challenged the contribution of various mechanisms to the inflammatory reaction. It is therefore questioned what is the evidence of deregulated effector and suppressor forces in human diabetes and the prevalent rodent models: NOD mice and BioBreed rats. Although most mechanistic information on T1D has been gathered in rodent models, the differences from and similarities to human disease impose further difficulties on elucidation of the mechanisms of diabetic autoimmunity.[1–7]

Discussion of immune deregulation in diabetes starts from hypotheses on the nature of emergence of this autoimmune reaction. On the one hand, the prevalent

Abbreviations: CD25, high-affinity α-chain of the IL-2 receptor; CD62L, selectin; FoxP3, X-linked forkhead/winged helix; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; NOD, non-obese diabetic; nTreg, naturally occurring Treg; PLN, pancreatic lymph nodes; T1D, type 1 diabetes; TCR, T-cell receptor; TGF-β, transforming growth factor-β; Treg, regulatory T cells

concept suggests that autoreactive T-cell clones escape from negative selection and expand to initiate and propagate inflammatory insulitis.[8] This hypothesis has evolved alongside characterization of the mechanisms of immune activation and discrimination between self and non-self primarily in the thymus, though peripheral sensitization may also occur as a result of particular characteristics of innate immunity in neonates.[9] Alternatively, it has been suggested that sensitization against the islets is a universal physiological process inadequately contained by suppressor subsets in subjects that will develop the disease.[10] This hypothesis has evolved from the concept that the immune homunculus includes numerous anti-self reactions that are effectively suppressed.[11] According to both scenarios, the persistent imbalance of immune homeostasis characteristic of autoimmunity leads to progressive loss of β-cell mass and hyperglycaemia associated with severe complications. Eruption and propagation of diabetic autoimmunity have been extensively investigated, debated and reviewed, questioning the equilibrium between deregulated effector and suppressor arms of the immune reaction.[12–22] It is questioned whether inflammatory insulitis does indeed involve characteristic aberrant immune mechanisms or has the configuration of a physiological immune reaction once the disease is launched.

Effector cells

Although the inflammatory process in T1D has been associated with a number of intrinsic aberrations in T cells and antigen-presenting cells,[12–17] some defects in effector cell activity fall within the normal functional range of T cells that acquire reactivity against wrong self-antigens.[18–22] Among the variable subsets of immune cells participating in destructive insulitis, earlier classifications of T helper type 1 (Th1) and Th2 cells and the associated cytokine profiles[23–26] have been intensively debated and interpreted.[27–29] It is basically questioned whether quantitative and functional shifts between T-cell subsets cause[30] or reflect secondary consequences of the inflammatory reaction.[31] More recent studies have described the roles of Th17 and follicular helper cells in T1D, emphasizing the participation of multiple cell types in the process of destructive insulitis.[32–34]

Is inflammatory insulitis perpetuated by effector cell accumulation?

One of the proposed mechanisms of immune deregulation involved in T1D pathogenesis is dominant activity of pathogenic cells without true deficit in regulatory mechanisms. Accumulation of infiltrating lymphocytes to a critical threshold results in progressive destruction of the islets apparent in and correlating with the tissue histology.[35] Supporting evidence evolves from the similar effector potential of T cells in male and female NOD mice, which display significant variations in disease severity, the pace of progression and incidence of overt hyperglycaemia.[36] Likewise, $CD8^+$ T cells display similar behaviour in healthy human controls and people with diabetes,[37] though the latter present increased expansion capacity and phenotypes of chronic sensitization.[38] A similar scenario of reduced quantitative T-cell infiltration has been reported in the disease-resistant congenic strain of non-obese non-diabetic mice,[39] attributed primarily to decreased proliferation and increased sensitivity of effector cells to apoptosis compared with NOD mice.[40] If accumulation of effector cells is a mere feature of propagation of a chronic disorder such as T1D, it is questioned whether the activity of diabetogenic cells is stable or increases during the course of disease.

Does pathogenic activity of effector cells increase with age?

Aggressive cytotoxic behaviour of effector cells has been attributed to qualitative differences evolving in various stages of the disease,[41] associated with accelerated islet destruction in late stages of insulitis.[42] Most islet-infiltrating T cells display gradual expression of co-stimulatory molecules (i.e. CD28, CD54, CD80), enhanced interferon-γ (IFN-γ) secretion and reduced interleukin-4 (IL-4) production, sensitivity to inhibition by transforming growth factor-β (TGFβ) and apoptosis, implying that effectors of inflammatory insulitis become more aggressive with age.[40,42–44] These features however, do not dissociate between increased pathogenic activity caused by physiological amplification of cytotoxic T-cell activity within the inflammatory infiltrates and particular characteristics of diabetogenic effectors. It is difficult to evaluate the function of effector cells based on immunophenotype, considering the vast secondary variations through the protracted course of the disease.[31] For example, the $CD4^+/CD8^+$ ratios and expression of activation markers (i.e. CD69, CD62L) are rather constant in $CD4^+$ $CD25^-$ splenocytes from NOD and wild-type mice at different ages.[45] Equal efficacy of adoptive disease transfer using cells negative for CD25 and CD62L from young (6 weeks), older (~12 weeks) and new-onset diabetic NOD mice argues in favour of propagation of insulitis by mere accumulation and sensitization of effector cells within the islets.[45]

Which T cells are recruited and activated?

Several mechanisms of recruitment of pre-activated and naive T cells to the islets have been proposed to play a dominant role in disease progression. One of the basic questions addresses the nature of T cells infiltrating the islets: driver clones pre-sensitized against selected antigens versus bystander naive lymphocytes. On the one

hand, some evidence shows that T-cell infiltration into the islets is restricted to subsets reactive against selected antigens.[46] Propagation of inflammation as a cell-autonomous event invokes that driver clones of pre-sensitized T cells are continuously attracted to the islets and fuel local inflammation.[47] On the other hand, naive bystander T cells are efficiently sensitized and activated within the islets *in situ* and contribute to disease progression.[48–50] The likely scenario is co-existence of both mechanisms of T-cell activation: injury inflicted by pre-sensitized T cells triggers changes in islet configuration and milieu that facilitate recruitment and activation of bystander naive/effector T cells.[51,52] Possibly, both mechanisms converge and make variable contributions to disease progression within the wide heterogeneity in activity and specificity of effector T cells composing the inflammatory infiltrates.[53,54] Consequently, T cells with acquired islet-specificity are variably found in peripheral circulation at various time-points of the inflammatory process, suggesting that both driver clones and bystander T cells contribute to disease propagation.[46,55]

Interpretation of the nature of diabetogenic effectors is difficult within the heterogeneous inflammatory infiltrates and the dynamic changes in composition of the islet microenvironment. At the first level, additional T-cell subsets are progressively recruited to the inflammatory reaction. For example, low-affinity[56–58] and anergic [59–61] T cells are reactivated *in situ* by repeated and sustained antigen-specific sensitization and local inflammation. At the second level, cytokines and vacant niches for lymphocyte expansion exert differential effects on spontaneous and homeostatic expansion of T cells with variable affinities to selected epitopes.[62] High-affinity T cells acquire cytolytic activity and become more aggressive in the process of expansion[63–65] and low-avidity subdominant clones rebound and become dominant under conditions of lymhopenia.[66] At the third level, antigen-specific T cells display variable pathogenic potentials. For example, T cells sensitive to insulin epitopes are more aggressive and are preferentially accumulated within the islets compared with T cells sensitive to glutamic acid decarboxylase-65.[67] At the fourth level, epitope spreading is a mechanism of persistent T-cell sensitization against expanding antigenic targets.[68–70] Within the promiscuous process of antigen recognition,[71] reactive T-cell clones with diverse sensitivities[72] are progressively sensitized against additional epitopes of common islet antigens along the course of disease.[63,73]

Is islet-selective migration associated with T-cell activation?

Various T-cell subsets migrate to and incorporate in the islets, where they undergo subsequent proliferation *in situ*.[47–53] Cell retention within the islets is mediated either by adhesion molecules or specific antigen encounter in the context of MHC. Chemotactic migration of naive/effector T cells is determined by expression of chemokine receptors for cognate ligands displayed by the islets, lymph nodes and the associated vascular endothelium. Most receptors are dynamically expressed in circulating T cells as a consequence of repeated exposure to sites of inflammation. For example, CCR5 is not detected on the surface of naive/effector T cells *in vitro* and is up-regulated following migration to the islets,[18] where it plays a significant role in cell trapping upon recognition of the cognate ligand.[74] Likewise, Ly6C, a glycophosphatidylinositol-linked cell surface receptor, is induced by T-cell receptor (TCR) ligation *in vitro* and in the islet infiltrates[75] and augments production of IL-2 and IFN-γ.[76] Participation of Ly6C in formation of the immune synapse triggers migration arrest and mediates retention of the cells within target tissues.[77] Additional adhesion molecules involved in chemotaxis, retention and stimulation of T cells include integrins and CXCL10 (CCR3 ligand).[78,79] In contrast, T cells with acquired antigen-specificity bypass chemotactic and adhesive interactions and are retained within the islets through establishment of MHC-dependent interactions.[80,81] Both modes of lymphocyte trapping are evidently associated with further activation of lymphocytes and conversion to cytotoxic T cells that foster disease progression in later stages of inflammation.[74,79,82] However, these physiological mechanisms are neither pathological nor specific to autoimmune disorders such as T1D.

Is pathogenic cell activity induced by proliferation?

Experiments performed in transgenic NOD mice expressing a viral peptide in the islets showed persistent evolution of diabetes in the presence of reduced T-cell repertoires.[83] Predisposition of the diabetes-prone mouse to lymphoid (predominantly CD8) expansion was deduced from progressive accumulation of TCR-restricted T cells in the transgenic NOD strain that was not shared by wild-type mice lacking the cognate viral antigen. In fact, inherent and induced relative lymphopenia has been suggested to predispose to autoimmunity and contribute to its evolution.[84–86] Physiological expansion occurs through clonal competition that reduces the diversity and generates dominant clones, resulting in preferential enrichment of antigen-reactive T cells.[87,88] Furthermore, fast cycling T cells induce early diabetes *in vivo*[50] as the result of aggressive behaviour through acquisition of cytotoxic T-cell characteristics such as enhanced IL-2 production,[89] and inefficient suppression.[90] However, fast proliferation of T cells in NOD mice is constant at various ages[89] and may reflect reduced sensitivity to modulation by TGF-β and IL-10 signalling.[41,43]

Does diabetogenic cell resistance to apoptosis cause disease progression?

Pathogenic cells are regulated through several negative loops consisting of activation-induced cell death, direct cytokine inhibition and suppression mediated by regulatory T (Treg) cells. Persistence of autoreactive cells in NOD mice might be caused by reduced sensitivity to negative regulation by apoptosis[91,92] gradually accentuated with age[93] through defective Fas expression in cytotoxic T cells.[94] In contrast, competent Fas expression, stable sensitivity of T cells to negative regulation by activation-induced cell death in NOD mice throughout the course of disease, suggests that variation in sensitivity to apoptosis is not one of the factors that contribute to progressive inflammation.[95]

Concerted propagation of inflammatory insulitis

Do islet antigens drive peak inflammation?

The interface between islets and immune cells amplifies the inflammatory reaction, indicating that islets serve as a driving force for propagation of autoimmune reactivity. Common islet antigens including pro-insulin, insulin, glutamic acid decarboxylase-65 and islet-specific glucose-6-phosphatase catalytic subunit-related protein[96–98] drive continuous and repeated T-cell stimulation *in situ*.[48–50] Expansion of cytotoxic cells within the islets is sufficient in propagation of destructive insulitis[36] in the absence of regional lymph nodes,[99] and β-cell death per se further stimulates the autoimmune reaction through exacerbation of effector T-cell activity.[100]

The role of lymph nodes and auxiliary cell subsets

Lymph nodes continuously propagate the inflammatory reaction through generation and expansion of reactive clones following transport of antigens from the injured islets.[101–103] Some effector cells are released to peripheral circulation[46] and the major fraction of cytotoxic T cells undergoes repeated rounds of stimulation and migrates back to the pancreatic islets.[104–106] The inflammatory reaction is amplified within the lymph nodes by several subsets of antigen-presenting cells including macrophages, dendritic cells and B lymphocytes.[103,107,108] In variance from the crucial role of regional lymph nodes in initiation of inflammatory insulitis, they are rather dispensable in the late stages of inflammation[85] because autoreactive T cells are efficiently expanded within the islets *in situ*.[48–50,100]

The role of cytokines in propagation of inflammation

The inflammatory islet microenvironment amplifies islet lysis mediated by cytokines produced by cytotoxic cells and the injured islets.[42] Immune cells are the primary source of pro-inflammatory cytokines responsible for accumulation of IL-1β, IFN-α, IFN-γ, TGF-β and tumour necrosis factor in the islets as well as the regional lymph nodes.[109,110] Despite significant difficulties in exact determination of the cytokine profile of the inflammatory microenvironments,[111] dominant β-cell secretion of pro-inflammatory cytokines is a paradoxical phenomenon that exacerbates destructive insulitis.[42]

Aberrant or physiological activity of diabetogenic cells?

Altogether these data present significant variability, redundant and synergistic mechanisms that amplify inflammatory insulitis. Enhanced T-cell migration to and retention in the islets, acquired antigenic specificity, repeated stimulation, expansion, activation, conversion to cytotoxic activity and dual elaboration in the islets and draining lymph nodes are co-factors that contribute to amplified diabetogenic effector activity and perpetuate destructive insulitis. However, despite the detection of apparently aberrant behaviour of immune cell subsets in NOD mice, the scenario depicted here is characteristic of any physiological immune reaction targeting selected antigens to extinction.

Regulatory T cells

The concept of suppressor mechanisms regulating autoimmune diabetes has been explored[112,113] prior to phenotypic identification of Treg cells according to expression of the high-affinity α-chain of the IL-2 receptor (CD25).[114] Within the heterogeneous population of suppressor cells,[115,116] the best characterized are naturally occurring Treg (nTreg) cells originating from the thymus and expressing the transcription factor X-linked forkhead/winged helix (FoxP3).[117,118] The significance of these cells is emphasized by the substantial increase in efficacy of adoptive disease transfer into immunocompromised mice following depletion of $CD25^+$ Treg cells.[39,119–121] Consistently, depletion of endogenous $CD25^+$ T cells in wild-type mice elicits autoimmunity with differential organ-specific expression[122] and age-dependent acceleration of insulitis in NOD mice.[123]

Is diabetes progression caused by deficient Treg cell numbers?

Quantitative aspects of Treg cells that might exacerbate insulitis include inherent reduction in numbers and progressive decline with age. All possible trends have been described so far, including decreased, stable and increased frequencies and absolute Treg cell numbers. This quite complex analysis should consider the phenotypic variability of Treg cell subsets, changes in phenotype and function at

different time-points within changing microenvironments and the site of evaluation, i.e. pancreatic infiltrates, lymph nodes, spleen. Interestingly, Treg cells are quite rare in the pancreata of people with diabetes.[124]

Several studies have reported reduced absolute numbers and frequencies of Treg cell subsets characterized by high CD25, CD62L and FoxP3 expression in secondary lymphoid tissues and pancreatic lymph nodes (PLN) of NOD mice compared with age-matched BALB/c[125–127] and C57BL/6 wild-type strains.[128] Decreased Treg cell numbers in NOD mice and humans[115] were associated with predisposition to autoimmunity and were considered to contribute substantially to aggravation of inflammatory insulitis in late stages of the disease.[127,129] A distinction should be made between fractional and absolute Treg cell estimates. The lower absolute Treg cell numbers could originate from inherent defects in production, maintenance and/or excessive susceptibility to apoptosis. Alternatively, the decrease in relative frequencies of $CD25^{high}$ and/or $FoxP3^+$ Treg cells might result from dilution as the result of excessive accumulation of effector cells.

Other studies showed similar Treg cell frequencies in NOD and wild-type mice,[130] and have not confirmed a decline in Treg cell numbers along the course of insulitis in mice[43,130–132] and humans.[133–135] Quite stable $CD4^+$ $CD25^+$ Treg cell numbers and FoxP3 expression frequencies were also detected in secondary lymphoid tissues and PLN of NOD mice,[131] with no significant variations between 8 and 16 weeks of age.[43] Other studies showed a rise in Treg cell frequency in PLN and pancreatic infiltrates in aged euglycaemic NOD mice, which subsides after the onset of overt hyperglycaemia.[136] The rise in peripheral Treg cells may reflect a rebound phenomenon associated with disease eruption,[137] which occurs simultaneously at the central and peripheral levels.[138] Nevertheless, the surge in Treg egress from the thymus[139] and elevated levels of suppressor cells in PLN and islet infiltrates[136] are largely insufficient in preventing progression of the inflammatory process.

Is inflammation fostered by deficient Treg cell function?

Similar to the dispute over quantitative aspects of effector and suppressor T cells in NOD mice, the functional sufficiency of Treg cells has been considered as a possible cause of disturbed homeostasis. Several studies have reported decreased suppressive Treg cell activity in diabetic rodents[43,126] and humans.[133,135] Inherent deficiency in Treg cell function in NOD mice may be caused by defective evolution in the thymus[140] as well as the capacity to acquire suppressor activity in the periphery.[141] In addition, flaring of destructive insulitis in the late stages of inflammation may be caused by a progressive decline in Treg cell function with age.[43,126,142,143] Impaired suppressive function as a possible characteristic of the diabetes-prone strains is considered to operate through reduced surveillance and inhibition of effector cells. For example, perturbed reciprocal interactions that maintain the pools of dendritic and regulatory cells has significant impact on progression of autoimmunity.[144,145] In contrast, other studies confirmed neither decreased Treg-mediated suppression in NOD mice nor progressive decline with age[126,128,131,134,146] and crossover studies showed equal efficacy of Treg cells from young and old mice to suppress effector cell activity *in vitro*.[41,89]

Which Treg cells operate at the site of inflammation?

The major source of $CD4^+$ $CD25^+$ $FoxP3^+$ nTreg cells is the thymus. In fact, NOD mice are characterized by higher basal Treg cell output from the thymus compared with wild-type mice,[138] and the response of NOD thymus to stimulation displays one order of magnitude overshoot in Treg cell production.[139] Treg cells are expanded *in situ*,[137,147–149] and although they cycle at faster rates under resting conditions,[150–152] proliferation is markedly slower than effector cells upon stimulation in the periphery.[153] Adaptive Treg cells are continuously produced at sites of inflammation and within the regional lymph nodes and produce IL-2,[154,155] in contrast with the down-regulation of this cytokine by FoxP3 in thymic nTreg cells.[156,157] Therefore, IL-2 is not only essential to peripheral Treg cell development and function but also serves as a chemotactic factor that is involved in active recruitment of circulating suppressor subsets to the site of inflammation.[158] Indeed, there is evidence of directed Treg cell traffic from the mesenteric to pancreatic lymph nodes and further to the islets, resulting in reciprocal distribution between these lymphoid tissues.[127,136]

Are Treg cells antigen-specific?

It has long been recognized that antigen-specific Treg cells have superior suppressive capacity compared with polyclonal Treg cells, both *in vitro* and *in vivo*.[155,159] Like effector cells, Treg cell activity is antigen-specific[160–162] and the sustained TCR engagement required to initiate Treg cell proliferation gives the antigen-sensitive clones an expansion advantage.[163–165] Notably, TCR stimulation triggers fast Treg cell cycling, exceeding the proliferation of effector cells *in vivo*,[150,151,164] and proliferative anergy is only characteristic of arbitrary culture conditions.[163]

Is diabetes promoted by excessive Treg cell sensitivity to apoptosis?

A major potential cause for decreased suppressor activity is excessive Treg cell susceptibility to apoptosis in NOD mice[152] and humans.[166–168] In the tradition of controversy,

other studies could not confirm these observations, demonstrating that murine and human Treg cells are relatively resistant to apoptosis.[155,169,170] In fact, Treg cell sensitivity to apoptosis is equivalent to effector cells in NOD and wild-type mice under resting conditions,[95] but these subsets are differentially affected by the cellular and cytokine composition of the environment.[171] For example, dissociated influence of IL-2 includes increased susceptibility of effector cells to activation-induced cell death and improved Treg cell survival,[95,171] fostering local expansion and function of Treg cell subsets.[146,158] Similar to IL-2,[156,157] FoxP3 down-regulates Fas-ligand expression and desensitizes Treg cells to receptor-mediated activation-induced cell death, awarding Treg cells a survival advantage.[172]

Discrepant observations of sensitivity to apoptosis probably originate from variability in Treg cell subsets,[116] the local conditions within the inflammatory environment[165] and the composition of cultures used for viability assays.[171] For example, Treg cell death is influenced by variations in rates of proliferation such as increased sensitivity to apoptosis within a generalized lymphoproliferative state in mice deficient in Fas-ligand.[152] In addition to susceptibility to apoptosis associated with cytokine deprivation,[173] IL-2 deficiency is partially responsible for proliferative anergy and high levels of apoptosis *in vitro* and *in vivo*.[158,171,174] These data would suggest that low IL-2 levels detected in the inflamed islets in the late stages of insulitis impair Treg cell development and function[175] and sensitize Treg cells to apoptosis.[171] This contention is challenged by the increased numbers of Treg cells egressing from the thymus and detected in the inflamed islets in the late stages of destructive insulitis[130] and the functional proficiency of suppressor cells.[41,89]

Aberrant or physiological activity of suppressor cells?

Despite various observations in diabetes-prone species, there is no conclusive evidence of inherent quantitative and qualitative aberrations in naturally occurring and adaptive Treg cells. It appears that immune homeostasis responds to the ongoing chronic inflammatory disorder by compensatory increase in thymic Treg cell output and directed traffic through the lymph nodes to the site of inflammation. Experimental evidence demonstrating functional competence of suppressor cells within the physiological adaptation range of immune homeostasis does not provide a clear answer to the cause of immune imbalance leading to destructive insulitis.

Is inflammation enhanced by deregulated interactions between effector and regulatory cells?

As a pivotal cytokine essential for development and function of Treg cells, IL-2 secreted by naive/effector, cytotoxic T cells and dendritic cells determines the size of suppressor cell pool through several mechanisms.[145,176] Enrichment of the inflammatory infiltrates with IL-2 attracts nTreg cells to the site of inflammation,[158] promotes proliferation *in situ*[177] and contributes to conversion of naive T cells to adopt suppressor phenotypes.[178–180] As a coupling mechanism between the intensity of inflammation and Treg cell numbers and function,[176,181] a decay in IL-2 concentrations in the late stages of insulitis may amplify inflammation[175] or may reflect termination of the immune reaction due to extinction of the target tissue.[165] Coupling between IL-2 production by effector cells and Treg cell function is altered by IL-21, which is a partial analogue of IL-2 and promotes destructive insulitis.[182,183] High IL-21 levels render effector T cells less sensitive to Treg cell-mediated suppression[184,185] and reciprocally, IL-21 receptor deficiency impairs antigen-presenting cell function and recruitment of diabetogenic T cells to the islets.[184,186] The mechanism appears to involve the suppression of IL-2 production in cytotoxic T cells by IL-21, with partial compensation of IL-2 deficiency by IL-21 in effector cells but not in regulatory cells.[187]

Relative resistance of effector T cells to Treg-cell-mediated negative regulation is considered to contribute to immune dysfunction that promotes inflammatory insulitis in NOD mice[40,43,89,132] and people with diabetes.[188–190] If the effector activity of diabetogenic cells is stable along the course of disease[45] and regulatory cells are functionally proficient,[41,89,185] then reduced capacity of Treg cells from young NOD mice to suppress disease transfer with cells from diabetic mice suggests disturbed interaction between these subsets. Likewise, fast proliferation and progressive decline in the sensitivity of effector cells to inhibition by IL-10 and TGF-β with age are possible causes of gradual intensification of inflammatory insulitis.[41,43,50] The large Treg cell numbers required to arrest inflammation and the higher efficacy when interventions are performed early in the course of disease are suggestive of relative functional incompetence of Treg cells facing aggressive effector cell activity.[119–121] Hence, similar to the unsettled debate of whether diabetogenic effectors are more aggressive in the distal stages of inflammation, their responsiveness to Treg-cell-mediated suppression remains to be resolved.

What is the meaning of quantitative and qualitative measurements?

Quantitative and qualitative evaluation of immune cell subsets is generally performed at experimental end-points, which reflect a near steady-state condition within a continuous time axis of the inflammatory process. Analysis of effector and regulatory subsets using current technologies ignores the modulation of the microenvironment,

peripheral interconversions, phenotypic promiscuity and instability, migration, rates of proliferation and susceptibility to apoptosis. Furthermore, analysis is compromised by the obligatory search for phenotypes, which are continuously modulated by local activity, expansion, influence of cytokines and other cell subsets.[15,191] Even most precise measurements are questionably accurate, and may not disclose the behaviour within pathophysiological environments,[151,171] but may reflect consequences rather than causes of the observed immune reaction.[31]

Discrepancies in various measurements are affected by numerous factors. First, cellular distribution is often characterized in independent inbred NOD colonies, which may accentuate some features of the particular immunological makeup of the founders. Second, Treg cell subsets are highly variable and represent dynamic populations particularly under conditions of inflammation, with promiscuous expression of CD25 and FoxP3 as Treg cell markers.[115,116] Functional competence of suppressor cells is largely independent of phenotype, with regulatory functions displayed by $CD25^-$ $FoxP3^+$,[192] $CD25^+$ $FoxP3^-$,[193,194] $CD25^-$ $FoxP3^-$[195] and Th17 Treg cells.[196,197] On the one hand CD25 is shed without loss of suppressive Treg cell function[164,198–200] and on the other hand, adaptive Treg cells evolve continuously in the islets and regional lymph nodes[154,155,178] under the stimulatory influence of IL-2 and antigen-presenting cells.[146,179,180] Likewise, FoxP3 as a key factor of nTreg cells is dispensable in the suppressive activity of some Treg cell subsets[201] and is transiently expressed in effector cells.[202] Adaptive Treg cells with differential characteristics from those of thymic nTreg cells are continuously produced at the periphery, either from common precursors of naive and regulatory cells,[203–205] including Th17 effector and suppressor subsets,[206] or interconversion of naive/effector T cells to adopt suppressor phenotype and function.[207–209] Reciprocally, Treg cells may convert to adopt effector activities.[210] For example, low-avidity $CD8^+$ T cells can be converted into autoregulatory T cells[211] and reduce the intensity of inflammatory insulitis.[212]

Third, the highest concentrations of Treg in NOD mice are found in the islets (compared with LN and spleen), emphasizing directed migration to and accumulation in the sites of inflammation.[136] Directed and selective migration is recognized in islet grafts in NOD mice, where selected TCR rearrangements prevalent in the pancreas are variably found in the grafts, peripheral lymphoid tissues and circulation.[46,55] In addition, increased Treg cell contents of PLN[213] are mirrored by reciprocal decreases in mesenteric LN of NOD mice, emphasizing dynamic redistribution between various lymphoid tissues in the course of disease.[136]

Fourth, cytokine environments generated by the islets, and diabetogenic and suppressor cells have a decisive impact on the composition of the inflammatory infiltrates.[51,52] First and foremost, IL-2 is an essential factor for Treg cell development and function,[155,174–177] which has the capacity to recruit nTreg from the periphery[158] and support *in situ* evolution of regulatory clones.[147,178–180] The susceptibility of effector and suppressor cells to apoptosis is modulated by the composition of the environment through relative cytokine deprivation and/or selective provision of cytokines, as well as co-stimulatory factors and cell-to-cell interactions.[150,155,166–174] Although *in vitro* assays are essential for the elaboration of various aspects of T-cell biology and suppression assays serve as the gold standard for identification of suppressor subsets, such experimentation has limited capacity to predict the behaviour of immune cells *in vivo*.[95,151,158,165,169,170]

Fifth, measurements are affected by variable proliferation rates of effector and suppressor T cells within sites of inflammation and regional lymphatics,[83–90] with a considerable advantage of effector cells.[214] Being unable to positively identify diabetogenic cells, their cycling rates are rather deduced from the bulk measurements, disregarding the fact that selective high proliferation rates may be driven by the abundance of cognate target antigens of diabetogenic clones. Treg cells cycle at faster rates under baseline unstimulated conditions compared with naive/effector T cells.[150–152] The drive for resident Treg cell proliferation within inflammatory infiltrates is based on the absolute dependence on TCR engagement, and antigen presentation is evidently most abundant within the islets.[5,165] Other Treg cell subsets are recruited to the site of inflammation through mechanisms such as IL-2 gradients[158] that further stimulate their proliferation.[51,52]

Concluding remarks

Facing the variable experimental data and interpretation, the question of whether autoimmunity in T1D is a feature of inherent dysfunction of effector and suppressor cells remains open. Besides being activated against an essential endocrine tissue responsible for glucose homeostasis, inflammatory insulitis is largely propagated by physiological mechanisms of cytotoxic cell activation. Suppressor subsets are insufficient in containing inflammation, which takes a chronic course of progressive destructive insulitis. What we usually measure is the dominant impact of the microenvironment on the local state of activation, which impacts the dynamics of T-cell numbers, expansion, function, sensitivity to apoptosis and interactions between effector and suppressor cells.[215–217] Despite the invaluable importance of *in vitro* assays towards understanding T-cell function, better definition of the kinetics of effector and regulatory cells in autoimmunity warrants the development of additional instrumental tools to monitor their behaviour in real time with molecular resolution.

Disclosures

The author has no conflict of interest to declare.

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