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Cytopenia and Autoimmune Diseases: a Vicious Cycle fueled by mTOR Dysregulation in Hematopoietic Stem Cells

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Abstract

A long-standing but poorly understood defect in autoimmune diseases is dysfunction of the hematopoietic cells. Leukopenia is often associated with systemic lupus erythematous (SLE) and other autoimmune diseases. In addition, homeostatic proliferation of T cells, which is a host response to T cell lymphopenia, has been implicated as potential cause of rheumatoid arthritis (RA) in human and experimental models of autoimmune diabetes in the NOD mice and the BB rats. Conversely, successful treatments of aplastic anemia by immune suppression suggest that the hematologic abnormality may have a root in autoimmune diseases. Traditionally, the link between autoimmune diseases and defects in hematopoietic cells has been viewed from the prism of antibody-mediated hemolytic cytopenia. While autoimmune destruction may well be part of pathogenesis of defects in hematopoietic system, it is worth considering the hypothesis that either leukopenia or pancytopenia may also result directly from defective hematopoietic stem cells (HSC). We have recently tested this hypothesis in the autoimmune *Scurfy* mice which has mutation *Foxp3*, the master regulator of regulatory T cells. Our data demonstrated that due to hyperactivation of mTOR, the HSC in the *Scurfy* mice are extremely poor in hematopoiesis. Moreover, rapamycin, an mTOR inhibitor rescued HSC defects and prolonged survival of the *Scurfy* mice. Our data raised the intriguing possibility that targeting mTOR dysregulation in the HSC may help to break the vicious cycle between cytopenia and autoimmune diseases.

Keywords

Cytopenia; homeostatic proliferation; CD24; mTOR; hematopoietic stem cells; autoimmune diseases; inflammatory cytokines; rapamycin

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Introduction

Autoimmune diseases are caused by activation of self-reactive T and B cells that escape a multitude of mechanisms for immune tolerance [1–3]. Paradoxically, autoimmune diseases are also associated with immune deficiency and infections. For instance, at least two-thirds of patients with common variable immunodeficiency exhibit signs of autoimmune diseases [4–7]. In addition, patients with primary immune deficiency, such as DiGeorge syndrome, also exhibit signs of autoimmunity [8–12].

The cause and effect between autoimmune diseases and immunodeficiency remains unresolved. It is generally agreed that autoreactive antibodies may cause elimination of leukocytes and thus contribute to immune deficiency [4, 13]. Methotrexate and occasionally, prednisone, the popular drug for autoimmune diseases, are known to cause cytopenia [14–18]. On the other hand, lymphopenia has been shown to cause homeostatic proliferation (HP) of T cells [19–22]. HP has been suggested as a direct cause of autoimmune diseases in the NOD model of type I diabetes [23] and fatal autoimmune diseases in mice devoid of regulatory T cells [24]. One can thus envisage a vicious cycle between autoimmune diseases and immunodeficiency. How to untangle the web between autoimmunity and immunodeficiency is not only of interest for fundamental understanding of immunology, but also of practical significance in treatment of both autoimmune diseases and immunodeficiency. In this presentation, we will review literature in this less explored area and present our recent studies that address the impact of autoimmune diseases on hematopoiesis and the molecular pathway underlying such impact.

A vicious cycle between autoimmune diseases and cytopenia

The link between autoimmune diseases and defects of hematopoiesis system has its root in long-standing clinical observations. In the SLE patients, the cytopenia has emerged as a major hematologic criterion. The American College of Rheumatology (ACR) has the laboratory finding of hemolytic anemia, leukopenia, lymphopenia and thrombocytopenia as a diagnostic marker for SLE [25]. Likewise, cytopenia has been observed in rheumatoid arthritis patients and those with Sjogren's syndrome [15, 26–28]. In addition to generalized cytopenia, more selective defects such as T cell lymphopenia have been reported in rheumatoid arthritis [29] and multiple sclerosis [30].

It is of interest to consider the cause-effect relationship between autoimmune diseases and cytopenia. A well-established autoimmune disease in both adults and children is autoimmune hemolytic anemia, in which autoreactive antibodies are abnormally produced and mediate elimination of both erythrocytes and/or leukocytes [4, 13]. In addition, it is increasingly clear that drugs frequently used for autoimmune patients, such as methotrexate and occasionally prednisone, may have cytopenia as a major adverse event [14–18]. A largely overlooked issue is whether autoimmune diseases may cause defective hematopoiesis. This issue will be revisited in the next section.

On the other hand, cytopenia may also be a fundamental cause of autoimmune diseases. An intriguing link between cytopenia and autoimmune diseases is T cell HP. HP refers to the ability of T cell to mount proliferation in response to paucity of T cells in the host. Physiologically, homeostatic proliferation occurs during the neonatal period. It has been suggested that such proliferation may complement T-cell lymphopoiesis in the thymus to fill the periphery lymphoid organs [20].

Importantly, homeostatic proliferation not only increases the number of T cells in the host, but also fundamentally changes the T cells in at least two ways. First, since HP is primarily driven by self-antigens [19, 21, 22], it is to be expected that homeostatic proliferation would

increase the overall autoreactivity of T cells. This has been confirmed in mice with neonatal thymectomy [31]. Second, T cells that have undergone HP acquire features of memory T cells and thus have lower activation threshold [32, 33]. Both features suggest that homeostatic proliferation may increase the risk of autoimmune diseases. To test this notion, we used mice with a fetal autoimmune disease, called *Scurfy* [34–36]. The *Scurfy* mice were chosen as they are known to exhibit both cytopenia and severe autoimmune diseases [34–36]. Moreover, subsequent studies have identified a similar X-linked autoimmune disease, known as IPEX for immune dysregulation, polyendocrinopathy, enteropathy, and x-linked syndrome [37]. The genetic bases for both diseases were identified about 10 years ago, as inactivating mutations of the FOXP3 gene [38–41]. As the first step to determine if T cell production was defective in the thymus, we analyzed T cell development during the perinatal period. We showed that, in the *Scurfy* mice, the production of T cells in the thymus was reduced as proliferation of T cell progenitors was hampered by an increased Erbb2 expression in the thymus [42]. Corresponding to defective T cell production, the *Scurfy* mice had exacerbated homeostatic proliferation [24]. Since increased survival of the *Scurfy* mice can be achieved only by adoptive transfer of a combination of regulatory T cells and non-regulatory T cells [24], homeostatic proliferation of T cells must be suppressed to prevent the fatal autoimmunity in the *Scurfy* mice.

In order to test this hypothesis by genetic manipulation, one needs to identify a T-cell intrinsic regulator for homeostatic proliferation. In this context, we have reported that a functional CD24 gene on T cells is critical for homeostatic proliferation in a lymphopenic host [43]. To test whether a similar requirement also holds true in the *Scurfy* mice, we adoptive transferred a mixture of WT and CD24-deficient T cells to the *Scurfy* mice. As shown in Fig. 1a, while wild-type T cells mounted a vigorous proliferation, $CD24^{-/-}$ T cells were largely undivided. Thus, much like the lymphopenic host, the homeostatic proliferation in the *Scurfy* mice also requires CD24 expression in T cells. The requirement for CD24 in homeostatic proliferation in the *Scurfy* mice provides us with a model to evaluate its contribution to the pathogenesis of autoimmune diseases in the *Scurfy* mice. We crossed the CD24-null alleles into the *Scurfy* mice and monitored survival of *Scurfy* mice with different CD24 genotypes. As shown in Fig. 1b, CD24-deficiency significantly extended the survival of the *Scurfy* mice. These data make a compelling case that homeostatic proliferation is a missing link between lymphopenia and autoimmune diseases

Apart from the *Scurfy* model, studies by others have demonstrated that T lymphopenia is associated with exacerbation of autoimmune diseases in type I diabetes in the NOD mice [23]. More importantly, the development of diabetes can be prevented by adoptive transfer of naïve T cells [23]. Corresponding to mouse data, defective T cell production and homeostatic proliferation was observed in RA patients [29, 44].

The link between lymphopenia and autoimmune diseases is strengthened by genetic studies in mice, rats and humans. Lymphopenia was observed in the Y chromosome-associated lupus in mice [45]. In the BB rat, the immune-associated nucleotide (Ian)-related genes are associated with lymphopenia and risk of type I diabetes [46, 47]. More importantly, DiGeorge syndrome, which is a prototype of primary immune deficiency due to defective T cell production, is associated with autoimmune diseases, including juvenile arthritis and Grave's disease [8–12, 48].

Taken together, a compelling case can be made that cytopenia may be an important cause of autoimmune diseases (Fig. 2). While lymphopenia provides the most compelling link between autoimmune diseases and cytopenia, it is also likely that additional associations with cytopenia can exacerbate autoimmune diseases. For instance, neutropenia is often associated with infections [49]. Infections may initiate or exacerbate autoimmune diseases

through both activation of Toll-like receptors [50] and/or through molecular mimicry [51, 52].

Hematopoietic stem cells and autoimmune diseases

As outlined above, autoantibodies and drug side effects are two accepted causes of cytopenia in autoimmune patients. It is largely unresolved whether autoimmune diseases may affect the function of HSC. The most important clue that autoimmune diseases may affect the HSC functions comes from clinical experience with aplastic anemia, a pancytopenia attributed to stem cell defects. Aplastic anemia is caused by defective stem cell function and manifests as defective production of both erythroid, myeloid and lymphoid cells. Most cases of aplastic anemia are considered idiopathic. Transplantation is recommended when histocompatible donors are available. Since this is not an option for most patients with aplastic anemia, immune suppression therapy, typically a combination of anti-thymocyte globulin (ATG), which eliminates T lymphocytes, and cyclosporine A, a commonly used immune suppressant, are adopted. Since immune suppression results in complete response in 50–70% of patients, it is generally accepted that most acquired aplastic anemia is a result of concurrent autoimmunity [53].

To directly demonstrate a link between autoimmune diseases and HSC function, we first analyzed the hematopoiesis in the *Scurfy* mice, which is devoid of regulatory T cells [54], and developed fatal autoimmune diseases and pancytopenia [34–36]. We observed a progressive loss of bone marrow cellularity that closely correlated with the progression of autoimmune diseases. Interestingly, the number of HSC ($Flt2^{-}Lin\text{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}CD34^{-}$) temporally expanded in the *Scurfy* mice at three weeks of age when the autoimmune diseases initiated but dropped precipitously by 4 weeks of age when autoimmune symptoms reached their peak, to a level that is 5–10 fold lower than wild-type littermates. In order to compare HSC activity in the bone marrow of WT and *Scurfy* mice, we harvested bone marrow from 1, 3, and 4 week old mice and carried out competitive transplantation. Our data demonstrated that while bone marrow cells from day 7 old *Scurfy* mice were as competent as WT cells in long-term hematopoiesis, bone marrow cells from 3 and 4 week old *Scurfy* mice had greatly diminished hematopoiesis. The defects were observed in all lineages of lymphocytes and myeloid cells. These data provide direct evidence that autoimmune diseases have severe effects on both the number and function of HSC [55]. This model is also valuable for therapeutic intervention of cytopenia associated with autoimmune diseases.

mTOR hyperactivation as the underlying cause for defective hematopoiesis in autoimmune diseases

Mammalian targets of rapamycin (mTOR) have emerged as a key cellular sense for environmental changes, including nutrition, energy, inflammatory stimuli and growth signals such as hormones [56–59]. mTOR is stimulated by signals that activate AKT, which inactivates TSC1/TSC2 complex [57, 60–62]. Conversely, PTEN, a multi-functional negative regulator of cellular signaling and genomic stability, is known to inhibit mTOR activation, perhaps through inactivation of AKT [63]. TSC function is maintained by GSK but abrogated by Wnt signaling pathway [58]. In addition, mTOR sense cellular energy level through AMPK, which is in turn activated by energy-deprivation [59]. Inactivation of TSC complex by IKKβ links mTOR to inflammation [64]. The levels of free amino acids are sensed by RAGA/B complex, which in turn activates mTOR [65]. mTOR forms two distinct signaling complexes, known as TORC1 and TORC2, by interacting with either Raptor or Rictor, respectively [66, 67]. While the TORC1 is activated by AKT, TORC2 regulates activation of AKT1. Moreover, TORC1 and TORC2 are differentially affected by TSC complex. While inactivation of TSC increases TORC1 activity, deletion of TSC appears to

inactivate TORC2 [68, 69], perhaps through a negative feedback mechanism. As depicted in Fig. 3, mTOR pathway has now emerged as one of the best characterized central pathways for cell-environment interaction, including HSC function. Since hematopoiesis is dynamically regulated by the environment, the role for mTOR in HSC function is of great interest.

Two groups have reported that targeted mutation of the Pten gene in the HSC results in transient expansion and their loss of stem cell activity as demonstrated by dysregulation in hematopoiesis in the host and lack of hematopoiesis in bone marrow transplantation studies [70, 71]. Since Pten is a negative regulator of mTOR, it has been suggested that functional loss caused by Pten may be due to hyperactivation of mTOR [70, 71]. However, other studies have raised the possibility that this is achieved by dysregulation of FOXO and genomic instability [72]. To address this issue, we tested the HSC function after inactivation of TSC, which is a more specific regulator of mTOR activity. Our data showed that deletion of the Tsc1 gene results in loss of quiescence and stem cell activation in the HSC, even though loss of HSC function does not correspond to reduction of cells with HSC markers [73]. The defects can be attributed to mTOR hyperactivation as treatment with rapamycin restores the stemness of the HSC. More importantly, mTOR activation causes increased production of radical oxygen species and mitochondrion biogenesis, which is responsible for the defective stem cell function.

Given the broad similarity in stem cell behavior in autoimmune mice and those with *Tsc1* deletion, we tested if mTOR activation is responsible for the stem cell defects in the autoimmune mice. We have provided several lines of evidence for the hypothesis [55]. First, we observed that inflammatory cytokines, such as IL-6 and TNFα, which were highly elevated in the *Scurfy* mice, induced activation of mTOR within 30 minutes. Second, we showed that HSC in the *Scurfy* mice had highly elevated levels of mTOR activation, as revealed by the phosphorylation of mTOR and its downstream substrate S6. Third, we showed that short-term treatment of rapamycin significantly restored bone marrow cellularity and increased production of lymphoid, myeloid and erythroid lineages in the bone marrow. Fourth, we reported that rapamycin treated bone marrow cells showed vastly improved activity in long-term reconstitution in competitive bone marrow transplantation. Last but not least, we observed a very significant improvement of survival of *Scurfy* mice by short-term treatment with rapamycin.

The genetic basis of *Scurfy* mice is the mutation in *Foxp3* gene. In human, the *FOXP3* mutations result in the syndrome of immune dysregulation, polyendocrinopathy, autoimmune-enteropathy (IPEX; OMIM304930) which is a fatal X-linked recessive disorder of early childhood. Protean symptoms of IPEX are severe secretory enteropathy causing failure to thrive, early onset insulin-dependent diabetes mellitus, and eczema [74–77]. Impressively, Bindl, et al reported that sirolimus successfully controlled the gastrointestinal and dermatologic symptoms of IPEX and reduced the systemic inflammatory reaction in three patients for up to 5 years without significant side effects [78]. Given the lack of effective treatment for IPEX patients, it is surprising that only few follow up clinical reports on using sirolimus in IPEX and IPEX-like children have been reported with variable results since the initial report [79, 80].

Implications for the treatment of hematopoiesis defects in autoimmune diseases in humans

Taken together, our studies have demonstrated that mTOR activation is an underlying cause of hematopoiesis defects in autoimmune patients. The above discussion highlights the fact that cytopenia is both a cause and effect of autoimmune diseases (Fig. 2). Therefore, how to break the vicious cycle of cytopenia and autoimmune diseases has significant implication in

the treatment of autoimmune diseases. Since mTOR hyperactivation is a root cause of HSC defects in autoimmune diseases, an obvious question is whether it is feasible to use rapamycin to restore hematopoiesis in autoimmune patients.

Perhaps the first issue is that of safety. A number of transplantation studies have led to a labeling of cytopenia and lipidemia as rapamycin side effects [81, 82]. However, close examination of the trials suggests that the side effects were observed in patients with multiple drug combinations [82, 83], but rarely in rapamycin monotherapy [84, 85]. Even in multi-drug combinations, the side effect was observed in patients with trough concentrations equal or greater than 16 ng/ml [86]. These studies raise the possibility that when doses and drug combinations are carefully managed, it is possible to implement a regimen to use rapamycin for autoimmune diseases. Importantly, in a number of pilot studies, rapamycin appears to have conferred clinical benefits for patients with SLE [87] and type I diabetes [84]. However, to our knowledge, no clinical trial has been conducted to test the concept that hematological defects in autoimmune diseases can be corrected with administration of rapamycin.

It is important to bear in mind that our proposed use of rapamycin to correct hematological defects in autoimmune diseases is based on reprogramming of HSC rather than immune suppression. As such, one may expect a long-lasting therapeutic effect after a short treatment window. In our experience with animal models, short-term treatment of rapamycin during the perinatal period has a long-term effect in adult mice [55]. Thus, for survival studies, the treatment lasted for only one week but the effect was observed for several months after the treatment. For the test of long-term HSC function, the treatment lasted for only two weeks and the impact on long-term HSC could be observed in a new host that received no rapamycin. This is consistent with the notion that rapamycin reprogrammed HSC to increase its stemness. The long-term impact of transient treatment suggests that it may be feasible to identify a therapeutic window to avoid the adverse effects of rapamycin.

Conclusions and future directions

Autoimmune diseases and leukopenia form a vicious cycle. The leukopenia is caused by both direct autoimmune destruction and drug toxicity of leukocyte and inflammation-induced aging of HSC. Leukopenia exacerbates autoimmune diseases by increasing the risk of infection and by inducing homeostatic proliferation. Recent studies suggested the possibilities that environmental features and epigenetics may also be important in pathogenesis of leukopenia in autoimmune diseases [88–90]. Since the HSC defect was caused by mTOR hyperactivation, it may be possible to break this vicious cycle through mTOR targeting. Further studies are needed to evaluate the feasibility of restoring hematopoiesis in cytopenic autoimmune patients through judicious use of mTOR inhibitors and other drugs available for the specific indications. A successful restoration of hematopoiesis in autoimmune patients will not only fulfill an unmet medical need, but also provide us an opportunity to evaluate the contribution of cytopenia to the progression of autoimmune diseases.

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Highlights

- Cytopenia and autoimmunity paradoxically co-exist in patients with autoimmune diseases;
- Cytopenia promotes autoimmune diseases through increased risk of infection and lymphopenia-driven homeostatic proliferation;
- Autoimmune diseases cause mTOR dysregulation and loss of stemness of HSC;
- mTOR inhibitors may break the vicious cycle between cytopenia and autoimmune diseases.

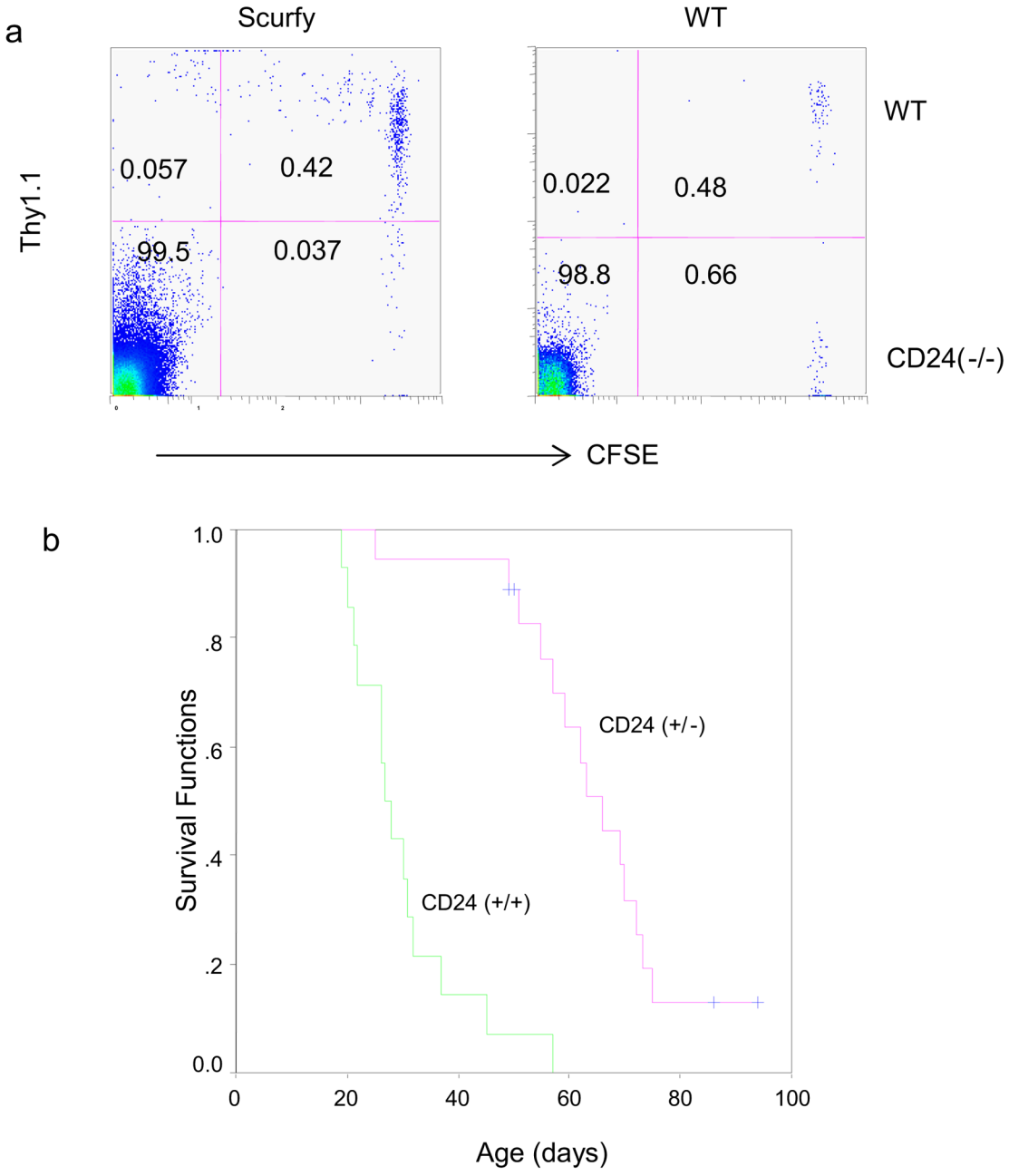

Fig. 1.

Genetic evidence for a critical role for CD24-mediated homeostatic proliferation in the pathogensis of autoimmune diseases in the *Scurfy* mice. a.. CD24-dependent homeostatic proliferation of $Foxp3^{WT}$ T cells in the *Scurfy* mice. 4×10^6 total T cells from either WT Thy1.1$^+$ B6 or Thy1.1$^-$ CD24-deficient B6 mice were mixed at a 1:1 ratio and injected into four day old *Scurfy* B6 mice or wild type littermates. Four days later, the recipient mice were sacrificed and the spleen and lymph node cells were stained with anti-CD4, Thy1.1 antibodies. Data shown are profiles of gated CD4 T cells in the lymph nodes and have been repeated twice. Note that in the WT mice, donor T cells did not dilute CFSE regardless of CD24 genotype. In contrast, WT but not CD24-deficient T cells divided in the *Scurfy* host.

CD24-deficiency abrogated homeostatic proliferation of T cells in the *Scurfy* mice. b. Heterozygous deletion of CD24 is sufficient to prolong survival of the *Scurfy* mice. Life span of $CD24^{+/+}$ and $CD24^{+/-}$ $Foxp3^{sf}$ mice. The mice that have not reached the endpoint of analysis are shown as censored samples, marked by a cross. An extremely significant difference was observed between the life spans of the two strains of mice ($P<0.00001$).

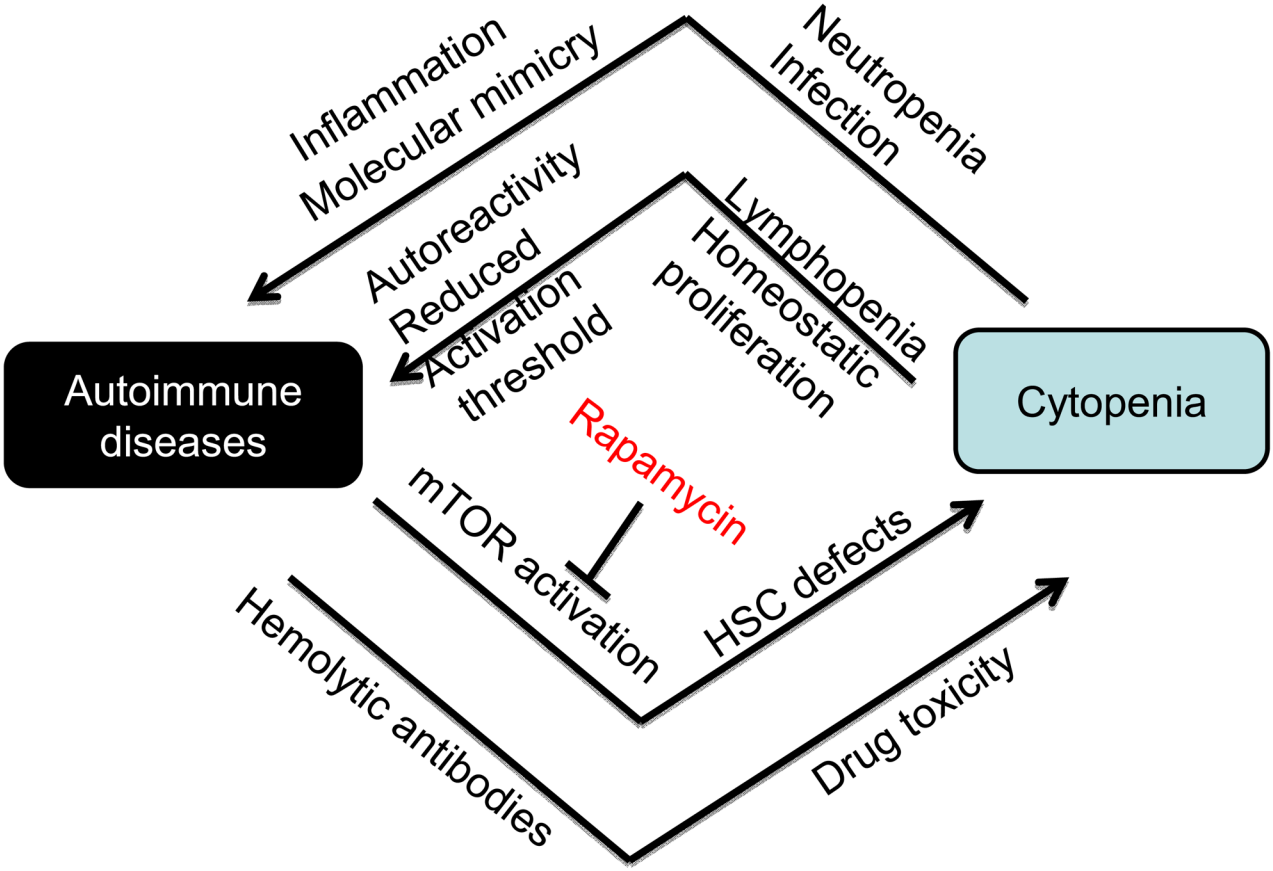

Fig. 2.

A vicious cycle between cytopenia and autoimmune diseases. Cytopenia, as observed in autoimmune patients, has been shown to be caused by autoreactive hemolytic antibodies, drug toxicity or HSC defects associated with mTOR-hyperactivation. Cytopenia may exacerbate autoimmune diseases by increased infection and lymphopenia. Infection has been shown to exacerbate autoimmune diseases, both through molecular mimicry and activation of TLR. On the other hand, since lymphopenia-induced HP requires self MHC-peptide complex, homeostatic proliferation may increase the frequency of autoreactive T cells. Furthermore, since HP converts naïve T cells into memory-like T cells with a lower activation threshold, HP will likely facilitate activation of autoreactive T cells. Given the central role of mTOR activation in HSC defects, we propose that rapamycin may be used to break the vicious cycle between cytopenia and autoimmune diseases.

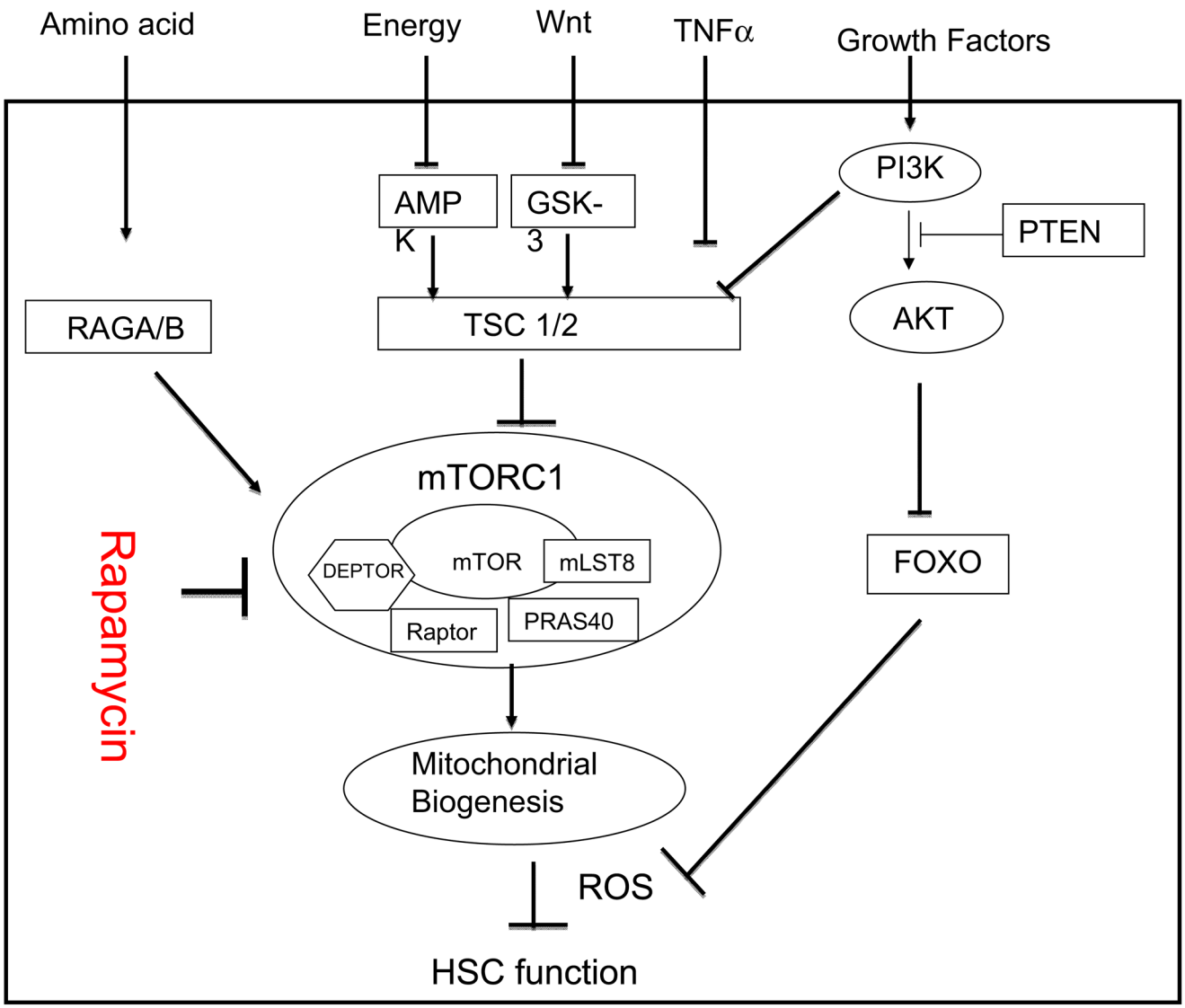

Fig 3.

A putative molecular mechanism underlying the hematopoietic defects in autoimmune patients. mTOR is negatively regulated by the TSC1/2 complex, which senses energy levels, inflammatory environments, growth signals from Wnt and other growth factors. Although apparently down-stream of the TSC1/2 complex, amino acid levels also regulate mTOR activity through the GTPase RAGA/B complex. Hyperactivation of mTOR disrupts the quiescence and function of HSC through increased mitochondrial biogenesis and ROS production. By suppressing TORC1 activation, short-term treatment of *Scurfy* mice with rapamycin resulted in long-term restoration of HSC function. Therefore, it is worth exploring whether the drug may be used to restore hematopoiesis in autoimmune patients.