Sympathetic Nervous System Regulation of Th Cells in Autoimmunity: Beyond Th1 and Th2 Cell Balance

Dianne Lorton[1]* and Denise L Bellinger[2]

[1]College of Arts and Sciences, Kent State University, Kent OH 44304, USA
[2]Department of Pathology and Human Anatomy, Loma Linda University, School of Medicine, Loma Linda, California, 92350, USA

Abstract

Mechanisms that cause autoimmune disease are complex and include interactions with genetic, environmental, immunologic, and neural-endocrine factors. These events are often separated by many years, suggesting that disease onset requires a triggering event that if understood, could be targeted therapeutically. How tolerance is broken and disease onset is initiated remain enigmatic. Psychological stressors are implicated in the development and progression of autoimmune diseases. First, severe life stressors are strongly associated with disease onset in up to 80% of patients. Second, the major stress pathways, the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis function become pathological in autoimmune diseases. Finally, there is a common "trifecta" of dysregulated immune functions, elevated SNS activity, low parasympathetic and low HPA-axis responsiveness across most autoimmune diseases. Understanding the changes in bidirectional cross-talk between these systems leading to this "trifecta" is key to fully understanding autoimmune diseases. These neuro-endocrine systems normally function to regulate immune responses and restore immune system homeostasis after immune challenges. Here, we focus on our current understanding of the imbalance of the CD4$^+$ T cell subtypes thought to drive autoimmunity and how dysregulation of the cross-talk between the SNS and the immune system impacts CD4$^+$ Th cell subtype balance in the autoimmune disease, rheumatoid arthritis (RA). Our lab has shown that elevated SNS tone and altered nerve to β$_2$-adrenergic receptor (β$_2$-AR) signaling occurs in lymphocytes in immune organs where arthritogenic CD4$^+$ T helper cells develop. In an animal model of RA, these receptors no longer signal via cAMP, the canonical signaling pathway for β$_2$-ARs. Instead, β$_2$-AR signaling is shifted towards signaling pathways expected to promote the generation of arthritogenic CD4$^+$ T cells. These findings indicate that dysfunctions in SNS to immune system communication are pathological events that could trigger the onset of RA and, by extension, other autoimmune diseases.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that afflicts ~1.5 million Americans [1-3] – mostly middle-aged or older, and mostly women, but also children and young adults [4]. RA causes chronic inflammation and synovial hyperplasia in the joints that destroys articular cartilage, and leads to bone loss and joint deformities. In addition to the joint pathology, systemic pathology often occurs as part of the disease course [5]. Systemic pathology may involve various tissues and organ systems, including the skin (rheumatoid nodules), cardiovascular system (vasculitis), lungs (interstitial lung disease), kidneys, hematologic system (Felty's syndrome), eyes and nervous system [5-8].

While the etiology of RA is not fully known, the clinical association of the major histocompatibility complex (MHC) class II allele, HLA-DRB1, with RA, and the presence of dysfunctional CD4$^+$ T cells in the joints of RA patients provide compelling evidence for a central role of CD4$^+$ T cells in the pathogenesis of this disease [9,10]. Studies using animal models of RA have further solidified the role CD4$^+$ T cells play in disease induction and progression [11]. These studies have provided a more specific understanding of the CD4$^+$ T cell subsets, the key cytokines they produce [12,13] and/or their signaling molecules [14-16] that are critically involved in disease initiation and progression of RA. Collectively, both patient and animal studies indicate a dysregulation of CD4$^+$ T cell differentiation and expansion that lead to an imbalance in CD4$^+$ T helper (Th) cell subtypes, specifically Th1, Th2, Th17, and T regulatory (Treg) cells, key components in RA pathology. However,

the mechanisms that lead to this imbalance in Th cell subsets in RA are not clear.

One participant that regulates the balance of CD4$^+$ Th cells is the sympathetic nervous system (SNS) [17-19]. This "super" system functions to maintain homeostasis in essentially every organ system of the body. A successful immune response requires the coordinated functions of many systems of the body (e.g., cardiovascular and energy metabolism) [20-23]. The SNS coordinates these organ systems so that the body can respond to a threat immediately. It subsequently returns the functions of the involved organ systems back to normal after the threat is eliminated.

Immune cells release cytokines that serve as messengers to the brain, and provide immune system functional information that is utilized by the SNS to coordinate all the systems required for an appropriate immune response [18,19] (Figure 1). Conversely, sympathetic nerves reside in the parenchyma of lymphoid organs (LOs) and release norepinephrine (NE) in response to increases in circulating and local

*Corresponding author: Dianne Lorton, PhD, College of Arts and Sciences, Kent State University, 850 University Esplanade, Bowman Hall 124A, Kent, OH, 44242, USA, Tel: 330-672-2255; Fax: 330-672-3720; E-mail: ddifran7@kent.edu
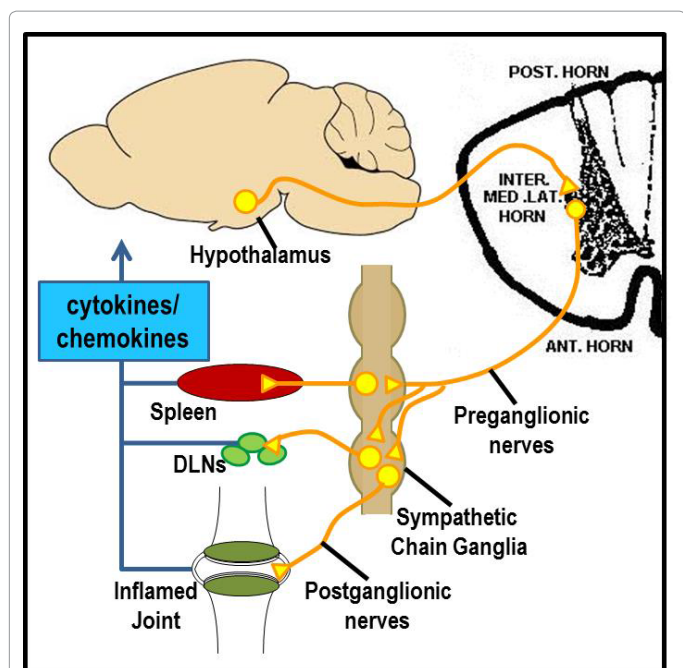
Figure 1: Sympathetic-immune communication relevant to rheumatoid arthritis. Neural circuits for sympathetic regulation of immune functions in secondary lymphoid organs and tissue immunity (e.g., synovial tissue in an articulating joint). The brain receives immune system information in the form of circulating cytokines and chemokines, and from activated sensory neurons. The hypothalamus responds by sending the appropriate signals to areas of the brainstem that regulate the firing rates of sympathetic neurons (not shown). Neurons in these brainstem areas send their axons to the preganglionic sympathetic neurons located in the intermediolateral cell column of the thoracic and lumbar spinal cord. These sympathetic neurons are the first neuron of a two neuron chain. Preganglionic neurons send their axons to post-ganglionic neurons located in sympathetic ganglia. Axons of post-ganglionic neurons extend into the parenchyma of secondary lymphoid tissues (spleen and lymph nodes) to regulate immune functions. The SNS also sends postganglionic nerves to most organs and tissues of the body to modulate their essential functions, as well as, to regulate inflammatory and cell-mediated immune responses that may occur in each of these tissues due to infection or tissue damage. Of relevance to RA, the synovial tissue of articulating joints is innervated by sympathetic nerves, and is thought to play a role in inflammatory arthritis.
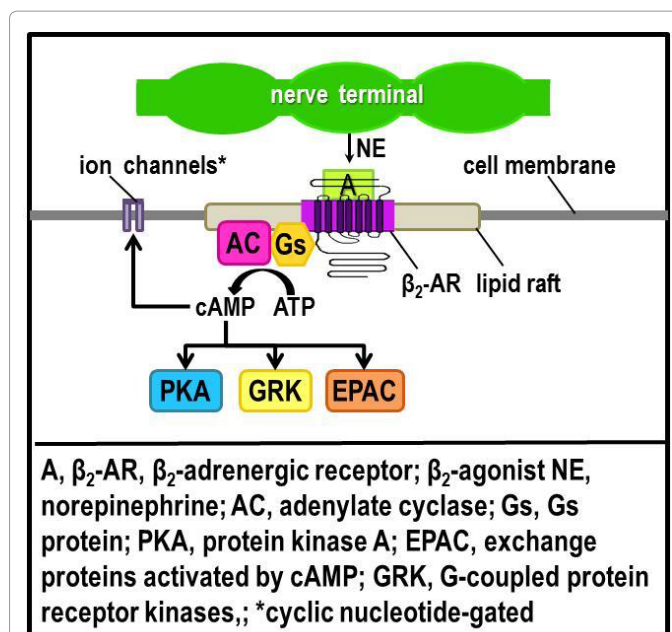

Figure 2: β_2-AR-mediated signal transduction in immune organs. Norepinephrine (NE) is released from sympathetic nerves. NE released from sympathetic nerves binds to β_2-ARs expressed in innate and adaptive immune cells to regulate immune cell functions. Binding of NE to β_2-ARs activates the G protein, Gs. Gs protein regulates the activity of adenylate cyclase (AC). Adenylate cyclase catalyzes dephosphorylation of ATP to cyclic AMP (cAMP). Cyclic AMP activates protein kinase A (PKA), G protein-coupled receptor kinases (GRK), and exchange protein activated by adenylyl cyclase (EPAC). PKA is a kinase that regulates the activity of several proteins by their phosphorylation. PKA phosphorylation of target proteins is responsible for many of the β_2-AR-induced cellular responses. Activation of GRK ultimately determines the later functions and fate of β_2-ARs, including the induction of alternative intracellular signaling, and receptor desensitization, internalization, recycling and/or trafficking to the lysosomes for degradation. Activation of EPAC leads to activation of the B-Raf/mitogen-activated protein kinase signaling pathway. Downstream effects of PKA, GRK and EPAC regulate diverse cellular processes, including gene transcription by transcription factors. Additionally, cAMP can bind to their binding sites on ion channels that express cAMP binding sites. Binding of cAMP to these cyclic nucleotides-gated ion channels then alter movement of ions across the channel to exert change in cell functions.

cytokines [24,25]. NE released from sympathetic nerves activates either α- or β-adrenergic receptors (ARs) that are expressed by immune cells.

The β_2-AR is the receptor subtype almost exclusively expressed in T cells [26]. β_2-AR stimulation activates a cascade of signaling messengers, including cAMP and protein kinase A (PKA). PKA phosphorylates numerous cellular proteins that ultimately modulate T cell functions [18,27] (Figure 2). By activating this signal transduction pathway, the SNS regulates the differentiation of naïve CD4+ Th cells, and augments or suppresses the functions of effector CD4+ Th cells. In RA and other autoimmune disorders, abnormalities in SNS functions contribute to the symptom burden linked to immune dysfunction – possibly triggering, but clearly mediating disease pathology.

Abnormal functions of the SNS include increased sympathetic nerve firing rates [28-34], changes in receptor functions [17,35-41], and dying back of sympathetic nerves in affected joints [42-49] (Table 1 and Figure 3). Similar defects in SNS functions are observed in animal models of RA and also show SNS neuropathy and dysfunction in LOs [50-53] that is currently not feasible to assess in RA patients. Still, the extent that SNS dysfunction contributes to the imbalance in CD4+ Th cell subtypes

1	↑ NE release and availability
2	LO-specific change in NE content
3	Sympathetic nerve neuropathy with subsequent nerve remodeling in splenic white pulp
4	Aberrant innervation of the red pulp, a site where activated immune cells (e.g., plasma cells) reside
5	Loss of neurotrophic factors in secondary lymphoid tissues
6	β_2-AR down-regulation and desensitization (peripheral blood mononuclear cells (PBMCs), spleen cells, and synovial immune lymphocytes)
7	β_2-AR down-regulation and desensitization (PBMCs, spleen cells and synovial immune immune lymphocytes)
8	Shift in β_2-AR signaling pathways activated (lymph nodes that drain the arthritic joints)

β_2-AR: β_2-Adrenergic Receptor; LO: Lymphoid Organ; NE: Norepinephrine; PMBCs: Peripheral Blood Mononuclear Cells; SNS: Sympathetic Nervous System

Table 1: Changes in SNS-Immune System Transmission.

observed in RA is not fully understood. Here, we will review the current understanding of the contribution of stress pathways, particularly the SNS to autoimmunity, CD4+ Th cells in regulating, inducing and promoting inflammatory joint disease and the potential impact of altered SNS regulation of these cells on disease processes.
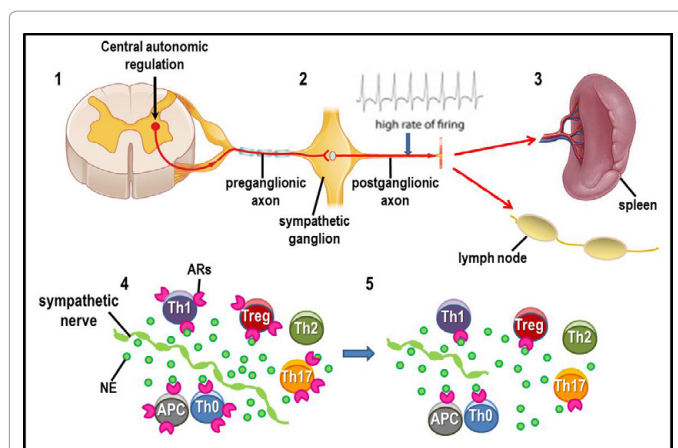
Figure 3: Sympathetic neurotransmission in the spleen from rodent models of RA. 1, 2) Increases in the firing rate of sympathetic neurons that supply the spleen. Preganglionic sympathetic neurons stimulated by descending axons from central regulatory nuclei (e.g., the rostroventral lateral medulla) drive hyperactivity. **2, 3)** The greater reactivity of the preganglionic neurons induces an increase firing rate in postganglionic sympathetic neurons (e.g., superior mesenteric-celiac ganglion) that send their axons to the spleen. Sympathetic nerves that enter the spleen (and lymph nodes) reside in T cells regions and at sites of antigen presentation and processing. **4)** Increased firing rates of sympathetic nerves induce greater release of norepinephrine (NE), increasing the interaction of NE with adrenergic receptors (ARs) expressed in antigen presenting cells (APC) and Th cells. Ligand binding to ARs can cause shifts in Th cell subset balance and functions. **5)** Increased nerve activity over time destroys nerves (referred to as autodestruction or "dying back"). Subsequently, sympathetic nerves respond with a sprouting response in which the nerves return, but with a different pattern of distribution in immune compartments, a process termed remodeling. The high frequency of nerve firing also increases NE concentrations/availability that induces decreases β_2-AR number and sensitivity and affects AR-mediated signal pathway activation and transduction. These events also occur in the arthritic joints (not shown).

The Link between Stress and Autoimmune Diseases

For decades, physical and psychological stress have been implicated by many animal and human studies to induce the onset of many autoimmune diseases. The majority of patients (up to 80%) report severe or chronic emotional distress and upheaval preceding the onset of autoimmunity (like RA) [54]. Stress is well known to aggravate disease activity (Figure 4A) [55-60]. These observations coupled with evidence of dysfunction in the major stress pathways, the autonomic nervous system (both sympathetic and parasympathetic components) [28-32] and the hypothalamic-pituitary-adrenal (HPA) axis dysfunction [61-69], provide compelling support that the nervous system is important in the onset of clinical disease. Most of these studies indicate an intriguing pattern of high SNS, and low parasympathetic nervous system activity combined with a hypoactive HPA axis [33,54,60,70] (Figure 4B). Observations that arthritis does not develop on the paralyzed side in hemiplegic patients with RA [71-75] further supports a causal role of the nervous system. Similarly, in animal models of RA (i.e., adjuvant-induced arthritis (AA)), the nervous system is required for arthritic spread to the contralateral side [76-78].

The SNS is well-known to regulate both inflammatory and cell-mediated immune responses, initially promoting these responses upon immune challenge; and later suppressing immune responses as the pathogen is cleared. In this manner the SNS modulates immune responses, as well as, coordinates the ensuing responses of other systems (e.g., cardiovascular, energy metabolism). This is required to mount an effective immune response and subsequently to restore homeostasis once the threat is eliminated. The chronic high SNS activity observed

in patients with autoimmune diseases suggests the normal cross-communication required to promote, then suppress cellular immunity to restore homeostasis is impaired. Discordant cross-communication between stress pathways and immune reactivity is likely causal for many stress-linked diseases (e.g., hypertension, cardiovascular disease).
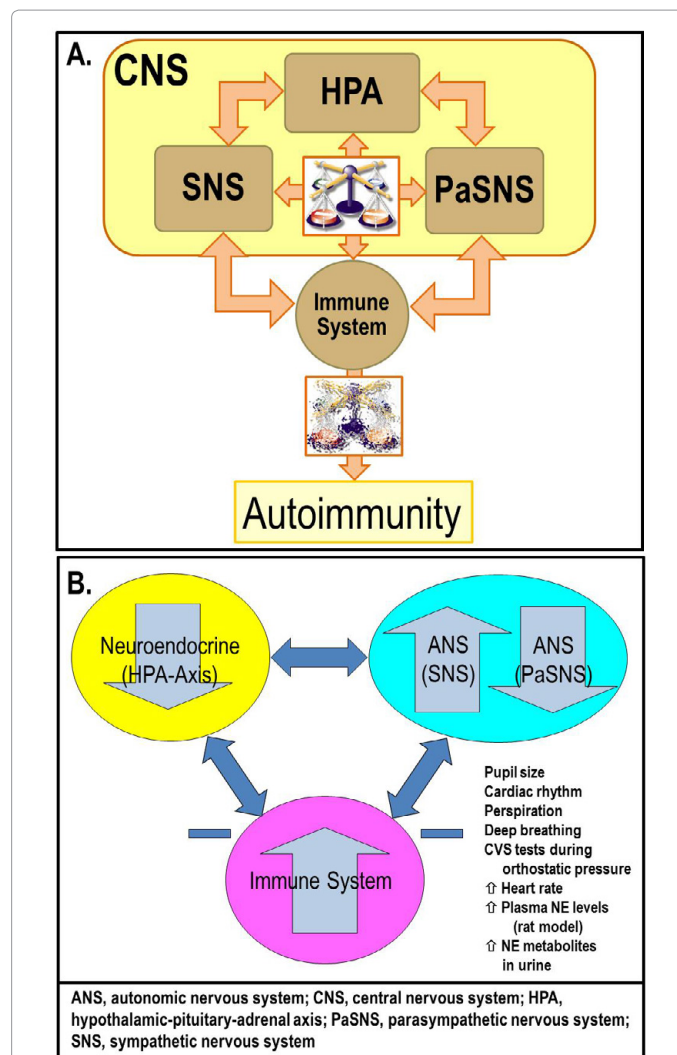

Figure 4: Imbalance of neural, hormonal and immune regulation in autoimmunity. A) The common patterns of neural-Immune dysregulation in autoimmune diseases, including RA, are illustrated. The stress system-composed of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic and parasympathetic nervous systems (SNS and PaSNS, respectively) regulate immune responses, promoting immune functions in responses to danger signals and pathogens, and inhibiting immune responses as these threats are removed and homeostasis is restored. In autoimmunity, homeostasis is not restored, and an imbalance in these cross-regulatory systems develops. **B)** Changes in the ability of the autonomic nervous system to appropriately control body functions, such as those listed on the right side of the panel for RA patients, are often observed in patients with autoimmune diseases. These findings indicate ANS dysregulation in these diseases. The observed pattern of dysregulation generally observed for each major regulatory system is indicated by the direction of the arrows. The SNS becomes hyperreactive, whereas the PaSNS becomes hyporeactive. Similarly, dampened responsiveness of glucocorticoid receptors to glucocorticoids in patients with RA and other autoimmune disorders indicates a hypo-functional HPA axis. Increased immune system activation (production of proinflammatory cytokines and auto-antibodies and increased activity of autoreactive T cells) may, in part, result for from lost inhibitory regulation by these neural and neuroendocrine pathways that are required to restore immune system homeostasis.

Disruption in any part of the bidirectional communication pathway can lead to disease development and progression [79]. The extent to which high SNS activity contributes/causes pathology in autoimmunity is unclear. Presumably, the high SNS activity contributes significantly to the immune dysregulation observed in these diseases. We [17,27,80] and others [71,81] provide substantial evidence to support the hypothesis that high SNS activity is a primary mediator of the dysregulated cell-mediated immunity in RA. However, the notion that immune dysregulation imposed by altered neural regulation could trigger the onset of clinical disease has received much less attention. A better understanding of when these stress pathways become dysregulated relative to each other, as well as relative to clinical measures of pre- and post-disease, are necessary to address these problems.

SNS regulation of the immune system is complex. It is not yet entirely clear how sympathetic nerves in secondary immune organs direct antigen-presenting cell (APC) functions and CD4+ Th cell differentiation and expansion [82-84]. Moreover, it is not clear what role the SNS plays in regulating auto-reactive CD4+ Th cells or the regulatory T (Treg) cells, cells that drive autoimmunity and maintain peripheral tolerance, respectively [85]. The presence of circulating autoantibodies can precede the clinical onset of disease by decades, indicating that tolerance is lost well before disease onset [85]. The extent that the SNS is involved in maintaining a new homeostatic "set-point" during preclinical disease is unclear. Finally, the role of the SNS in triggering disease onset remains unknown [85]. Still it is clear that the SNS is a major regulator of the differentiation and clonal expansion of CD4+ Th cells and the balance between the different CD4+ Th cell subtypes. Therefore, we hypothesize that changes in the SNS can indeed induce a loss of peripheral tolerance to induce clinical-onset of autoimmunity. We review new findings that support this role of the SNS in perpetuating immune dysregulation and loss of tolerance to promote and possibly trigger the onset of RA.

Aberrations in SNS Neurotransmission in Immune Organs: RA Rodent Models

Dysregulation of the 3-way cross-talk between the SNS, dendritic cells (DCs) and Th cells occurs in the pathogenesis of RA [81,86-88]. Its complexity is reflected by the disparate findings of drugs that target sympathetic nerves or their receptors on disease outcome of experimental arthritis (EA) [89-94]. Opposing SNS effects on disease outcome depend on the timing of SNS activation relative to the disease phase. The opposing effects support a transition phase between SNS-mediated disease promoting or ameliorating effects in early asymptomatic and late symptomatic phases of EA, respectively [76,89,91,94,95] (Figure 5). Dynamic and dramatic changes in the concentration of NE [51,89,91], sympathetic innervation [96,97] and neurotransmission [49,97-101] in secondary LOs and affected joints [49], further complicate the role of the SNS in RA development and progression. Still, noradrenergic (NA) nerves do differentially regulate immune responses in immune compartments relevant to disease development (e.g., draining lymph nodes (DLNs), spleen) and after disease onset (e.g., DLNs, spleen, skin, inflamed joint) [17,76,102]. Dissecting out LO-specific regulatory roles of NA nerves in Th cell functioning during different phases of disease is important for understanding the etiology and pathophysiology of RA, and may be translational to other types of autoimmune disorders. In this manuscript, we focus on the consequences of manipulating sympathetic signaling via treatment with a β-AR agonist on Th cell function during the effector phase of AA in disease-relevant immune organs. Identifying key events in neural-immune signaling that affect the handling of adjuvants by APCs and Th cell differentiation that
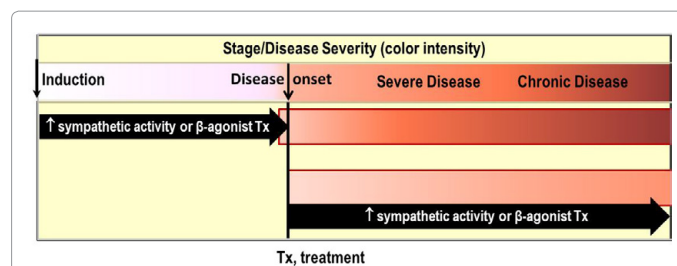

Figure 5: Effect of high sympathetic reactivity or β$_2$-AR treatment on inflammatory arthritis in rodent models. In both the collagen-induced and adjuvant-induced animal models of RA, treatments that mimic increasing sympathetic nerve activity (e.g., administration of β$_2$-AR agonists) have differential effects on disease severity depending upon the timing of treatment relative to disease onset. Treatment with β$_2$-AR agonists at the time of the disease-inducing challenge to disease onset increases the severity of disease at the time when disease becomes apparent and as the disease progresses from severe to chronic stages. In contrast, when β$_2$-AR agonists are administered beginning at disease onset, disease severity, including extent of joint inflammation and progressive joint destruction, is significantly reduced. (Tx: treatment).

triggers the breach in tolerance will provide new insights into the disease pathogenesis.

Th Cell Subsets and the Th Balance in Autoimmunity

Since the SNS regulates mechanisms that drive Th cell differentiation toward different phenotypes, we will briefly overview humoral and transcriptional regulation of Th cell differentiation. Both Th and Treg cells recognize peptides presented by APCs; however, each plays distinctly different roles in host defense under various conditions of immune activation [103]. Naïve Th cells (Th0) differentiate into a variety of Th effector cells under the direction of APCs. The most relevant for autoimmunity include Th1, Th2, Th17, and Treg cells. These Th cell subsets will be the focus of this section.

CD4+ Th1 and Th2 cells

The effector Th cell subsets are defined by unique developmental programs that control their differentiation and the specific cytokine profiles they produce. Based on the Th1-Th2 cell paradigm, the cytokine profile for each subtype counter-regulates differentiation of the other subsets as well as self-reinforcing mechanisms that promote and stabilize their own maturation [104]. For example, with antigen stimulation (viral/tumor antigens), interferon-γ (IFN-γ) and interleukin (IL-12) promote the differentiation of naïve T (Th0) cells to Th1 cells (Figure 6). This response occurs by activating a Th1 cell-specific transcriptional network regulated by the transcription factor, T-bet, that results from IFN-γ and IL-12 induction of STAT1 and STAT4, respectively [104-107]. In contrast, IL-4 directs CD4+ Th0 cells to differentiate into Th2 cells by inducing the transcriptional cascade regulated by the transcription factor, GATA3, in response to IL-4-induced STAT6 [108]. Development of each of these Th cell subsets is reinforced by Th1 or Th2 cell production of IFN-γ or IL-4, respectively (Figure 7). These feedback mechanisms further push subsequent differentiation of Th0 cells toward Th1 or Th2 cells, (IFN-γ or IL-4, respectively), and counter-inhibit effects on Th2 cell development (IFN-γ) and Th1 cell differentiation (IL-4).

CD4+ Th17 and Treg cells

Similarly, the more recently discovered Th17 cells are defined by their production of IL-17A and IL-17F. Th0 cell differentiation into Th17 cells requires transforming growth factor-β (TGF-β) and induction of
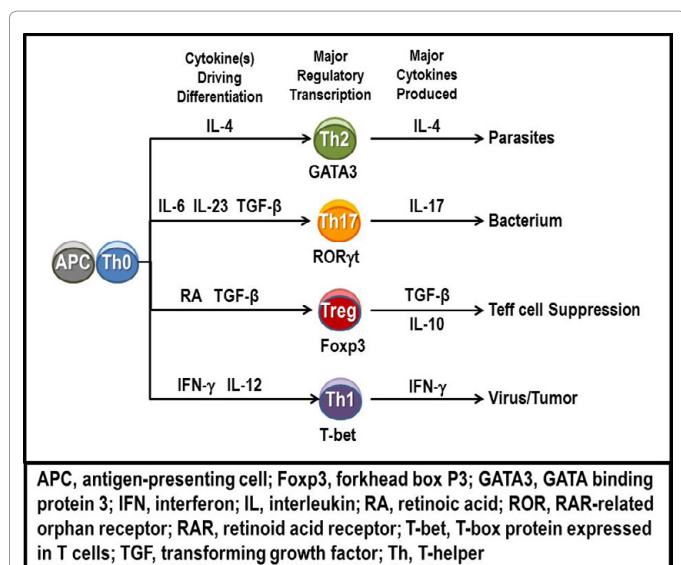
Figure 6: Th0 cell differentiation. CD4+ T-helper (Th) cell differentiation from naïve CD4+ Th cells activated by antigen-presenting cells (APCs). In response to different pathogens or abnormal host cells, APCs present pathogen/tissue specific antigens to Th0 cells that then induce a biased differentiation into functional Th cells with specific phenotypes. The dominant Th cell subtype that develops is dependent upon the cytokine milieu produced by the APCs and naïve/activated Th cells. Differentiation of each Th cell subset results from the induction of uniquely subset-specific transcriptional programs, as indicated and described in the text. The different Th cell subtypes that differentiate from naïve Th0 cells, their hallmark transcription factor that is expressed and production a specific cytokine profiles characteristic for each subtype is indicated.

Figure 7: Th cell subtype counter-regulation of the differentiation of the other Th cell subsets from activated naïve Th cells. Counter-regulation serves to allow the development of subset-specific immune responses necessary to eliminate the pathogen/tumor/antigen detected by the APCs and subsequently presented to Th0 cells. Antigen presentation to naïve Th0 cells drives their differentiation towards the most appropriate adaptive immune response. Red lines indicate inhibitory influences on Th cell differentiation and green lines illustrate lineage promoting effects. Transcription factor, downstream signaling factors, and cytokines that mediate Th cell subset regulation are indicated. (Green arrows, excitatory; red arrows, inhibitory).

the transcriptional program RORγt [109] (Figure 6). Consistent with the cross-inhibitory roles shown for Th1 and Th2 cells, IFN-γ and IL-4 are potent inhibitors of Th17 cell development and IL-17 impedes Th1 cell development [110] (Figure 7). Interestingly, another recently discovered Th cell subtype, inducible T regulatory (iTreg) cells, also requires TGF-β, as well as IL-4 and IL-10, for their differentiation from naïve Th0 cells [111]. Inducible Treg cells differ from natural Treg (nTreg) cells in that iTreg cells acquire CD25 expression outside of the thymus, typically induced by inflammation or disease processes, like autoimmunity. Th0 cells differentiate into a Th17 cell phenotype in the presence of TGF-β and IL-6 (produced by activated DC), or IL-23 (produced by IL-6-stimulated T cells) [112,113]. Like Th1- and Th2-cell driving cytokines, the cytokines required for Th17 cell differentiation form an autocrine feedback loop that further promote the differentiation and maturation of Th0 cells into Th17 cells [109,111] (Figure 7). Thus, the presence of pro-inflammatory signals from the innate immune system promotes the differentiation of naïve Th0 cells to Th17 cells [111].

In the absence of IL-6, TGF-β promotes Th0 cell differentiation towards Treg cell development by activating the FoxP3-mediated transcriptional program (Figure 6). In a non-inflammatory environment, antigen-induced activation of Th0 cells in a TGF-β-rich environment enhances iTreg cell development. Reciprocal regulation of Treg cells and Th17 cells also exists at the transcriptional level, as FoxP3 protein physically interferes with the association between RORγt and the IL-17 promoter [114-116] (Figure 7). IL-6 blocks FoxP3 interference with RORγt, initiating Th17 cell differentiation. Thus, IL-6 is pro-inflammatory by its promotion of Th17 cell differentiation and inhibition of Treg cell differentiation. The dual function of TGF-β in driving both Th17 and iTreg cell differentiation challenges the Th1-Th2 cell paradigm that distinct inductive cytokines are uniquely
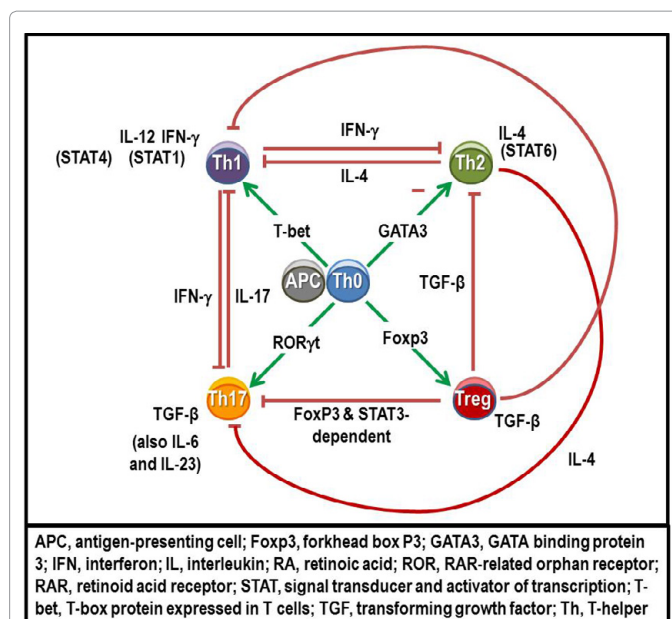
linked to each Th cell lineage. Indeed, induction of Th17 and Treg cell differentiation by the distinct transcriptional factors, RORγt and FoxP3, respectively, both depend on TGF-β signaling [114].

Sympathetic Regulation of Th Cell Balance

SNS regulation of Th1 and Th2 cell differentiation

The SNS regulates the differentiation of Th cells, including autoreactive cells, through the activation of α- and β_2-ARs expressed on their cell surfaces [35,36,49,95]. Sympathetic nerves are present adjacent to target immune cells in secondary LOs [42,97] and the immune cell infiltrates in the tertiary lymph nodules that occur in the affected joints [117]. In secondary LOs, NE released from sympathetic nerves activates β_2-ARs expressed in T cells. This receptor activation inhibits IL-2 production, which subsequently suppresses lymphocyte proliferation required for clonal expansion (reviewed in [18]). Activation of β_2-ARs on Th0/Th1 cells also inhibits cellular and promotes humoral immunity by regulating the phenotypic differentiation of CD4+ Th cells in response to challenge with T cell-dependent antigens [18]. β_2-AR stimulation inhibits IFN-γ production by receptor coupling to Gs protein, increased intracellular cAMP production and subsequent phosphorylation of PKA. Reduced IFN-γ limits Th0 cell differentiation towards the Th1 phenotype, and dampens IFN-γ-mediated inhibition of IL-4 production, promoting IL-4 synthesis by Th2 cells (Figure 8A). In this manner, the SNS provides a negative feedback mechanism to restore immune system homeostasis after antigen challenges that activate cellular immunity [18]. Additionally, β_2-ARs preferentially inhibit IL-12 and increase IL-10 production by APCs, which subsequently promotes Th2 and inhibits Th1 differentiation [17,102] (Figure 8B).
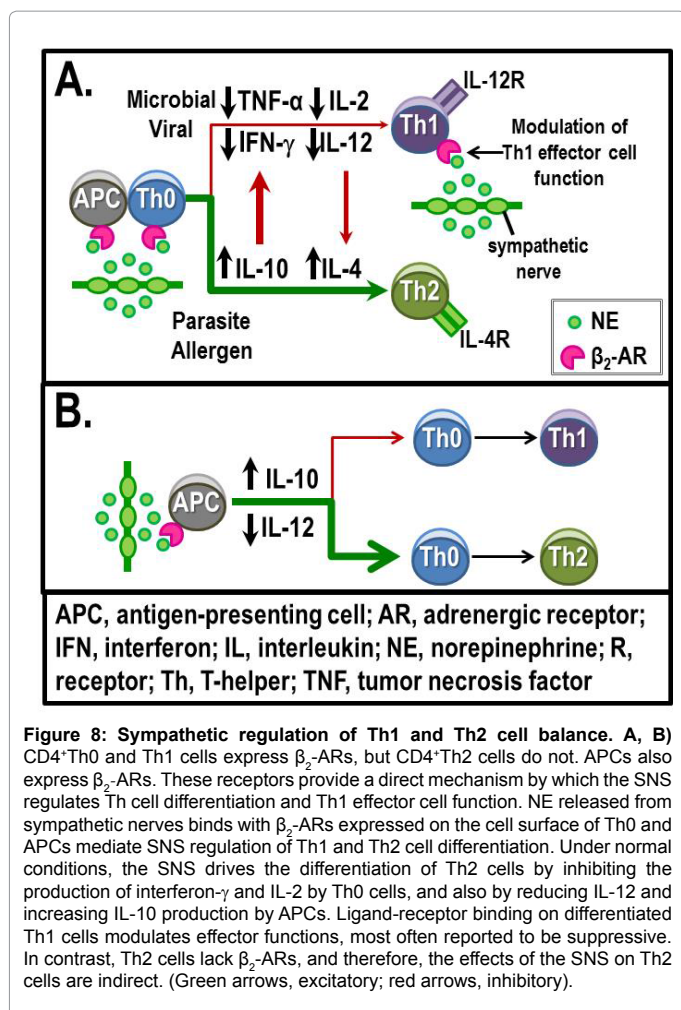
Figure 8: Sympathetic regulation of Th1 and Th2 cell balance. A, B) CD4+Th0 and Th1 cells express β₂-ARs, but CD4+Th2 cells do not. APCs also express β₂-ARs. These receptors provide a direct mechanism by which the SNS regulates Th cell differentiation and Th1 effector cell function. NE released from sympathetic nerves binds with β₂-ARs expressed on the cell surface of Th0 and APCs mediate SNS regulation of Th1 and Th2 cell differentiation. Under normal conditions, the SNS drives the differentiation of Th2 cells by inhibiting the production of interferon-γ and IL-2 by Th0 cells, and also by reducing IL-12 and increasing IL-10 production by APCs. Ligand-receptor binding on differentiated Th1 cells modulates effector functions, most often reported to be suppressive. In contrast, Th2 cells lack β₂-ARs, and therefore, the effects of the SNS on Th2 cells are indirect. (Green arrows, excitatory; red arrows, inhibitory).

SNS regulation of Th17 and Treg cell differentiation

Sympathetic regulation of the differentiation and expansion of Th17 and Treg cells is not as well studied as it is for Th1/Th2 cells. It is known that IL-2 inhibits Th17 cell differentiation from naïve Th0 cells [118], and promotes the clonal expansion of differentiated Th17 cells [119-122]. Since β₂-AR stimulation of T cells suppresses IL-2 production, sympathetic activation is expected to promote Th17 cell differentiation (Figure 9A, see ①). In contrast, β₂-AR-mediated IL-2 suppression is expected to inhibit clonal expansion of fully differentiated Th17 cells (Figure 9A, see ②). Moreover, under conditions that promote Th1 cell differentiation, the SNS is also expected to augment Th17 cell differentiation due to a β₂-AR-mediated suppression of IFN-γ and IL-12 production by Th1 cells [123] (Figure 9A see ③). Similarly, β₂-AR suppresses IFN-γ and IL-12, both of which are potent inhibitors of Th17 development.

In contrast to Th17 cells, IL-2 promotes the differentiation of Treg cells, and is required for their suppressor functions [120-122]. Thus, SNS-induced IL-2 suppression will likely inhibit Treg cell differentiation and their suppressor functions (Figure 9B). It is not clear how the SNS influences IL-23-promoting expansion of Th17 cells [124]. The predicted effects of the SNS via β₂-AR stimulation under conditions that promote Th17 or Treg cell differentiation and clonal expansion need to be confirmed.

No studies have directly examined the effects of the SNS on Th17 cell or Treg cell differentiation under conditions that would promote the differentiation of either of these Th cell subsets. While several studies indicate that Treg cells express β₂-ARs [125-127], there are no studies that indicate Th17 cells express β₂-ARs. However, the SNS can influence Th17 cell differentiation through indirect effects via ARs expressed on APCs, Th0, or Th1 cells (Figure 9C). ARs modulate cytokine production by DCs and affect their ability to prime Th cells [128]. The fate of naïve CD4+ Th cell responses is dependent upon cytokines produced by DCs that sense pathogen-associated molecular patterns (PAMPs) expressed in microbes and local microenvironmental factors. DC production of IL-10, IL-12, IL-23 or TGF-β/IL6 controls the differentiation of naïve CD4+ Th cells into Th2, Th1, Th17 or Treg cells, respectively [35,124,129-136].

NE released from sympathetic nerves regulates macrophage and DC production of both IL-12 and IL-23 [137] to potentially influence Th cell differentiation towards Th1 or Th17 cells (Figure 9C). Manni and coworkers [138] find that β₂-ARs stimulation shifts the balance from a Th1 to a Th17 cell response after DC priming with a toll-like receptor (TLR)2 agonist and/or a nucleotide-binding oligomerization domain receptor (NOD) agonist either *in vitro* or *in vivo*. The shift in Th1/Th17 cell balance is blocked in the presence of a β₂-AR antagonist. Stimulation of β₂-ARs in activated DCs increases *in vitro* production of IL-6, and inhibits IL-12 without affecting IL-23 secretion, a cytokine pattern favoring Th17 cell differentiation (Figure 9C), since both IL-6 and IL-23 are central mediators for the generation of Th17 cells [111,139]. Manni et al. cultured DLN cells from mice immunized with DCs stimulated *in vitro* with muramyl dipeptide and keyhole limpet hemocyanin in the presence of the β₂-AR agonist, salbutamol. They report an increase in IL-17 and reduction in IFN-γ. These findings are consistent with a shift in Th cells towards the Th17 cell subtype.
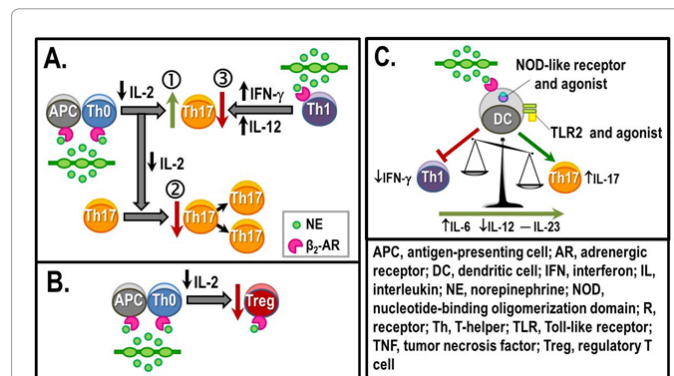
Figure 9: Effects of β₂-ARs stimulation on cytokine profiles and Th 1 and Th17 cell differentiation. The SNS regulates the differentiation of Th0 cells to Th17 and Treg cells, in part by modulating levels of IL-2. **A)** NE released from sympathetic nerves bind β₂-ARs expressed in naïve Th cells. Activation of Th0 cell β₂-ARs reduces the production of IL-2. IL-2 exerts an inhibitory effect on Th17 cell differentiation from naïve Th0. However, IL-2 does promote the clonal expansion of Th17 once these cells develop. By suppressing IL-2 production, the SNS is expected to promote Th17 cell differentiation, but inhibit clonal expansion of the Th17 cells once differentiated. **B)** Activation of β₂-ARs in APCs and Th0 cells reduces their production of IL-2. Since IL-2 promotes the differentiation of Treg cells, and is required for their suppressor functions, SNS suppression of IL-2 production is anticipated to inhibit Treg cell differentiation and their suppressor functions. **C)** Stimulation of β₂-ARs on dendritic cells (DC) that have been co-stimulated with a TLR2 and a NOD-like receptor agonist skews the immune response away from a Th1-type response but towards a Th17 response by increasing DC production of IL-6 and reducing DC production of IL-12, without changes in IL-23. (Green arrows, excitatory; red arrows, inhibitory).

This study did not examine effects of β_2-AR agonists on the generation of Treg cells directly. However, the findings that β_2-AR activation increases IL-6, a cytokine that inhibits Treg cell differentiation, would be expected to suppress Treg cell development in favor of Th17 cells (Figure 10). Suppression of IL-2 by β_2-AR stimulation would be anticipated to further suppress Treg cell differentiation in favor of Th17 cell development. Further, modulatory effects of the SNS on Treg cell functions are provided below.

Similarly, Kim and Jones [133] show that DCs treated with epinephrine and lipopolysaccharide enhance IL-17 and IL-4 but not IFN-γ production by CD4$^+$ T cells *in vitro*, a cytokine profile indicative of shift towards Th17 /Th2 cells and reduced Th1 cells. Notably, these findings indicate that the SNS via β_2-ARs expressed on APCs are involved in defense against extracellular bacteria and in diseases in which the pathology is driven towards Th17 cell polarization. These findings have both basic and clinical relevance for immune responses against extracellular bacteria and fungi, and the pathogenesis of autoimmune and inflammatory disorders. Thus, stimulation of β_2-ARs may enhance IL-17-type immune responses contrary to the concept that β_2-AR agonists suppress cellular immunity and promote humoral immunity. These findings need to be confirmed and expanded.

SNS and Treg cell functions

Elevating cAMP is one mechanism by which G protein-coupled receptors (GPCRs), like β-ARs, can promote Treg cell suppression of proliferation and responder Th cell functions [125-127]. NE, the major neurotransmitter of sympathetic nerves, induces cAMP production upon ligation with β_2-ARs, the major AR subtype expressed on T cells. Very little is known about sympathetic regulation of Treg cells or the influence of NE on their functions, including β-AR-induced intracellular cAMP concentrations. However, the data that is available indicate that sympathetic nerves can positively regulate suppressive activity of Treg cells via β_2-AR-mediated mechanisms. It is unknown whether regulation of cAMP in Treg cells by β_2-AR stimulations is responsible for greater Treg cell activity.
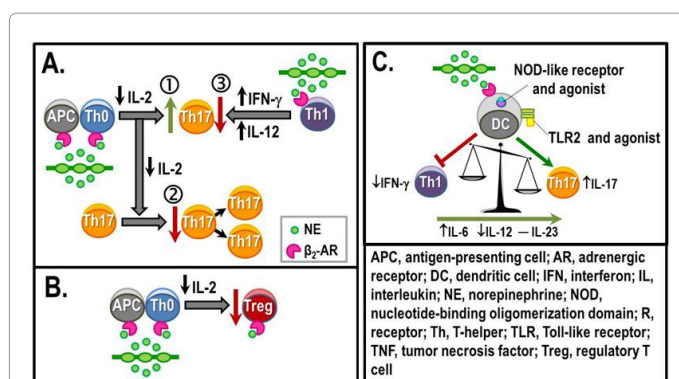

Figure 10. Sympathetic regulation of Th17 and Treg cell balance. Th0 and Treg cells express β_2-ARs; however, whether Th17 express this receptor has not been determined. APCs also express β_2-ARs. These receptors provide a direct mechanism by which the SNS regulates Th cell differentiation and Treg effector cell function. NE released from sympathetic nerves binds with β_2-ARs expressed on the cell surface of Th0 and APCs mediate SNS regulation of Th17 and Treg cell differentiation. Under normal conditions, the SNS drives the differentiation of Th17 cells by increasing the production of IL-6 and TGF-β by APCs. In contrast, activation of β_2-ARs in Th0 cells inhibits Treg cell differentiation by reducing IL-2. Further, downstream signaling pathways of β_2-AR in Treg cells, inhibit effects of TGF-β by suppressing TGF-β-induced activation of ERK- and JNK pathways. (Green arrows, excitatory; red arrows, inhibitory).
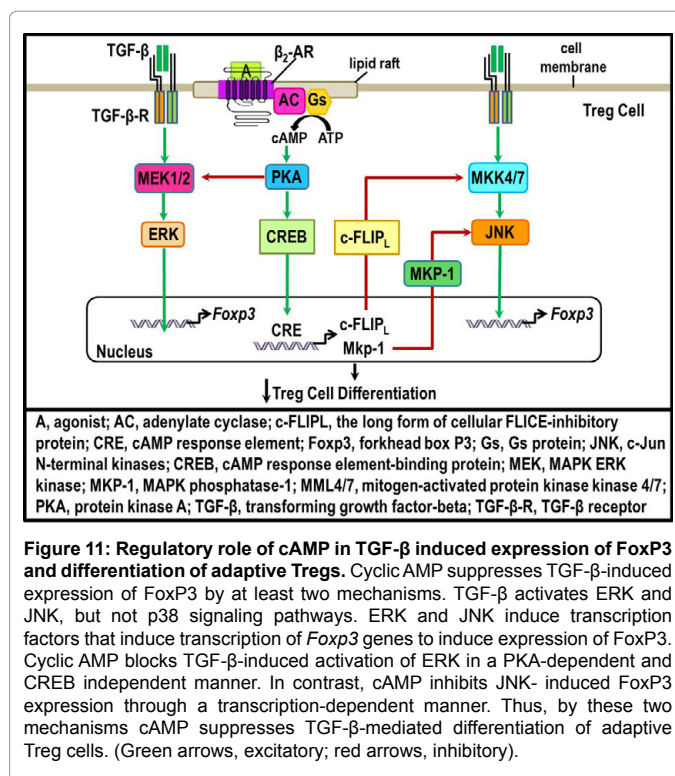

Figure 11: Regulatory role of cAMP in TGF-β induced expression of FoxP3 and differentiation of adaptive Tregs. Cyclic AMP suppresses TGF-β-induced expression of FoxP3 by at least two mechanisms. TGF-β activates ERK and JNK, but not p38 signaling pathways. ERK and JNK induce transcription factors that induce transcription of *Foxp3* genes to induce expression of FoxP3. Cyclic AMP blocks TGF-β-induced activation of ERK in a PKA-dependent and CREB independent manner. In contrast, cAMP inhibits JNK- induced FoxP3 expression through a transcription-dependent manner. Thus, by these two mechanisms cAMP suppresses TGF-β-mediated differentiation of adaptive Treg cells. (Green arrows, excitatory; red arrows, inhibitory).

SNS regulation of Treg cell number and function in secondary immune organs is indicated from a study by Bhowmick et al. [140] . They demonstrate that 6-hydroxydopamine-induced sympathectomy increases murine splenic and lymph node CD4$^+$FoxP3$^+$ Treg cell number by a TGF-β-dependent mechanism. Moreover, this research shows that the increase in Treg cell numbers coincides with an inhibition of the induction of experimental autoimmune encephalomyelitis. Interestingly, agents that elevate cAMP, including the β-AR agonist, isoproterenol, suppress TGF-β-mediated Treg cell differentiation by inhibiting the activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) [141] (Figure 11). Cyclic AMP-mediated inhibition of TGF-β occurs in a transcription-independent manner for ERK and a transcription-dependent manner for JNK. Therefore, the SNS can regulate Treg cell differentiation by activating β_2-ARs, which increases intracellular levels of cAMP in Treg cells to reduce TGF-β-induced FoxP3 expression. Thus, the SNS is well positioned to regulate the balance and functions of T cells by multiple mechanisms. However, pathology in the SNS has not been explored in the imbalance between Treg and autoreactive T effector cells in autoimmune diseases. Still, these findings implicate the importance of SNS dysfunction in understanding the loss of tolerance, and in triggering the onset of clinically apparent autoimmune diseases.

CD4$^+$FoxP3$^+$ Treg cells use multiple mechanisms to suppress immune responses (Figures 11 and 12), maintain self-tolerance, and efficiently regulate innate [122,142-143] and adaptive immunity [144,145]. Cyclic AMP signaling plays a significant role in this process. Compared with conventional CD4$^+$ T cells, Treg cells express high levels of cAMP, which can be transferred into activated conventional T cells via gap junctions [146,147] (Figure 12). Similarly, co-culturing Treg cells with activated murine DCs causes an increase in intracellular cAMP in Treg cells that suppresses DC function [148] (Figure 12). In Treg cells, high cAMP is induced by suppressing transcription of the
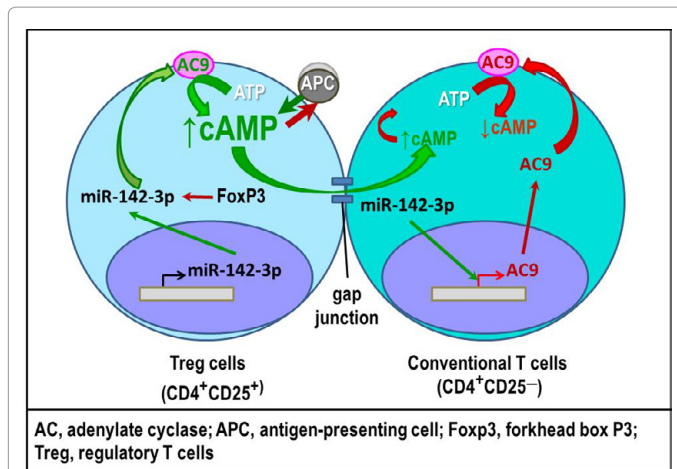
Figure 12: Cyclic AMP-mediated regulation of Th cells by Treg cells. Treg cells express high levels of cAMP compared with other Th cell subsets. Cyclic AMP generally suppresses Th cell functions. One mechanism in which Treg cells suppress effector Th cells is by the transfer of cAMP to other Th cell subsets via gap junctions. The mRNA, miR-142-3p, regulates the upper limits of cAMP expression in Treg cells (cell on the left) by targeting adenylate cyclase (AC) [9]. Greater transcription of miR-142-3p inhibits AC9 activity. FoxP3 downregulates miR-142-3p transcription, which disinhibits AC9 activity to increase cAMP levels in Treg cells. In contrast, miR-142-3p inhibits AC9 expression in other T cell subsets (shown on the right), which reduces cAMP production. (Green arrows, excitatory; red arrows, inhibitory).

microRNA, miR-142-3p, which increases the activity of adenylate cyclase (AC) 9 to phosphorylate ATP to cAMP (Figure 12, left side). FoxP3 down-regulates miR-142-p3 to keep the AC9/cAMP pathway active. In contrast, this microRNA limits the concentration of cAMP in other Th cell subsets by down-regulating AC9 transcription (Figure 12, right side). These regulatory features of Treg cells indicate that cAMP is crucial for naturally occurring Treg-mediated suppression.

Like naïve T cells, Treg cells express transcripts for $β_1$-AR, $β_2$-AR and $α_2$-AR, but not $α_1$-ARs. The predominant AR expressed in Treg cells is the $β_2$-AR. However, in comparison to naïve T cells, $β_2$-ARs expression is 5-fold lower in Treg cells [125]. $β_2$-ARs expressed in FoxP3+ Treg cells are functional, as they increase intracellular cAMP levels and induce PKA-dependent cAMP response element-binding protein (CREB) phosphorylation (Figure 13), and these responses are abrogated by $β_2$-AR blockade [125]. This is consistent with PKA mediating many of the downstream actions of cAMP through nuclear transcription, and PKA as a regulator of TCR signaling through C-terminal Src kinase (Csk) or by regulating gene transcription [149-153] (Figure 13). The activating transcription factor (ATF)/CREB family bind to CRE (cAMP-response elements) in the promoter region of many genes to mediate cAMP/PKA signal transduction. These CREB modulatory proteins are encoded by three genes, *Creb*, *Crem* and *Atf-1*. CREB, CREM and ATF-1 suppress or activate gene expression to control T cell functions (e.g., proliferations/ differentiation, cytokine/chemokine production, apoptosis and cell homeostasis).

Crem encodes many activators or suppressor isoforms, including ICER (inducible cAMP early repressor). ICER is a splice variant of CRE modulator (CREM) that negatively regulates CRE-mediated transcription, including the IL-2 gene. Treg cells suppress IL-2 transcription through ICER induction in T effector cells; ICER levels correlate with cAMP levels (Figure 13; feedback shown by red arrows). ICER signals autoregulatory feedback loops of transcription that govern the downregulation of early response genes critical for immune

responses [149-153]. Thus, the PKA–CREM/ICER signaling axis is considered a central pathway for cAMP-mediated T-cell suppression and Treg cell function. ICER can suppress proliferation, production of pro-inflammatory cytokines (IL-2, tumor necrosis factor-α (TNF-α), IFN-γ), anergy, and expression of FasL [154-156]. In Treg cells, ICER is a mediator of cAMP-induced suppression of *Il2* and *Nfat* gene transcription in T effector cells [146-158]. ICER binds to multiple nuclear factor of activated T cell (NFATc1)/ activation protein 1 (AP-1) sites within the *Il2* promoter, and correlates with reduced numbers of IL-2-expressing CD4+ T cells [149,150]. NFAT and ICER form inhibitory ternary complexes on several composite NFAT/AP-1 DNA binding sites. These ternary complexes repress cytokines in addition to IL-2, including TNF-α, IL-4, IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF) [159]. Thus, NFAT/ICER complexes mediate transcriptional attenuation of many NFATc1-driven cytokine promoters in conventional CD4+ T cells [160].

In contrast, Treg cells cannot induce NFATc1 at the transcriptional levels. They express low cytoplasmic levels that do not translocate
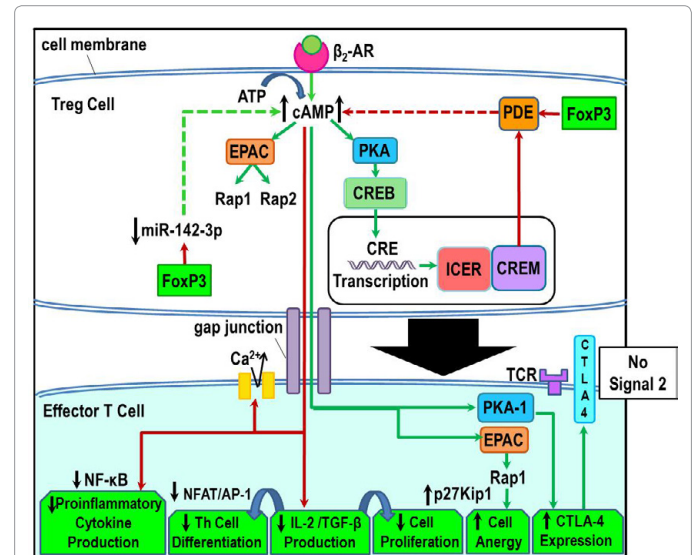

Figure 13: Cyclic AMP-mediated regulation of Th cells by Treg cells. Treg cells predominantly express $β_2$-ARs on their cell surface (top center). Ligand activation of these receptors stimulates cAMP production, which induces a cascade of PKA-dependent cAMP response element-binding protein (CREB) phosphorylation and subsequent transcription of ICER and CREM. CREM inhibits PDE activity, which prevents the breakdown of cAMP, and therefore promotes higher intracellular cAMP in Treg cells (upper right side). Elevated cAMP also increases EPAC activity which acts to increase Rap 1 and Rap 2 GTPase activity. Ras-proximate-1/2 (Rap1/2) is implicated in promoting Treg cell regulation of Th cells. FoxP3, a phenotypic marker of Treg cells, regulate intracellular cAMP concentrations by at least two mechanisms. FoxP3 inhibits PDE activity, promoting greater intracellular levels of cAMP. FoxP3 also down-regulates miR-142-3p (upper left side) to keep the AC/9/cAMP pathway active, a role that is opposite to that played by this microRNA in other Th cell subsets. The cAMP generated in Treg cells and transferred to Th cells via gap junctions (center of figure) can exert both suppressive and enhancing actions on Th cells. These suppressive and enhancing effects are illustrated in the lower left and right of the figure, respectively. Greater cAMP concentrations inhibit proinflammatory cytokine production by suppressing NF-κB and Th cell differentiation by blocking IL-2 and TGF-β production, via reduced NFAT/AP-1 binding to DNA binding sites (lower right). Lower influx of calcium is induced by cAMP, which negatively regulates NFAT protein kinase. Increased cAMP concentrations in Th cells induce PKA-dependent CTLA-4 upregulation, a negative regulator of contact-dependent T cell receptor (TCR) activation (lower right), preventing binding of CD28 (signal 2) necessary for T cell activation. EPAC transduces cellular actions of cAMP in Th cells that promote anergy via the activation of Rap1 (lower right). (Green arrows, excitatory; red arrows, inhibitory).

efficiently to the nucleus after CD3/CD28 stimulation [161]. Under these conditions, calcium flux is reduced, along with lower calcineurin activation and greater glycogen synthase kinase-3β, which negatively regulates NFAT protein kinase [161]. Thus, the suppression mechanisms in Tregs differ markedly from those generating NFAT/AP-1 and other NFAT complexes that activate cytokine promoters in conventional CD4+ T cells. Direct contact between Tregs and CD4+ T cells increases intracellular cAMP in Treg cells. Increased cAMP induces greater expression and nuclear localization of ICER/CREM, represses NFATc1 and IL-2 production, and interferes with their activity in T cell activation. IL-2 is an autocrine growth factor that increases the number and suppressive activity of Tregs.

Treg cells express lower levels of phosphodiesterase (*Pde*) genes than effector T cells. *Pde*) gene expression and protein increase when T cells are incubate with a β-AR agonist [147,153,162]. However, in Treg cells, FoxP3 prevents the degradation of cAMP generated from GPCR activation by repressing gene expression of *Pde3A* and *Pde4A10*. Consequently, *Pde3A* and *Pde4A10* repression promote higher cAMP levels that inhibit conventional T cell functions and amplify Treg cell function (Figure 13). Therefore, β$_2$-AR stimulation of Treg cells drives their suppressor function and maintains tolerance and dampens Th cell immunity by increasing intracellular cAMP. Houslay and Baillie [163] report β-arrestin recruitment of PDE4 desensitizes PKA-mediated suppression of β$_2$-AR signaling via cAMP, thus PDE4 promotes receptor signaling via cAMP.

Additionally, T effector cell suppression can be mediated by another cAMP mediator, EPAC (exchange protein elevated by cAMP) (Figure 13). EPAC is directly activated by cAMP and its activation induces Ras GTPase, Ras-proximate (Rap)1 and Rap2, implicated in enhancing negative regulation of Th cells by Treg cells [153,164-166]. *Epac* expression promotes the Treg anergic phenotype after anti-CD3 antibody stimulation, and in part, mediates cAMP-induced T effector cell suppression [167,168]. Naïve and activated Treg cells express higher levels of EPAC than T effector cells [153,164], suggesting a greater role for EPAC in Treg cells. Rap1 is activated by cytotoxic T lymphocyte antigen-4 (CTLA-4) engagement on T cells and mediates the subsequent inhibitory signal on IL-2 production [169,170]; EPAC-induced Rap1 may contribute to this response. CTLA-4 is a CD28-family receptor expressed on Tregs and other activated effector T cells. It negatively regulates T cell function and proliferation [171]. Mice deficient in CTLA-4 develop lethal lymphoproliferative disease [172,173].

Interestingly, β$_2$-AR stimulation with NE or a β$_2$-AR agonist promotes the conversion of naïve T cells to FoxP3+ T cells (iTreg cells). The number of FoxP3 iTreg cells increases two-fold when anti-CD3-stimulated naïve T cells are co-cultured with β$_2$-ARs [125]. These effects are cAMP-, PKA- and EPAC-dependent.

Moreover, β$_2$-AR signaling augments the *in vitro* suppressive function of Treg cells. Cultured at a Treg-naïve T cell ratio of 1:0.25, β$_2$-AR activation promotes Treg-mediated reduction in anti-CD3-stimulated CD4+ T cell proliferation and IL-2 mRNA levels [125]. Lower TCR-induced IL-2 transcription is linked to higher levels of ICER in Treg cells [146]. ICER expression depends on cAMP, PKA, and pCREB [147], intracellular mediators induced by β$_2$-AR activation that suppress Treg function (Figure 13). The effect of β$_2$-AR stimulation on T cell proliferation and IL-2 production is blocked by β$_2$-AR antagonist treatment, but not a specific α$_{2a}$-AR antagonist [125]. Treg cells from β$_2$-AR$^{-/-}$ mice display proliferative responses and IL-2 levels similar to that from unstimulated Tregs cells from wild type controls [125]. Collectively, these findings suggest that β$_2$-AR-induced cAMP signals

ICER-mediated reduction in IL-2 production and cell proliferation in activated CD4+ T cells.

Like other CD4+ T cells [174], β$_2$-AR stimulation of Treg cells up-regulates CTLA-4 in a PKA-dependent manner (Figure 13) [125]. CTLA-4 is an important negative regulator of contact-dependent suppressive functions of Treg cells and of T cell activation [175-177]. CTLA-4 binds to CD80 or CD86 expressed on APCs with greater affinity than CD28, thereby blocking costimulatory signals, and subsequent cell cycle progression and IL-2 production [178]. A similar suppressive effect on proliferation and IL-2 is mediated by cAMP (Figure 13) [146,179]. CTLA-4 expression is regulated by adaptive immune signaling at the level of transcription [175,180]). Blocking the binding of CTLA-4 to CD80 or CD86 prevents the accumulation of ICER, and rescues production of IL-2 [157,158,181]. Recent studies indicate that cAMP induces CTLA-4 in human CD4+ T lymphocytes [174,182]. Thus, these finding provide a possible mechanism for β$_2$-AR-mediated suppression of anti-CD3-stimulated proliferation and IL-2 secretion. Collectively, these findings support many mechanisms through which the SNS can regulate the suppressive function of Treg cells on conventional T cells via β$_2$-AR-cAMP signal transduction. Therefore, dysregulation of the SNS in rheumatic diseases could be a major player in triggering RA and regulating its disease course. In the next two sections, we discuss newly discovered complexities in the terminal differentiation of Th cell subsets. We believe this discussion is relevant to their regulation by the SNS. They raise important questions about whether Th effector cells are truly terminally differentiated and elude to possibly new Th cell subsets under SNS regulation.

Terminal Differentiation and Plasticity of Th17 and Treg Cells

During the host's defense against different pathogens, antagonism between differentiating CD4+ effector T cells are thought to sustain each lineage-specific response. However, recent findings of late-stage plasticity and lineage convergence, particularly with Th17 and iTreg cells, complicate our understanding of their mutually antagonistic and reciprocal nature. Th17 and iTreg cells challenge the Th1-Th2 cell paradigm that Th cells terminally commit to a specific lineage once a key transcription factor becomes dominant. Several recent studies indicate that both Th17 and Treg cells display developmental plasticity. Th cells committed to the Th17 lineage can shift to Th1-like cells in response to IL-12, indicating the ability to shift their phenotypic fate late in their differentiation. CD4+T cells can produce both IFN-γ and IL-17, especially at sites of inflammation. This cytokine profile is reported to occur in re-stimulated Th17 cells. Co-expression of both IL-23R and IL-12Rβ2 enables Th17 to respond to either IL-23 or IL-12 [183] (Figure 14). If re-stimulated with TGF-β alone, these cells regain high production of IL-17A and IL-17F. Interestingly, upon repeated stimulation of Th17 cells with TGF-β, a subset of Th17 cells co-produces both IL-17 and IFN-γ, despite the absence of IL-12 or IL-23 signaling. Further, re-stimulation of Th17 cells with IL-12 induces rapid transition to a Th1-like phenotype [183-185]. In contrast, re-stimulation with IL-23 induces IFN-γ-producing Th17 cells that fail to produce IL-17A. Plasticity is a property of Th17 cells that does not extend to IL-17+ memory T cells. They maintain the Th17 cell phenotype regardless of being cultured with IL-12. Thus, IL7+ memory T cells achieve phenotype stability; the mechanisms by which they do so are unclear. Shifts in Th17 cell phenotype towards IFN-γ-producing cells have clinical relevance in models of RA, colitis [183], antigen-specific models of ocular inflammation [186], and models of diabetes [187,188]. In the latter case, pancreatic islet injury depends on the production of
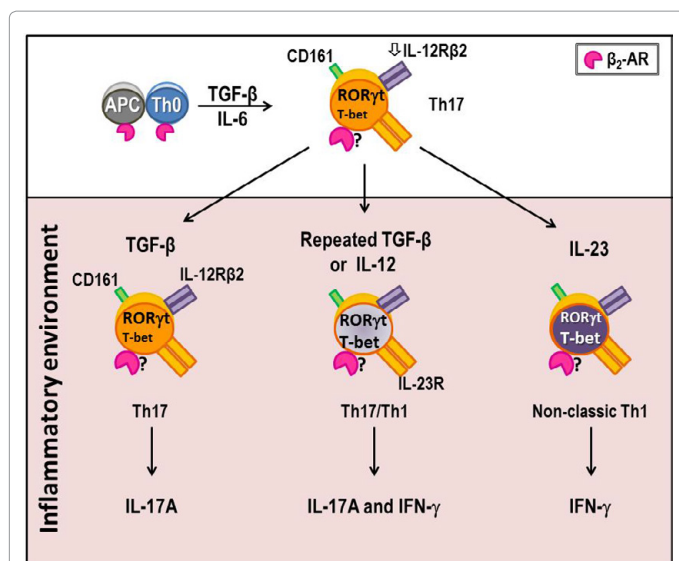
Figure 14: Late Th17 cell plasticity. Naïve Th cells that have been activated by antigen in the presence of TGF-β and IL-6 differentiate into Th17 cells via activation of the RORγt transcription program. IL-6 induces Th17 cells to produce IL-12, which subsequently up regulates RORγt expression. In an inflammatory environment, re-stimulation with TGF-β causes Th17 cells to regain their high production of IL-17A. However, re-stimulation with IL-12 leads to the expression of IL-23R in addition to low levels of IL-12Rβ2. In the repeated presence of relatively high TGF-β (compared to IL-23 or IL-12), Th 17 cells derived from Th0 cells produce high levels of IL-17A and IL-17F. If IL-23 is the dominant cytokine, then T-bet expression is increase and these cells further differentiate to IL-17A and IFN-γ producing Th17/Th1 cells. Interestingly, either repeated stimulation with TGF-β in the absence of IL-12 or IL-23 or a dominant presence of IL-12 can induce shift the transcriptional program towards higher activation of T-bet, resulting in Th17 cells that produce both IL-17A and IFN-γ. In contrast, Th17 cells restimulated in an inflammatory environment with IL-23 induce a differentiation shift towards non-classic Th1 cells that produce IFN-γ due to activation of the T-bet transcription program. All of these Th17 subtypes express CD161, a traditional marker of Th17 cells. Thus, Th17 cells are responsive to both IL-23 and IL-12. Th17 cells exhibit high late plasticity, depending upon the balance of TGF-β, IL-23 and IL-12 in the milieu.

IFN-γ, indicating that IFN-γ production by Th17 cell is pathogenic in some autoimmune models.

An increasing number of studies indicate iTreg cells also display substantial late developmental plasticity under conditions of elevated inflammatory cytokines. Both natural and iTreg cells have been reported to produce IFN-γ and IL-17 [189-192]. Under *in vitro* conditions, stimulation of mature Treg cells with IL-6 can induce IL-17, decrease FoxP3 and initiate RORγt expression. These findings suggest that mature Treg cells can further differentiate into Th17-like cells in an inflammatory environment [190,193] (Figure 15). In contrast, reciprocal conversion of Th17 cells into Treg-like cells has not been described. This may indicate that Tregs-to-Th17 cell and Th17-to-Th1 cell conversion are irreversible. Regarding Th17 and Treg cells, most findings that support their plasticity derive from *in vitro* studies. The extreme polarizing conditions that enable clearer phenotypes and more homogeneous T cell populations could exaggerate the potential for plasticity of these cell types. Some *in vivo* studies confirm the potential for their plasticity under more physiologic conditions. However, further studies are needed to determine whether similar Th cell plasticity can occur *in vivo,* and if so, its implications for health and disease. Phenotypic plasticity of Th17 and Treg cells has important implications in autoimmune diseases, including RA. With respect to the SNS, no studies have examined β2-ARs regulation of Th17-to-Th1 or Treg-to-Th17 phenotypic plasticity.

Th Cell Balance, Tolerance, and RA

RA is thought to arise from a loss of immunologic tolerance to self-antigens that result in an aberrant immune response towards self-proteins. Tolerance is maintained and autoimmunity avoided by central and peripheral tolerance mechanisms [194,195]. Central tolerance is a complex developmental process. In this process, high-affinity self-reactive T and B cells are eliminated by negative selection in the thymus and bone marrow. Self-reactive T and B cells with low affinity towards self-antigens survive negative selection and join the mature T cell repertoire. Peripheral tolerance exerted by the suppressive activity of Treg cells, normally keep potentially self-reactive immune cells that escape negative selection in check. Thymic-derived nTreg cells (CD4+CD25hiFoxP3+) [196,197] and naïve CD4+ Th cell-derived iTreg cells [198,199] prevent autoimmune diseases in humans [200] and mice [201]. Treg cells maintain self-tolerance by suppressing effector T cells in the periphery [121-202]. An imbalance between CD4+ effector T cells that target self-proteins and CD4+ Treg cells is proposed to precipitate onset of autoimmune diseases. For example, activated self-reactive Th17 cells increase, while Treg cell numbers and/or their functions decline in RA patients with active disease compared with healthy controls [203,204].

Treg cells critically maintain peripheral tolerance by suppressing antigen-specific immune responses of activated self-reactive Th cells

APC, antigen-presenting cell; Foxp3, forkhead box P3; IL, interleukin; R, receptor; ROR, RAR-related orphan receptor; RAR, retinoid acid receptor; TGF-β, transforming growth factor-β; Th, T-helper; Treg, regulatory T cells
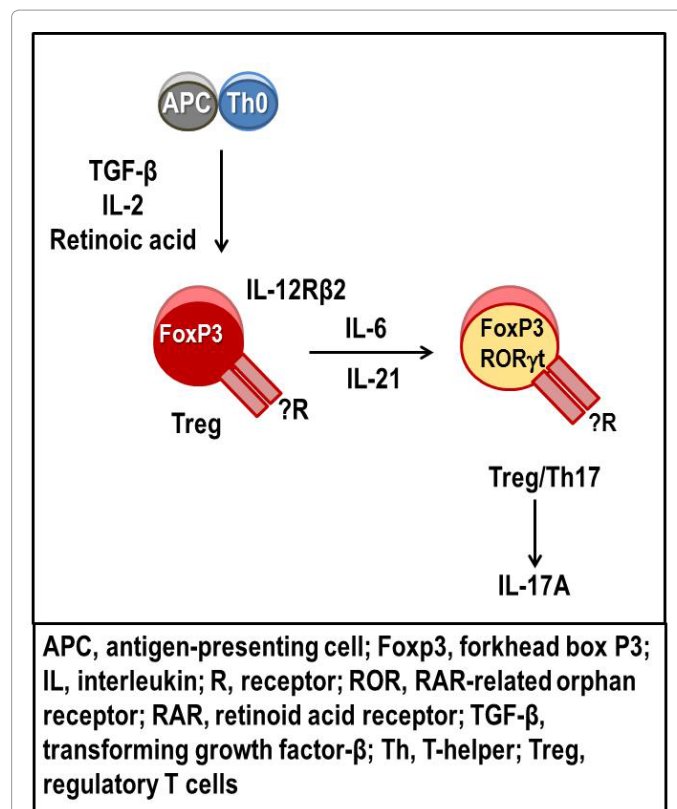
Figure 15: Late Treg cell plasticity. Multiple reports indicate that inducible Treg cells can readily switch to IL-17-producing cells upon stimulation by inflammatory cytokines. Tregs stimulated with IL-6 and IL-21 can be induced to express IL-17. Under these conditions expression of FoxP3 is down-regulated. These findings indicate that mature Treg cells can be subverted to Th17-like cells after commitment to the FoxP3 transcriptional program. There is, at present, no evidence that the reciprocal conversion of Th17 cells towards a Treg cell phenotype occurs.

[205,206]. These cells make up about 10% of the peripheral CD4⁺ T cells. They constitutively express CD25 (IL-2Rα), FoxP3, CTLA-4, and glucocorticoid-induced TNF-related protein (GITR) [207-209] – signaling proteins that serve important functional roles in Treg cells. For example, simply blocking CTLA-4 abrogates Treg cell function *in vivo* [210,211]. CTLA-4 blockade prevents the cell-to-cell contact between Treg and Th effector cells that is required for Treg-mediated immunosuppression of T effector cells [212,213]. In contrast, GITR activation stimulates T effector cell functions and inhibits Treg suppressor activity, which potentiates immune responses against pathogens [208,214,215]. Further, studies indicate that GITR activation can break Treg cell anergy and stimulate proliferation of Treg cells [207-209].

The mechanisms responsible for lost tolerance in autoimmune diseases like RA are not entirely clear. Available evidence supports that peripheral tolerance is broken due to an imbalance between effector CD4⁺ Th cells and CD4⁺ Treg cells [216]. However, the initiating factors, and whether the imbalance includes Th1 and/or Th17 effector cells, are still debated. Early studies indicate Th1 and Th2 cell imbalance is skewed toward Th1 reactivity in animal models of RA, like collagen-induced arthritis (CIA) and AA [217,218]. For example, IFN-γ-expressing CD4⁺ T cells home to the inflamed arthritic joints. However, CD4⁺ T cells fail to release high levels of IFN-γ in the synovium of patients with RA [219-222]. In fact, neutralizing Th1 cells and/or their key cytokine, IFN-γ, yield paradoxical results on disease outcome, depending upon the animal model and timing of the intervention. Neutralizing IFN-γ activity in the pre-onset or early stages of CIA was protective, whereas, late administration exacerbated disease [223,224]. Genetic manipulation of IFN-γ in autoimmune arthritis models reveals equally paradoxical results of increased or decreased disease susceptibility/severity depending on the model [225-229].

Recent discovery of Th17 and iTreg cells and the tolerance function of Treg cells have shifted the focus of research towards understanding Th17-Treg cell functional imbalance. However, it is likely that this is an over simplification of the disease process. Th1 cells are important in the initial disease stages and/or for disease induction as they are predominant in the pre-arthritic stage in animal models. Deleting Th1 cells prevent disease onset and their ability to adoptively transfer the disease to naïve animals in some animal models of RA [230-233]. Moreover, it seems that Th17 cell involvement in the pathogenesis depends on CD4⁺ Th1 cells [234].

Extensive Th1-Th17 cell cross-regulation supports dysregulation of both subpopulations, as well as, Th17 cells that express a Th1-like phenotype, in the pathogenesis of RA. Controversy remains over Th1-Th17 cell cross-regulation and late plasticity in Treg cell differentiation, but data continue to support an important role of Th1 cells in the etiology of RA. Th1 and Th17 lineages are more intertwined and complex than initially appreciated; the integrated counter-regulatory mechanisms, cooperativity and interdependency of these lineages supports this notion. The role of Th1 in autoimmune disease cannot be discounted, particularly prior to and at disease onset, and clearly warrants further investigation. Thus, it appears that Th17, Th17/Th1, and classic Th1 cells are all involved in joint pathology, with each having variable importance at different disease stages. Understanding how Th cell interactions induce and moderate clinically-apparent disease is critical for developing therapeutics that prevent disease in individuals at risk for RA.

Does the SNS Play a Role in the Loss of Tolerance in RA?

The SNS's primary function is the fine tuning of biological responses to ensure whole body homeostasis, which includes immune system functions. During an immune challenge, the SNS coordinates systemic responses (e.g., neural, immune, cardiovascular, renal, and metabolic, etc.) required for an appropriate immune response and subsequent return to homeostasis once the challenge is eliminated. One mechanism by which the SNS regulates immune function is by modulating the production of key cytokines that regulate the differentiation of CD4⁺ Th subpopulations. In this manner, the SNS has a role in determining the appropriate phenotypic balance of Th effector cell subtypes to effectively handle pathogens. In the absence of overt pathogens, the role of the SNS in maintaining tolerance to prevent autoimmunity is less clear. Treatments that destroy sympathetic nerves in immune organs do not induce autoimmunity. However, animal models of autoimmune diseases, like MS or RA, indicate that enhanced SNS signaling can exacerbate disease outcome during the preclinical phase, but reduce disease severity after disease onset [51,91,94,95]. Moreover, severe life stressors often precede RA induction, suggesting that increased sympathetic activity may be a trigger for disease onset [54,56,235,236].

Identifying factors that precipitate disease onset is highly relevant clinically. Patients at risk for autoimmune diseases can be identified by circulating autoantibodies (e.g., rheumatoid factor anti-citrulline antibodies) that precede onset of clinically evident disease by as much as 10 or more years [56,237-243]. Early expression of self-reactive CD4⁺ T cells and B cells that cooperatively produce these autoantibodies [244] precede onset of clinically obvious disease. Loss of tolerance in the pre-disease state provides an opportunity for identifying at risk patients for preventative treatment, if the onset-triggering event(s) is(are) identified.

Under conditions of acute psychological stress, sympathetic activation can induce effector Th cell/Treg cell imbalance that favors immune reactivity. Two studies in which healthy volunteers were recruited lend support to this notion. In one study [245], acute stress increases antigen-experienced effector T cells. In the other study [127] stress reduces circulating Treg cells, implying that the stress-induced increases in effector T cells are linked to a loss of Treg cell number and/or function [127,246]. Interestingly, greater expression of β-ARs in Th1 [246] and Treg [127] cells accompany these stress-induced effects on T cell subtype number/functions. The increase in circulating Th1 cells is consistent with the elevated IFN-γ production that occurs after the acute stress. These reports pertain to acute stress. Others postulate that chronic/severe psychological stress constantly drives elevated peripheral effector T cells, and promotes inflammatory and autoimmune conditions [247]. These stress-induced effects on immunity may provide a biological explanation for psychological stress exerting an influence on autoimmune diseases [60,248,249]. However, the mechanisms by which chronic stress drive self-reactive effector T cells and suppress Treg cell inhibition of autoreactive T cells are not clear.

The SNS suppresses the production of IFN-γ in Th1 cells by activating β₂-ARs expressed on their cell surface. β₂-ARs are GPCRs that classically regulate cellular functions by activating the cAMP-PKA signaling pathway (canonical pathway). This signaling pathway inhibits mitogen-activated protein kinase (MAPK) signaling pathways that drive IFN-γ production. β₂-AR-reduced IFN-γ production disinhibits its cross-regulatory drive on Th2 cells. Potential effects of β₂-AR-induced IFN-γ suppression on Th17 differentiation has not been assessed directly. However, lowering IFN-γ is expected to promote Th17 cell differentiation under Th17 cytokine-promoting conditions. Thus, the SNS could shift the immune response towards a humoral (Th2) or Th17

(cell-mediated) response. The SNS also regulates clonal expansion of Th cells through its ability to suppress the production of IL-2, a cytokine that expands effector Th cell clones. IL-2 is critically required for Treg cell suppression of Th effector cells, providing an indirect mechanism for SNS-regulated Treg cell inhibition of Th cell functions. Through these mechanisms, SNS is positioned as an important neural regulator of immunity, consistent with a homeostatic role across the course of an immune response.

Is the SNS Functionally Intact to Signal Th cells in Autoimmune Arthritis?

The activity of the SNS is increased in patients with RA. Thus, sympathetic nerve firing rates, release of NE, and transmitter engagement of ARs are elevated in sympathetic target organs. High NE concentrations or chronic β_2-AR stimulation can induce β_2-AR down-regulation. β_2-AR down-regulation impairs receptor coupling to the G protein, Gs, that is required to induce cAMP production (receptor desensitization). Increased SNS activity observed in RA patients may explain the decreased expression and desensitization of β_2-ARs on peripheral blood lymphocytes observed in these patients. This observation is further coupled with an impaired ability of catecholamines to stimulate β_2-AR-induced cAMP and to regulate immune functions [37,38,40,250]. These findings further underscore the concept of a dysfunctional SNS in these patients. For obvious reasons, the function of β_2-ARs has only been examined in peripheral blood leukocytes. The peripheral blood is not innervated by the SNS, thus, elevated circulating NE in the blood is the result of spillover from all tissues that are innervated by the SNS. Also, immune cells present in the blood do not exert their immune functions in this tissue, but are being transported to tissues where they exert these functions. Thus, immune cells obtained from the blood are unlikely to be the most relevant place to assess SNS neurotransmission.

Examining β_2-AR functions in lymphocytes in immune organs that participate in disease process are of greater relevance. Arthritogenic Th1 cells develop in the DLNs of arthritic limbs and the spleen and are capable of transferring disease in animal models of RA. We are using the AA model of RA in Lewis rats to determine the functional status of SNS neurotransmission and their impact on the production of IFN-γ, a key cytokine required for the development of Th1 cells in these relevant immune organs. Using the AA model, we've shown that *in vivo* β_2-AR stimulation induces greater *ex vivo* IFN-γ production in lymphocytes from lymph nodes that drain arthritic joints [17,35]. This finding supports the SNS as a driver of Th1 (or Th17 cells with a Th1 phenotype): Treg cell imbalance. Augmented IFN-γ production is specific to lymph node cells that drain the arthritic limbs, as IFN-γ production remains unchanged in splenocytes and peripheral blood mononuclear cells (PBMCs), and reduced (the classical response) in mesenteric lymph nodes cells after β_2-agonist treatment. The inability of β_2-ARs to alter IFN-γ in the spleen indicates a loss in the ability of the SNS to regulate immune homeostasis in this target tissue, and is consistent with our findings that β_2-ARs are down-regulated and desensitized in splenic lymphocytes [17,27,35]. Thus, the mechanism for regulating Th cell subtype balance is lost in the spleens of arthritic rats. Collectively, our data support that (1) β_2-AR stimulation can promote production of pathogenic T cells in lymph nodes that drain disease-relevant tissues; (2) SNS regulation of Th cell subtype balance is lost in the spleen; (3) β_2-ARs function differently in lymphocytes from lymph nodes that drain the affected limbs compared with lymphocytes from mesenteric lymph nodes or the spleen; and (4) aberrant β_2-AR-mediation of Th cell functions is specific to disease-relevant immune organs (Figure 16).
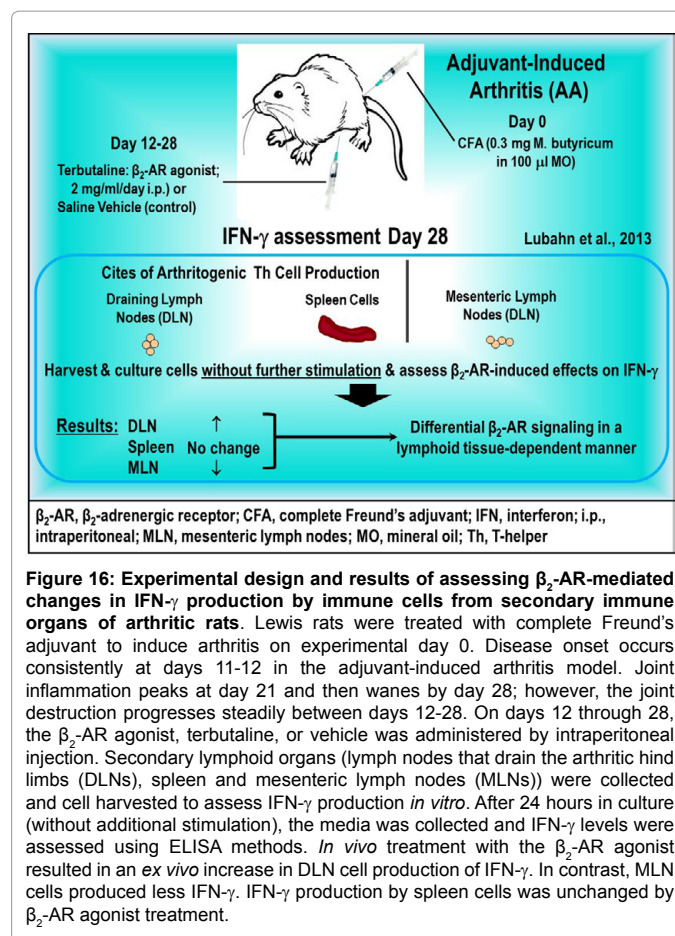

Figure 16: Experimental design and results of assessing β_2-AR-mediated changes in IFN-γ production by immune cells from secondary immune organs of arthritic rats. Lewis rats were treated with complete Freund's adjuvant to induce arthritis on experimental day 0. Disease onset occurs consistently at days 11-12 in the adjuvant-induced arthritis model. Joint inflammation peaks at day 21 and then wanes by day 28; however, the joint destruction progresses steadily between days 12-28. On days 12 through 28, the β_2-AR agonist, terbutaline, or vehicle was administered by intraperitoneal injection. Secondary lymphoid organs (lymph nodes that drain the arthritic hind limbs (DLNs), spleen and mesenteric lymph nodes (MLNs)) were collected and cell harvested to assess IFN-γ production *in vitro*. After 24 hours in culture (without additional stimulation), the media was collected and IFN-γ levels were assessed using ELISA methods. *In vivo* treatment with the β_2-AR agonist resulted in an *ex vivo* increase in DLN cell production of IFN-γ. In contrast, MLN cells produced less IFN-γ. IFN-γ production by spleen cells was unchanged by β_2-AR agonist treatment.

Molecular mechanisms for β_2-AR-mediated increases in IFN-γ [17,26,251,252] remain a mystery, as β_2-ARs typically activate the cAMP-PKA pathway, a pathway well known to suppress the production of IFN-γ. Our findings in the DLNs are not easily explained by signaling via the canonical cAMP-PKA pathway. There are a few studies in non-immune cells that may suggest some possible cAMP/PKA-mediated routes under specific conditions (reviewed in [253]), these are pathways that have not been clearly defined. We believe it is more likely these findings can be explained by the recently described ability of the β_2-AR to "switch" signaling towards MAPK pathways (reviewed in [27]). This is an important mystery to solve, as IFN-γ is a key regulator of Th cell differentiation, clonal expansion, and a cross-regulator of Th2, Th17, and Treg cell differentiation, with important implications for autoimmune pathophysiology. The Nobel Prize winning discovery in 2012 that GPCRs, which include β_2-ARs, can "switch" signaling pathways from the classical cAMP pathway to MAPK signaling pathways [254,255] is a timely discovery that may solve this puzzle.

β_2-ARs are dynamically regulated, in large part, by receptor ligand availability and receptor activation-induced PKA [256] (Figure 17). Similarly, G protein receptor kinases (GRKs) and β-arrestins also are critical regulators of β_2-AR signaling [256]. PKA and GRKs regulate β_2-AR function by site-specific phosphorylation of β_2-ARs (pβ_2-AR) at different serines [257-259]. High NE or agonist concentrations can cause β_2-ARs to become non-responsive to additional stimulation, a condition referred to as receptor desensitization. Receptor desensitization is induced by PKA-mediated pβ_2-AR (pβ_2-AR$_{PKA}$), and at higher agonist concentrations, by PKA- and GRK-induced pβ_2-AR
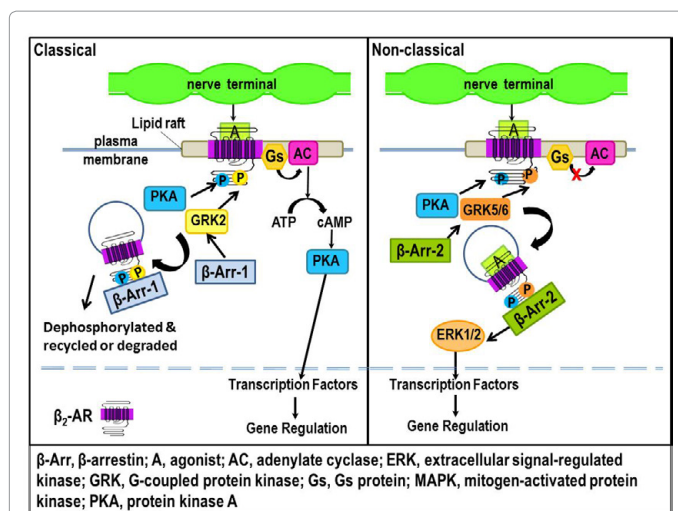
Figure 17: A-B. Classical and non-classical signaling of β_2-ARs. A) Agonist binding to β_2-ARs causes the dissociation of Gs subunits (Gsα and $\beta\gamma$). The Gsα subunit activates AC, which catalyzes the conversion of ATP to cAMP and the subsequent cAMP-induced activation of PKA. PKA activates transcription factors that subsequently regulate gene transcription to alter immune cell function. Receptor function is terminated by GRK2 phosphorylation of the receptor, which causes an uncoupling of Gs to AC. GRK2 recruits β-arrestin-1 to the receptor. Receptors phosphorylated by PKA and GRK2 are internalized via functions of β-arrestin-1. The internalized receptor is then either dephosphorylated and recycled to the membrane or transported to lysosomes for degradation. **B)** Under conditions of high agonist concentration, β_2-ARs can be phosphorylated by GRK5/6 rather than GRK2. Phosphorylation of β_2-AR by GRKs 5 or 6 desensitizes receptor signaling via cAMP-PKA by uncoupling the receptor from Gs. Phosphorylation of the receptor by GRK5/6 also induces β_2-AR internalization by recruiting β-arrestin-2 to the receptor. Once bound to the receptor, β-arrestin-2 acts as a scaffold for the sustained activation of the MAPK, ERK 1/2. Beta-arrestin-2 activation of ERK1/2 MAPK, in turn, increases the translocation of transcription factors into the nucleus to influence gene transcription and thus, alter cell functions.

[260-262]. The pβ_2-AR$_{PKA}$ alters receptor conformation, impairing Gs and enhancing Gi protein coupling that transiently activates the mitogen-activated protein kinases MAPKs, and extracellular signal-regulated kinase (ERK 1/2) [262]. In contrast, pβ_2-AR by GRK (pβ_2-AR$_{GRK}$), specifically GRK2, induces further receptor uncoupling with Gs protein, which prevents cAMP production, and recruits β-arrestin-1 to the receptor [262-264]. Binding of β-arrestin-1 to the receptor induces receptor internalization. Once the receptor is internalized, it is dephosphorylated and either recycled to the plasma membrane or degraded [265,266]. Receptor degradation reduces intracellular and/or cell surface expression of β_2-ARs, a condition referred to as receptor down-regulation. High SNS nerve activity resulting in high or sustained NE availability, as reported under chronic inflammatory conditions can induce β_2-AR desensitization and down-regulation.

Under conditions of high concentrations of β_2-AR agonists, the receptor also can be phosphorylated by PKA- and GRK5/6 (rather than GRK2) [267]. In contrast to GRK2, GRK5/6 recruits β-arrestin-2 to the receptor. Unlike β-arrestin-1, β-arrestin-2 serves as a scaffold/adaptor protein for MAPK activation [268,269]. In this manner, high β_2-AR agonist concentrations can induce a G protein-independent sustained activation of the MAPK, ERK [255,270,271]. Studies demonstrating activation of this pathway have mostly been demonstrated in cell lines. We have not found any studies that have determined the extent to which this non-classical signaling pathway for β_2-ARs can be activated in cells of the immune system. However, our work supports this non-classical signaling pathway for β_2-ARs activation in lymphocytes present in DLNs

from arthritic joints [27,35]. In contrast, β_2-ARs are down-regulated and desensitized in splenocytes, although arthritogenic T cells are derived from both sites. This suggests that microenvironmental factors may be important determinants in directing β_2-AR signal transduction in different lymphoid organs.

We are actively testing the hypothesis that disparate effects of β_2-AR agonists on IFN-γ production in splenocytes and DLN lymphocytes from AA rats is due to differences in receptor expression, differential phosphorylation, and signaling pathways activated by receptor stimulation (Figure 18). In splenocytes harvested at peak disease (day 21), β_2-ARs fail to induce an increase in intracellular cAMP production [35]. This is coupled with a decrease in affinity and number of cell surface β_2-ARs. These findings are consistent with receptor desensitization and down-regulation and with the inability of β_2-AR agonists to reduce IFN-γ production in splenocytes from arthritic rats.

Due to the limited number of cells that can be harvested from the DLN, cAMP assessments and receptor binding studies were not completed in this study. However, assessments of total β_2-AR expression (cell surface and intracellular) and receptors phosphorylated by PKA and GRKs using western blot analysis were completed for both spleen and DLN cells. Strikingly, different patterns of receptor phosphorylation and changes in receptor density were observed in splenocytes and DLN cells from rats with AA, despite both being sites shown to generate arthritogenic T cells [35].

In splenocytes, total expression of β_2-ARs (cell surface and intracellular) increases during peak disease (day 21), but declines during chronic severe disease (day 28). Although total expression of β_2-
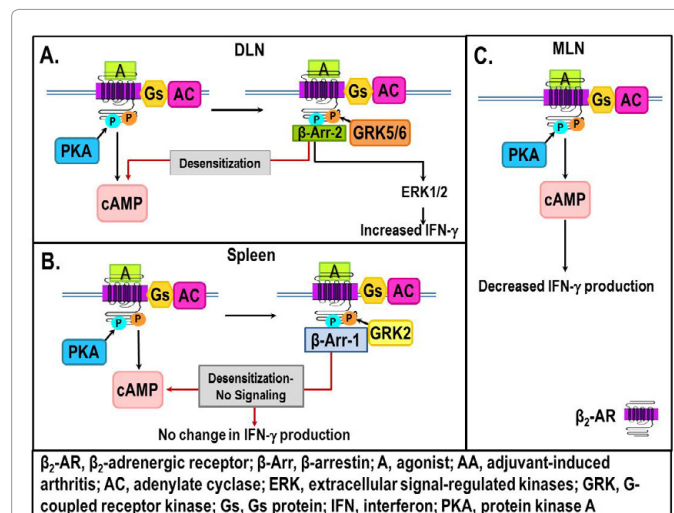
Figure 18: Proposed β_2-AR signaling pathways responsible for differential receptor regulation of IFN-γ production in immune cells from different secondary lymphoid organs in adjuvant-induced arthritis. A) In the DLN, where a β_2-AR agonist increased IFN-γ production, receptor phosphorylation patterns support phosphorylation of the receptor by PKA and GRK5/6. This phosphorylation pattern induces receptor uncoupling from Gs and recruitment of β-arrestin-2 to the receptor, which shifts signaling towards the ERK1/2 pathway. This shift in receptor signaling then promotes IFN-γ production. **B)** In spleen cells, the inability of a β-AR agonist to induce an increase in cAMP coupled with the pattern of receptor phosphorylation we observed is consistent with phosphorylation by PKA and GRK2. GRK2 phosphorylation uncouples the receptor from Gs and recruits β-arrestin 1 resulting in receptor desensitization. **C.** In contrast, MLN cell the ability of the β_2-AR agonist to suppress IFN-γ production in AA rats indicates receptors in these cells signal through the classical cAMP-PKA pathway.

ARs increases during peak disease, a reduction in cell surface receptor expression is observed with receptor binding assays on day 21 along with the decrease in total receptor expression on day 28. These findings suggest that β_2-ARs are internalized and traffick to lysosomes for degradation. We also find that receptor phosphorylation by PKA is reduced during severe and chronic disease stages. Receptor phosphorylation by GRK does not differ at severe disease, but increases during chronic disease. The lack of PKA-induced phosphorylation of the receptors is consistent with the receptor desensitization observed on day 21. The continued decrease in PKA-mediated receptor phosphorylation during chronic disease implies that β_2-ARs continue to be unresponsive. The pattern of reduced PKA-mediated phosphorylation coupled with the increase phosphorylation of the receptor by GRK, the inability of the receptor to increase intracellular cAMP, and reduced total β_2-AR expression suggests that the receptor is phosphorylated by GRK2, and subsequently targeted for lysosomal degradation. Thus, these findings are consistent with impairment of the SNS to reduce IFN-γ production in the spleen due to β_2-AR desensitization and down-regulation. While the data are intriguing, further studies are needed to confirm our findings and test our hypotheses more directly.
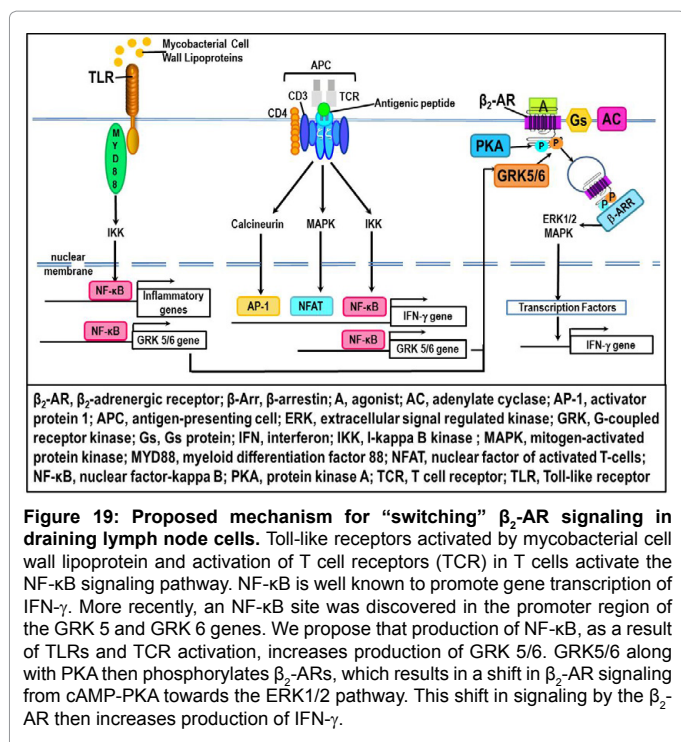
In contrast to the spleen, the total number of β_2-ARs in DLN cells does not differ between arthritic and non-arthritic rats during peak or severe disease stages [35]. Further, we find a striking two-fold increase in PKA-mediated phosphorylation of β_2-ARs during severe disease (day 21) followed by a significant decrease in PKA-mediated receptor phosphorylation during chronic disease (day 28) compared with non-arthritic control animals. Phosphorylation of β_2-ARs by GRK significantly increases during both the severe and chronic disease stages. These findings coupled with the observed β_2-AR-induced increase in IFN-γ in DLN cells from arthritic rats is consistent with receptor phosphorylation by PKA and GRK5/6, inducing a shift in signaling from the classical cAMP-PKA pathway for this receptor towards ERK1/2.

A similar shift in β_2-AR signaling away from cAMP toward ERK1/2 has been demonstrated for other cells types, but not yet shown in lymphocytes or other immune cells. Thus, in AA, different GRKs (i.e., GRK2 versus GRK5/6) may be responsible for the disparate phosphorylation of β_2-ARs by GRKs in spleen and DLN cells [255,259,272]. This hypothesis is consistent with the β_2-AR-induced increase in IFN-γ production in DLN without affecting IFN-γ secretion in spleen cells. Our data is also consistent with reports that phosphorylation of β_2-ARs by GRK5/6 shifts signaling into the ERK 1/2 cascade in HEK-293 cells treated with high physiological concentration of β-agonists [256,261,273]. Similarly, high SNS activity occurs in AA and RA. Elevated pro-inflammatory cytokines in inflammatory arthritis may also contribute to the phosphorylation of β_2-ARs by GRK, as IFN-γ and IL-6, or TNF-α, IL-1, and IL-12 induce different expression of GRKs and β_2-AR phosphorylation in PBMCs from healthy controls [274-276]. The SNS is crucial for maintaining homeostasis of the immune system, and particularly for regulating the balance of Th cell subtypes. Elucidating the mechanisms responsible for differential phosphorylation of β_2-ARs by PKA and GRKs, and their consequences for immune function in AA and other animal models of RA, are needed to understand disease-specific SNS-immune cross-regulation in RA. These studies are necessary to determine if β_2-ARs undergo down-regulation and desensitization or a shift in signaling towards ERK1/2, and if so, in which immune organs these events happen, when do they occur relative to different disease stages, and what are the consequences for disease pathology. Additional studies are also required to elucidate the mechanisms by which receptor-second messenger signaling shifts occur in DLN cells in the AA rat model of RA.

We propose that one mechanism for "signal switching" is by the activation of at least one type of pattern recognition receptor (PRR). PRRs are transmembrane receptors that detect microbial ligands of which TLRs are one family of these receptors. After TLRs detect microbial ligands they activate inflammatory signaling pathways [277-280]. In the AA model, complete Freund's adjuvant (CFA) is used to induce arthritis. It is composed of heat-killed mycobacterial cell wall that is emulsified in mineral oil. We have used the individual components of CFA to determine the role of each of its components in the altered β_2-AR signaling observed in this model. Rats were challenged with CFA, the cell wall in saline or mineral oil alone. As previously described, receptor phosphorylation by GRK is increased during severe disease in the DLNs of arthritic rats challenged with CFA. This finding is also observed in rats treated with the mycobacterial cell wall suspended in saline. In contrast, in rats treated with mineral oil, receptor phosphorylation by GRK is not increased [35]. These findings indicate a role for the mycobacterial cell wall component of CFA in driving phosphorylation of the β_2-ARs by GRKs. The mycobacterial component of CFA activates several TLRs (e.g., TLR2 and TLR4) [281,282]. Thus, our findings may implicate TLRs that are activated by the mycobacterial cell wall component in the regulation of β_2-AR phosphorylation by GRKs.

TLRs are critical regulators of the innate and adaptive immune response to mycobacterial cell wall components. The cell wall of Mycobacteria contain components which are recognized by TLR2 in combination with TLR1 and 6, TLR9 [283,284], and possibly TLR4 [285-288]. The mycobacterial cell wall contains a number of pro-inflammatory TLR2 ligands, including lipoproteins, mycolylarabinogalactan-peptidoglycan complex (the cell wall core structure), lipids, and lipoarabinomannan [289-293]. TLR2 works as a heterodimer with TLR1 or TLR6 in a complex with CD36 to recognize lipopeptides with different structures [294-298]. Further, TLR9 cooperates with TLR2 as a mechanism by which macrophages and DCs recognize mycobacterial cell wall TLR ligands. Thus, components of the mycobacterial cell wall bind to TLRs, and activate signaling pathways in innate immune cells and in T cells. Activation of TLRs leads to pro-inflammatory cytokine production, promotes maturation of APCs, antigen presentation, and T cell activation, which are now considered to be major factors in the development of autoimmunity. In addition to the better known TLR-induced maturation of APCs and pro-inflammatory cytokine/chemokine production, recent studies indicate TLRs are expressed in CD4^{+} and CD8^{+} T cells. In the presence of TCR activation, stimulation of TLRs expressed in T cells can function as a co-stimulatory signal to activate effector T cells and Treg cells [298]. This co-stimulation lowers the strength of the T cell signal that is required for proliferation and survival. These findings indicate an additional complexity in the mechanisms by which tolerance is broken and immune responses against self-antigens can be promoted.

Additionally, our findings that rats challenged with either CFA or mycobacterial cell wall alone promote phosphorylation of β_2-ARs by GRKs in the DLNs, suggest that the cell wall component of CFA also alters the ability of the SNS to restore immune system homeostasis, and thus, the susceptibility to autoimmunity. While no studies have directly determined the mechanism by which mycobacteria cell wall components can alter β_2-AR signaling in either APCs or T cells, activation of TLRs in APCs induces the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway (Figure 19). Further, activation of TCRs turns on many intracellular signal pathways, including MAPKs (p38, ERK, and JNK), PKC, and pathways that increase intracellular calcium (reviewed in [27]). These pathways

Figure 19: Proposed mechanism for "switching" β₂-AR signaling in draining lymph node cells. Toll-like receptors activated by mycobacterial cell wall lipoprotein and activation of T cell receptors (TCR) in T cells activate the NF-κB signaling pathway. NF-κB is well known to promote gene transcription of IFN-γ. More recently, an NF-κB site was discovered in the promoter region of the GRK 5 and GRK 6 genes. We propose that production of NF-κB, as a result of TLRs and TCR activation, increases production of GRK 5/6. GRK5/6 along with PKA then phosphorylates β₂-ARs, which results in a shift in β₂-AR signaling from cAMP-PKA towards the ERK1/2 pathway. This shift in signaling by the β₂-AR then increases production of IFN-γ.

β₂-AR, β₂-adrenergic receptor; β-Arr, β-arrestin; A, agonist; AC, adenylate cyclase; AP-1, activator protein 1; APC, antigen-presenting cell; ERK, extracellular signal regulated kinase; GRK, G-coupled receptor kinase; Gs, Gs protein; IFN, interferon; IKK, I-kappa B kinase; MAPK, mitogen-activated protein kinase; MYD88, myeloid differentiation factor 88; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor-kappa B; PKA, protein kinase A; TCR, T cell receptor; TLR, Toll-like receptor

activate the intracellular transcription factors, activating transcription factor 2 (ATF-2), nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and, notably, NF-κB. Interestingly, in cardiac myocytes, recent studies indicate a possible NF-κB binding site in the promoter region of GRK5 [299]. If a similar NF-κB binding site resides in the promoter region of the GRK5 gene in APCs and/or T cells, this could provide a mechanism for TLR induced promotion of GRK5-mediated phosphorylation of β₂-ARs and subsequent β-arrestin-2-mediated shift in β₂-AR signaling from cAMP/PKA to ERK1/2 activation (Figure 19). If this is true for CD4⁺ Th0, Th1 or Th1-like Th17 cells, it would provide a mechanism that explains the observed increase in IFN-γ production in DLNs during severe arthritis when Th cells are treated with a β₂-AR agonist after CFA challenge. Obviously, future studies are needed to test this hypothesis.

Of significance for RA, the ERK 1/2 signaling pathway increases IFN- production by CD4⁺ Th cells from rats with AA; these cells are arthritogenic and drive disease processes [243,300,301]. It is unclear whether stimulation of β₂-ARs in T cells can increase IFN- by activating ERK 1/2 under conditions of chronically elevated NE release. If so, then it would provide a mechanism to explain the increased IFN-γ production [302-308] after β₂-AR stimulation in activated splenic T cells [26] shortly after the onset of CIA [91,309] and DLN T cells on day 28 after CFA challenge in AA rats [17]. So far our findings are consistent with our hypotheses, but further work is required to definitively prove that changes in receptor signaling explain difference in IFN-γ production in DLN cells and splenocytes. These findings support that the imbalance between Th cell subtypes in autoimmune arthritis may result from faulty receptor regulation (desensitization, spleen) and non-canonical (DLN) β₂-AR signaling at sites where autoreactive T cells differentiate.

Both faulty and non-canonical β₂-AR signaling can be induced by high agonist concentrations or chronically elevated SNS activity [224]. Chronic hyperreactivity of the SNS, at least partially explains the striking association in ~80% of patients with a chronic or severe life stressor prior to onset of clinical disease. However, it is unclear whether chronically high SNS activity induces the faulty and/or non-canonical β₂-AR signaling, or if faulty β₂-AR signaling is responsible for severe or chronic stress-triggered onset of autoimmune disease. Furthermore, autonomic dysregulation reported in RA, juvenile chronic arthritis, and arthritic animals (reviewed in [310]) supports elevated sympathetic nerve activity. While receptor functions in lymph node and spleen cells are not available, the finding of altered β₂-AR function in PBMCs of RA patients and AA rats are consistent with an increase in SNS activity. β₂-AR numbers are reduced and their functional responses blunted in PBMCs collected from RA patients compared with normal age-matched controls [37,38,40,99]. Diminished expression and activity of GRKs in PBMCs also occur in RA patients [39] and in animal models of RA [14]. In RA patients, GRK-2 and GRK-6 levels in PBMCs are reduced, whereas, GRK-5 levels are not altered. Given that the receptor-ligand concentration and GRKs regulate β-AR functions, these findings indicate that sympathetic signaling in immune cells is dysregulated in RA and animal models of RA in a manner that contribute to disease pathology. Therefore, chronically elevated sympathetic tone in patients with RA [33,101,311,312] may contribute to conditions that lead to a shift from GRK2- to GRK5-mediated phosphorylation of β₂-ARs that result in β₂-AR-induced ERK 1/2 signaling and the promotion of arthritogenic CD4⁺ Th cells.

Summary and Conclusion

Mechanisms that cause autoimmune disease are complex, and defects in both the immune system and SNS are involved. Tolerance is often broken many years before the onset of disease occurs, but the mechanisms that conspire to break tolerance and trigger disease remain enigmatic. Psychological stressors acting through defective neural and endocrine pathways are implicated; these pathways critically regulate and maintain immune system homeostasis. Therefore, the nervous system is a main player in directing immune mechanisms in the disease process. Convincing evidence indicates an imbalance in the differentiation of specific CD4⁺ Th cells subsets as a key mechanism for breaking tolerance and disease onset. Differentiation of naïve CD4⁺ Th cells and their clonal expansion and effector functions are regulated by the SNS. Norepinephrine released from activated sympathetic nerves signal via β₂-ARs expressed in all CD4⁺ Th cell subsets. The induced rise in intracellular cAMP concentrations that result from β₂-AR stimulation directs cellular activities required for Th cell subset differentiation. Typically, β₂-AR-cAMP-PKA signaling inhibits cell-mediated immunity and promotes humoral immunity and tolerance in healthy individuals.

Still, we have many gaps in understanding SNS regulation, particularly Th17 and Treg cell development/differentiation and effector functioning, as they are relatively newly discovered T cell subsets. Moreover, there is no information regarding SNS regulation of post-differentiational plasticity in Th17 and Treg cells that further convert these populations into unique phenotypes in RA and other autoimmune diseases. Our research indicates SNS hyperactivity in immune organs and β₂-AR signal switching in Th cells conspire to promote development and differentiation of autoreactive Th cells in inflammatory arthritis.

Interestingly, the pathophysiological mechanisms are uniquely secondary immune organ-dependent. In the spleen, β₂-AR neurotransmission in lymphocytes is dramatically suppressed by receptor down-regulation and desensitization due to high sympathetic nerve activity. This indicates a loss of a negative feedback loop that is

required to maintain and/or restore Th cell balance in the spleen, with the likely consequence of Th cell subset differentiation into arthritis-inducing T cells. In contrast, in the DLNs, our data supports a shift in lymphocyte β_2-AR signaling towards signaling pathways expected to promote the generation of arthritis-inducing CD4$^+$ Th cells. The mechanisms underlying cellular adaptation in β_2-AR signal switching are not understood. Our results linking chronically heightened sympathetic nerve activity with faulty and/or non-canonical signal transduction in immune cells merits further investigation as a mechanism for autoimmunity. Additionally, altered β_2-AR signaling in immune cells may involve PRRs, such as TLRs. Mycobacterial components of disease-inducing adjuvant activate subclasses of these receptors, and research by other investigators report that β_2-AR ligands can affect TLR signal transduction. Collectively, these data are intriguing, and underscore the importance of further research that directly tests their role in the pathophysiology of RA.

The functional significance of β_2-AR signal switching in immune cells is its downstream derailment from immune inhibition of inflammation and cellular immunity, and redirection to β_2-arrestin-MAPK-mediated inflammation and cell-mediated immunity that drive disease. Such a shift in signaling prevents the innate function of the SNS – restoring and maintaining immune system homeostasis. Moreover, β_2-AR signal switching in immune cells may explain many of the controversial findings whereby β_2-AR agonists are reported to both inhibited and enhance specific types of immune functions. In these cases, more attention should be directed to both the context and the timing of the immune response under study.

Acknowledgment

Conflicts of Interest

The authors declare no conflicts of interest.

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