REVIEW

Medical Treatment Can Unintentionally Alter the Regulatory T-Cell Compartment in Patients with Widespread Pathophysiologic Conditions

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Regulatory T cells (Tregs) are non-redundant mediators of immune tolerance that are critical to prevent autoimmune disease and promote an anti-inflammatory tissue environment. Many individuals experience chronic diseases and physiologic changes associated with aging requiring long-term medication. Unfortunately, adverse effects accompany every pharmacologic intervention and may affect overall outcomes. We focus on medications typically prescribed during the treatment of prevalent chronic diseases and disorders, including cardiovascular disease, autoimmune disease, and menopausal symptoms, that affect >200 million individuals in the United States. Increasing studies continue to report that treatment of patients with estrogen, metformin, statins, vitamin D, and tumor necrosis factor blockers are unintentionally modulating the Treg compartment. Effects of these medications likely comprise direct and/or indirect interaction with Tregs via other immune and parenchymal populations. Differing and sometimes opposing effects on the Treg compartment have been observed using the same medication. The length of treatment, dosing regimen and stage of disease, patient age, ethnicity, and sex may account for such findings and determine the specific signaling pathways affected by the medication. Enhancing the Treg compartment can skew the patient's immune system toward an anti-inflammatory phenotype and therefore could provide unanticipated benefit. Currently, multiple medicines prescribed to large numbers of patients influence the Treg compartment; however, how such effects affect their disease outcome and long-term health remains unclear. (*Am J Pathol* 2020, 190: 2000–2012; <https://doi.org/10.1016/j.ajpath.2020.07.012>)

Currently, a number of medications, including estrogen, metformin, statins, vitamin D, and tumor necrosis factor (TNF) inhibitors, are being prescribed to millions of individuals for extended periods because they provide clinical benefits and improve quality of life. According to the Centers for Disease Control and Prevention, “Chronic diseases are defined broadly as conditions that last one year or more and require ongoing medical attention or limit activities of daily living or both. Chronic diseases including heart disease, cancer, and diabetes are the leading causes of death and disability in the United States” (CDC, www.cdc.gov/chronicdisease/about/index.htm, last accessed May 27, 2020). Such diseases are also major drivers of the nation's approximately \$3.5 trillion expenditures in annual health care costs. Modifiable behaviors, such as poor nutrition

(including diets low in fruits and vegetables and high in sodium and saturated fats), minimal or absent physical activity, excessive alcohol consumption, and tobacco use, contribute to many chronic diseases and conditions, including cardiovascular disease, diabetes, and vitamin D deficiency (CDC, www.cdc.gov/chronicdisease/about/index.htm, last accessed May 27, 2020).

Regulatory T cells (Tregs) are a unique subset of lymphoid cells responsible for controlling responses by the

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adaptive immune system and maintaining immunologic self-tolerance. In the human immune system, these cells are defined by a consensus phenotype, including expression of CD4, CD25, and CD127^{lo} on the cell surface together with expression of the nuclear transcription factor FOXP3⁺—the hallmark of mouse Treg cells.^{1,2} The cytokine IL-2 is essential for the survival and proliferation of this T-cell subset because the absence of IL-2 receptor signaling results in a loss of Tregs and onset of autoimmune symptoms.³ Experimental studies in mice found that the absence of this population after knockout of the *FOXP3* gene resulted in rapid onset in 100% of animals of autoimmune pathology.⁴ Such findings correlate with the clinical disorder immunodysregulation polyendocrinopathy enteropathy X-linked, which occurs in individuals with mutated *FOXP3* genes.⁵ Notably, a change in numbers and/or function have been reported in several autoimmune and inflammatory disorders, including type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and graft-versus-host disease (GVHD).^{6,7} Accordingly, experimental studies and clinical trials are under way, involving cell therapy and *in vivo* manipulation of Tregs in attempts to ameliorate these diseases. Early studies have provided promising results in T1D and GVHD, encouraging continued research to better understand.^{8,9}

Within the past decade, there has been significant expansion in development and implementation of pharmaceutical compounds to treat patients, which is advancing the ability of the medical community to ameliorate disease. As more effective compounds have been identified together with increasing medical knowledge, more successful treatment of many conditions has occurred; hence, patients with chronic disease are living longer. One consequence of this increased lifespan is that individuals will be prescribed many medications and therefore become exposed to multiple drugs for extended periods. Unfortunately, unintended and often adverse effects accompany virtually every pharmacologic intervention and may have a great effect on overall outcomes. In this context, one study in 2013 reported that >600 US Food and Drug Administration (FDA)—approved compounds used for treating inflammation, hypertension, heart disease, and infections were systematically evaluated and approximately 180 increased Treg counts.¹⁰ This review focuses on a number of medications typically prescribed during the treatment of prevalent chronic diseases and physiologic conditions that affect >200 million persons in the United States. Estrogen, metformin, statins, vitamin D, and TNF inhibitors (Table 1^{11–32}) were selectively chosen because i) they are widely used, ii) each is administered for an extended period, and iii) there are data in the literature documenting their unintentional effect on the Treg compartment. Notably, these medications are not being prescribed with the intent to ameliorate the condition via manipulation of the patient's Treg compartment. Other drugs that do not meet these criteria, such as cytotoxic and chemotherapeutic agents, will not be discussed in this review.

Estrogen and Progesterone

In addition to their role as natural hormones, estrogens are used as medications, for example, in menopausal hormone therapy (MHT) and hormonal birth control.¹¹ Estrogen therapy has been used alone or in combination with progesterone as the most effective treatment for menopausal symptoms, including night sweats, hot flashes, and vasomotor symptoms. MHT with estrogen also may have positive effects on preventing osteoporosis or cardiovascular disease, particularly for women with early-onset primary ovarian insufficiency. Importantly, the MHT for women with cardiovascular or breast cancer risks varies, depending on risk and benefit considerations.¹¹ Regarding birth control, two types of medications available: pills that contain estrogen and progestin and progestin-only pills. Overall, the estimate of the number of women in the United States using MHT and hormonal birth control is approximately 15 million (CDC, <https://www.cdc.gov/nchs/products/databriefs/db327.htm>, last accessed May 29, 2020; National Heart, Lung, and Blood Institute, https://www.nhlbi.nih.gov/files/docs/pht_facts.pdf, last accessed May 29, 2020).

As well as regulating reproductive functions, estrogen can also affect the development and activation of immune responses and together can modify bone metabolism.^{33,34} In the context of the present review, it is well established that this hormone can alter the Treg compartment. Under physiologic conditions during the menstrual cycle of fertile nonpregnant women, an increase of CD4⁺CD25⁺FOXP3⁺ Treg levels in peripheral blood was observed in the late follicular phase. This expansion was associated with high serum levels of estradiol and was followed by a significant diminution of Tregs in the luteal phase.¹² In contrast, in women with recurrent spontaneous abortions or after menopause, lower Treg counts and decreases in their suppressive function were detected at both the follicular and the luteal phases compared with fertile women.¹² In postmenopausal women, the occurrence of osteoporosis has been associated with estrogen deficiency, which leads to osteoclast-mediated bone resorption.³⁵ The protective role of estrogen in bone metabolism has been proposed to be by regulating Treg function on osteoclast differentiation and bone resorption. Supporting this hypothesis, *ex vivo* treatment of human Tregs with physiologic levels of estradiol resulted in an increase in Treg frequency, activation, and secretion of IL-10 and transforming growth factor (TGF)- β .^{36,37} This latter effect also promoted an increase in Treg-mediated suppressive effects on osteoclast differentiation, preventing greater bone resorption.³⁷

The molecular mechanism by which estrogen expands Tregs was studied in primary human Tregs from peripheral blood of healthy males and cervical cancers from patients.¹³ Inhibition of estrogen receptor (ER) α with a specific antagonist impaired the tumor-infiltrating Tregs by blocking FOXP3 expression, thereby inhibiting their function. Using co-immunoprecipitation, the authors found that estradiol

Table 1 Summary of Medications Discussed in This Review Article That Can Affect the Treg Compartment in Patients

Medication	Disease or condition	Mechanism of action	Major biological effect	Changes in Treg compartment	References
Estrogen	Menopause, premature ovarian failure, or birth control	Targeting ERs (ERα/ERβ)	Reduces menopausal symptoms (eg, night sweats and hot flashes). regulate menstrual cycle	Frequency, numbers, phenotype, and function	11–13
Metformin	Type 2 diabetes	Inhibits mitochondrial respiratory chain complex I and activates AMPK	Decreases hepatic glucose production, lowering blood glucose levels	Frequency and numbers	14–17
Statins	Cardiovascular disease, dyslipidemia	Inhibits HMG-CoA reductase	Reduces cholesterol	Frequency, numbers, migration, phenotype, and function	18–21
Vitamin D	Bone disease (osteoporosis, osteomalacia, skeletal fractures)	Targets vitamin D receptor	Regulates calcium, phosphorus, and bone metabolism	Frequency, migration, phenotype, and function	22–27
TNF inhibitors	Autoimmune disease	Blocks TNF-α cytokine	Reduces inflammation	Frequency, phenotype, and function	28–32

AMPK, AMP-activated protein kinase; ER, estrogen receptor; TNF, tumor necrosis factor; Treg, regulatory T cell.

drives FOXP3 expression and promotes function in the cancer-derived Tregs by direct interaction between the estradiol:ERα complexes with the *FOXP3* promotor.¹³ Furthermore, chromatin immunoprecipitation analyses of male blood Tregs revealed that ERα binds to four different sites along the *FOXP3* locus, including regions upstream and within the core of the promotor and conserved non-coding DNA elements 2 and 3. Computational analyses of enriched regions found eight putative estrogen response elements and predicted the forming of a loop that can trigger the *FOXP3* promotor^{13,38} (Figure 1).

Preclinical studies in estradiol-treated and pregnant mice have also found that estrogen drives Treg expansion and enhances their suppressive function by signaling through ERα.³⁹ Of note, CD4⁺ T cells express significantly higher levels of ERα compared with ERβ.⁴⁰ Estradiol treatment protected mice from experimental autoimmune encephalitis, a mouse model of human MS, by increasing *Foxp3* expression within the CD4 compartment.³⁹ Furthermore, treatment with this hormone increased the intracellular levels of checkpoint inhibitor programmed death (PD)-1 in FOXP3⁺ Tregs,⁴¹ which may contribute to their functionality. Accordingly, in ER knockout mice, PD-1 expression and Treg suppressive function were diminished.⁴¹ Moreover, failure of Treg function in patients with MS was attributed to further reduced expression of ERβ, which might contribute to the pathogenesis of this disease.⁴² Most studies reported that estrogen alleviates the symptoms of autoimmune and inflammatory diseases. However, in IBD the role of estrogen and ER is unclear. In a mouse model of IBD, ERα deficiency in T cells reduced the pathologic findings by lowering Th1 and Th17 responses and favoring *Foxp3* expression and consequently induced Treg (iTreg) differentiation.⁴³ In humans, oral contraceptive use was associated with the development of IBD,⁴⁴ although in a more current

study, MHT protected women from IBD.⁴⁵ These findings indicate that in patients with IBD, estrogen can mediate immunostimulatory or immunosuppressive effects, depending on cell type examined and context of disease. Therefore, the significance of estrogen regarding Tregs in IBD remains to be determined.

Progesterone effects on Tregs have been less studied. For instance, this hormone promoted *in vitro* differentiation of naive cord blood fetal T cells, but not peripheral blood adult T cells, into iTregs. Mechanistically, progesterone increased IL-2/STAT5 signaling while repressing IL-6–mediated STAT3 activation, facilitating T-cell differentiation into Tregs over Th17 cells.⁴⁶ Preclinical studies revealed that progesterone expanded systemic and local uterine CD4⁺CD25⁺ Tregs and enhanced their suppressive activity during midterm pregnancy in mice.⁴⁷ Although fewer data have been reported, androgens significantly augment Treg levels both *in vivo* and *in vitro*. A functional androgen response element was identified within the FOXP3 locus, and an androgen-dependent increase of FOXP3 expression was found in human T cells from women but not from men, indicating sex-specific androgen signaling.⁴⁸

In total, estrogen and, to a more limited extent, progesterone appear to be drivers of Tregs, indicating that woman receiving MHT and taking birth control medications are likely to experience long-term changes in this lymphoid subset. Nonetheless, it is currently unknown whether these changes will result in a decrease in autoimmune disease or other clinical effects.

Metformin

Type 2 diabetes (T2D) is characterized by insulin resistance, which impairs cellular uptake of glucose and is accompanied

Figure 1 Schematic model for estrogen receptor (ER) and vitamin D receptor (VDR) signaling in regulatory T cells (Tregs) and CD4 T cells. Estrogen (**left panel**) binds to the intracellular ER, which dimerizes (homo/heterodimers), undergoes translocation to the nucleus, and then interacts with estrogen response elements (EREs), inducing the expression of several genes, including *FOXP3*, *PD1* (*PDCD1*), *IL10*, and *TGFB*. $1\alpha,25$ -dihydroxyvitamin D₃ [$1\alpha,25(\text{OH})_2\text{D}_3$] (**right panel**) binds to the VDR, interacts with the retinoid X receptor (RXR), forming a heterodimer that engages vitamin D responsive elements (VDREs) and upregulating the expression of *FOXP3*, *CTLA4*, *CD25* (*IL2RA*), *PD1*, *Helios* (*IKZF2*), and other genes.

by hyperglycemia. Chronic hyperglycemia leads to damage of blood vessels that ultimately can affect multiple organs, leading to serious health consequences. According to the CDC, approximately 30 million individuals in the United States have T2D, in part related to the obesity epidemic, making T2D one of the most common diseases that primarily affects older individuals.

Persistent, low-grade, obesity-induced inflammation is thought to be a major mechanism that contributes to insulin resistance and T2D.⁴⁹ The accumulation of fat enhances a chemotactic gradient to attract monocytes and lymphocytes. Key proinflammatory cells that dominate adipose tissue from obese individuals include macrophages, Th1 and Th17 CD4⁺ T cells, CD8⁺ cytotoxic lymphocytes, and natural killer cells. These cells produce TNF- α , IL-6, C-reactive protein, interferon (IFN)- γ , and IL-17, among other cytokines, to contribute to adipose tissue inflammation. Brown adipose tissue in lean mice contains a high proportion of a specialized subset of Tregs that depends on peroxisome proliferator-activated receptor- γ for their development and proliferate to adipose-derived IL-33.^{50,51} These immunosuppressive Tregs dominate the CD4⁺ T-cell compartment in lean mice, whereas they are at lower proportions in fat from obese mice. Indeed, fat in lean mice might have a higher ratio of Tregs:Th1 cells, and immunotherapy that increases Tregs in the fat limits insulin resistance and metabolic disorders in a mouse model of T2D.^{52,53} Collectively, these data suggest that Tregs within visceral fat are beneficial to limit inflammatory responses that promote insulin resistance and T2D.

Metformin is an extensively used drug in patients with T2D and exhibits pleiotropic effects that help to lower blood glucose levels.¹⁴ Metformin is readily taken up by hepatocytes through Oct1 to concentrate it in the mitochondria to inhibit complex I of the respiratory chain, which lowers ATP production and increases the ratio of ADP:ATP and AMP:ATP. These changes act to activate AMP-activated

protein kinase (AMPK), and all act in concert to antagonize other mitochondrial-associated enzymes to lower production of glucose and lipids, while increasing insulin sensitivity of the hepatocytes. A relatively high concentration of metformin is found in the intestine and acts to increase glucose metabolism and thus lowers glucose uptake. This drug also supports a shift in the somewhat distinctive gastrointestinal microbiome in T2D toward a microbiome more commonly associated with healthy individuals. In addition, metformin increases glucose uptake and insulin sensitivity in muscle cells.

In limited studies, the peripheral blood mononuclear cells of patients with T2D produced a modest decrease in Tregs, with somewhat lower suppressive activity.⁵⁴ Human visceral adipose tissue in morbid obesity and lean adipose tissue from healthy individuals had low numbers of Tregs.⁵⁵ Another important issue, therefore, is the extent that metformin affects Tregs because these cells are generally important to maintain self-tolerance and, more specifically, have been implicated in limiting inflammation associated with T2D, as discussed above. *In vitro* studies found that metformin inhibits proliferation of purified T lymphocytes in part by affecting T-cell receptor signaling, suggesting a potential to inhibit T-effector cells.^{56,57} In mouse models of experimental autoimmune arthritis, IBD, experimental autoimmune encephalomyelitis, and systemic lupus erythematosus, metformin lowers the instances of autoimmunity by decreasing Th17 cells while increasing Tregs.^{15,58,59} Metformin acts to inhibit STAT3 while increasing AMPK, which in turn, inhibits the mammalian target of rapamycin (mTOR)-1 pathway; these molecular consequences help to explain the shift in the Th17:Treg balance to one that is more regulatory (Figure 2^{60,61}). In IBD and experimental autoimmune encephalomyelitis, metformin is associated with decreased mRNA levels for TNF- α (TNF), IL-1 β (IL1B), IL-6 (IL6), IL-8 (CXCL8), vascular endothelial growth factor (VEGFA), IL-17

Figure 2 Metabolic reprogramming of T cells, leading to greater regulatory versus effector phenotype: schematic model for metformin and statin involvement. Regulation of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway by AMP-activated protein kinase (AMPK) after metformin administration promotes fatty acid metabolism, which is the preferred energy pathway used by regulatory T cells (Tregs) but not naive or T effector cells. One study reported that human Treg cells from patients taking statins exhibited decreased phosphorylation of mTOR⁶⁰ and a preclinical investigation noted that simvastatin demethylates the *Foxp3* promoter locus in mice.⁶¹ Th, T helper cells.

(IL17A), and retinoid-related orphan receptor γ (RORC) but increased mRNA level for FOXP3 as well as increased Treg counts,^{15,59,62} consistent with lower inflammation. In the Roquin^{san/san} mouse model of systemic lupus erythematosus, these effects of metformin on STAT3, AMPK, and mTOR1 also inhibit autoantibody production by inhibiting the development of plasma cells.¹⁶ Metformin-dependent activation of AMPK inhibition of glycolysis and glutamine metabolism also favor metabolic reprogramming of T lymphocytes to favor Tregs.^{17,63} For obese patients with MS, treatment with metformin leads to a significant reduction of the number of new brain lesions and is accompanied by increased Treg numbers in peripheral blood mononuclear cells.⁶⁴ Collectively, these studies, which have been largely performed in mice, suggest that another favorable aspect of the use of metformin in patients with T2D is its ability to directly enhance immunosuppressive Tregs while limiting T-effector cells, particularly Th17 cells (Figure 2). Indeed, T cell–derived IL-17 is another inflammatory cytokine linked to obesity and T2D.⁶⁵ On the basis of these findings, speculation is that metformin may limit obesity-associated inflammation in part through enhancing Treg levels. However, more direct evaluation of patients with T2D will be required to confirm this hypothesis.

Statins

Statins are widely used to reduce the risk of cardiovascular diseases and stroke, and it is estimated that approximately 30% of people >40 years of age and approximately 35 million people in the United States are taking a statin (<https://www.drugs.com/article/statins-benefits-and-risks.html>, last accessed May 29, 2020). The benefit is a result of their capacity to inhibit the synthesis of cholesterol. This process occurs when statins block HMG-CoA reductase, a key regulatory enzyme of cholesterol biosynthesis.¹⁸ By inhibiting HMG-CoA reductase, statins diminish the hepatocyte cholesterol content together with an increase in LDL receptor expression, which results in increased clearance of LDL-C from the bloodstream and subsequent reduction in circulating LDL-C. In addition, statins have

non–lipid-related pleiotropic activity, such as improving endothelial cell function, reducing risk of dementia, stabilizing atherosclerotic plaques, diminishing proliferation, and having anti-inflammatory, immunomodulatory, and antithrombotic properties.⁶⁶ These cholesterol-independent effects of statins have been attributed to their capacity to inhibit the production of nonsteroidal isoprenoid compounds (eg, farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate), which are intermediate metabolites in the cholesterol synthesis pathway.^{67,68}

Although all statins reduce serum LDL in a dose-dependent manner and share a common mechanism of action, they differ in their chemical structure, pharmacokinetic profiles, and efficacy.⁶⁹ Currently, seven statins are on the market, including atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, simvastatin, and pitavastatin and are used as single agents or as combination products. The most prevalent and well-documented adverse effect of statins is the development of muscle symptoms, which can present as myopathy, myalgia, myositis (with elevated creatine kinase), and the most severe, fatal or nonfatal rhabdomyolysis. Other adverse effects include neurologic, hepatic, and renal toxic effects. In addition, statin treatment was associated with new-onset T2D, and this adverse effect appears to be more common in patients with preexisting risk factors (eg, increased body mass index, impaired fasting glucose, or elevated glycated hemoglobin level).⁷⁰

Beyond the cholesterol-reducing effect, statins also have additional beneficial effects as a result of their regulation of the innate and adaptive immune systems.⁷¹ As immune modulating agents, statins reduce proinflammatory cytokines, inhibit antigen-presenting cell maturation and function, diminish the adhesion and activation of monocytes, and impair T-cell activation by inhibiting the induction of major histocompatibility complex 2 expression by IFN- γ .^{72,73} Notably, among the CD4⁺ T-cell subsets, several studies have recently indicated that treatment with statins may increase Treg numbers, migration, differentiation, and/or suppressive function.¹⁹ For, example, in one study, patients (38 to 75 years old) with acute coronary syndrome who were treated with low-dose atorvastatin (20 mg/d) for 4 weeks had an augmented frequency of Tregs in peripheral

Table 2 FDA-Approved Medications That Target TNF- α

TNF- α inhibitor medications	Initial approval	Compound	Target
Etanercept* (Enbrel)	1998 for rheumatoid arthritis	Fusion protein: human TNFR2-IgG	TNF- α
Infliximab* (Remicade)	1998 for Crohn disease	Chimeric mouse-human IgG1 κ -mAb	TNF- α
Adalimumab* (Humira)	2002 for rheumatoid arthritis	Human mAb	TNF- α
Certolizumab (Cimzia)	2008 for Crohn disease	PEGylated Fab fragment of a humanized mAb	TNF- α
Golimumab (Simponi)	2009 for rheumatoid arthritis	Fully human IgG1 κ -mAb	TNF- α

*Binds to soluble and transmembrane TNF- α .¹¹²

Fab, antigen-binding fragment; FDA, US Food and Drug Administration; mAb, monoclonal antibody; PEG, polyethylene glycol; TNF, tumor necrosis factor; TNFR2, tumor necrosis factor receptor 2.

blood (approximately two times), and functionally, their *in vitro* suppressive capacity was increased when compared with the same patient group before treatment.²⁰ In a prospective randomized study, administration of a high dose of rosuvastatin before the procedure in patients with acute coronary syndrome receiving percutaneous coronary intervention also found increased Treg levels.⁷⁴ Studies in Asia recently reported that acute coronary syndrome is characterized by increased effector cells and decreased Tregs and that statins increase the frequency of CD4⁺CD25⁺ T cells, although FOXP3 expression was not examined.⁷⁵

In a setting of acute myocardial infarction, patients were evaluated and found that atorvastatin treatment (80 mg) resulted in increased Treg frequency in peripheral blood and infarct-related coronary arteries accompanied by higher FOXP3 and TGF- β (TGFB) mRNA levels compared with the control group.²¹ Moreover, in a retrospective study, patients with hypercholesterolemia treated with simvastatin (20 mg) or pravastatin (10 to 40 mg) for 4 or 8 weeks, respectively, exhibited increased frequency of CD4⁺CD25^{high} cells with upregulation of FOXP3 expression in the peripheral blood.⁷⁶ It has been proposed that increased levels of Tregs in atherosclerotic lesions suppress Th1 and Th2 effector responses, reducing their contribution to the pathogenesis of atherosclerosis.⁷⁷ Similar effects of statins on Treg phenotype and levels have been reported in several human *ex vivo* and mouse *in vivo*/*in vitro* studies, supporting the notion that statins are positive regulators of this cell population.^{76,78} In addition to enhancing Treg levels, statins can also affect Treg cell migration. In a murine model of skin delayed-type hypersensitivity response to *Candida albicans* treated with lovastatin, upregulation of CCL1 occurred, and subsequent Treg recruitment to the inflamed tissues was detected.⁷⁹ Dosage may be important because it was reported that higher concentrations of atorvastatin (10 μ M) inhibited Treg *in vitro* suppressive function and altered Treg phenotype by reducing the expression of Foxp3, the key regulatory molecule cytotoxic T-lymphocyte-associated protein (CTLA)-4, and PD-1.⁸⁰ Lastly, one study found that simvastatin treatment promoted generation of mouse Foxp3-expressing iTregs *in vitro* by inducing demethylation of the mouse *Foxp3* promotor and by releasing and potentiating the TGF- β signaling pathway (Figure 2).⁶¹

In addition to statin treatment for cardiovascular diseases, the effect of these compounds on Treg populations has also

been reported in patients with cancer and autoimmune disease as well as in healthy individuals. Briefly, in patients with colorectal cancer, atorvastatin treatment ($n = 25$; 10, 20, or 40 mg) led to an increase in FOXP3⁺ Treg infiltration with no differences in TGF- β expression within tumor tissues.⁸¹ Patients with RA treated with atorvastatin (20 mg/d) for 12 weeks exhibited increased Treg counts and restored suppressive function.⁶⁰ The molecular mechanism by which atorvastatin promotes the generation of human Tregs was attributed to the specific inhibition of phosphatidylinositol 3-kinase–Akt–mTOR and ERK signaling pathways (Figure 2).⁶⁰ In healthy individuals (18 men without cholesterolemia), administration of lovastatin or atorvastatin for 45 days resulted in increased Treg frequency and FOXP3 mRNA levels by day 30. However, at day 45, Treg counts returned to baseline, although FOXP3 and TGF- β mRNA expression remained high. This inflammation-independent study found a positive correlation between Treg levels and HDL-C, suggesting a role of the latter in Treg homeostasis.⁸² In total, studies with statins have found that the Treg compartment in patients undergoes enhancement as assessed by multiple criteria. It appears that these changes are associated with reduced pathogenesis in cardiovascular and other diseases.

Vitamin D

The human daily requirement of vitamin D is obtained primarily from exposure to sunlight and from diet. High-energy solar UVB photons penetrate the skin, causing the release of vitamin D₃, which undergoes two consecutive hydroxylations to become 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], the active vitamin D metabolite.⁸³ The first hydroxylation [ie, 25(OH)D₃] occurs primarily in the liver and the second, to 1 α ,25(OH)₂D₃, primarily in the kidney but also in immune cells.⁸⁴ Vitamin D₃ photoproduction is influenced by latitude of residence, season, and skin color.⁸⁵ Vitamin D₃ is required to maintain skeletal and mineral homeostasis and thereby prevents bone disease. Hence, insufficient levels of this hormone exacerbate osteoporosis, augment the risk of skeletal fractures, and cause osteomalacia.²² Notably, vitamin D deficiency has also been associated with increased risks of a broad range of chronic

inflammatory and autoimmune illnesses, including common cancers, cardiovascular disease, Crohn disease, T1D, MS, and RA.^{86,87} The most reliable marker of vitamin D status is the measure of 25(OH)D₃ in serum, which is the main circulating metabolite of vitamin D. In recent years, several reports have proposed levels of vitamin D considered to be deficient, and accordingly, recommendations to supplement deficient states have been prescribed.⁸⁷ It has been reported by the Endocrine Society in the United Kingdom and supported by the Institute of Medicine in the United States that individuals with <20 ng/mL (50 nmol/L) of 25(OH)D₃ are identified as vitamin D deficient.⁸⁷ However, controversy remains, and unanimity within the field is lacking regarding reference values defined by the Institute of Medicine.⁸⁸ Readers should refer to several articles to further clarify the issues involved.^{88–90}

1 α ,25(OH)₂D₃ exerts its functions by binding to the vitamin D receptor (VDR), triggering genomic and nongenomic responses that are mediated by nuclear VDR and caveolae-associated VDR of the plasma membrane, respectively.⁹¹ VDR is a DNA-binding transcription factor, and after binding to 1 α ,25(OH)₂D₃, it interacts with the retinoid X receptor, forming an active signal transduction complex that binds to vitamin D response elements (VDREs), inducing the transactivation or repression of up to 500 primary target genes per tissue.^{91,92} Through this process, vitamin D leads, in addition to established effects on mineral and bone metabolism, to upregulation of *FOXP3*, *CTLA-4*, *Helios* (*IKZF2*), suppression of IL-17, and consequently to Treg stimulation and Th1/Th17 suppression (Figure 1).^{23,91–94}

The effects of vitamin D on the innate and adaptive immune system are mediated by the expression of the VDR in many immune cells, including T and B lymphocytes, macrophages, dendritic cells, and natural killer cells.^{24,95,96} The effects of VDR agonists on dendritic cells reduce the expression of costimulatory molecules (CD40, CD80, and CD86) and major histocompatibility complex 2 and enhance secretion of IL-10, leading to induction of Tregs.^{97,98} VDR expression exhibited a maturation stage-specific pattern with greatest expression on human naive and memory T cells.²⁵ We are not aware of a research study demonstrating the expression of VDR on Tregs, so it remains controversial whether the actions of vitamin D₃ on this cell population are via a direct and/or indirect manner. Regardless, accumulating evidence supports the notion that vitamin D could prevent autoimmune diseases, allograft rejection, and GVHD by expanding Tregs and inhibiting Th17 cells.^{26,99} A positive correlation was identified between vitamin D status and CD4⁺FOXP3⁺ Tregs in the serum and the airways of patients with moderate to severe asthma.¹⁰⁰ Moreover, four of five trials in patients with autoimmune diseases, including T1D, MS, and Addison disease, reported vitamin D₃ supplementation (dose range: 2000, 4000, 7000, or 14,000 IU/d for >3 months) increased circulating Treg levels and suppressive function.¹⁰¹ In patients with ulcerative colitis, *ex vivo* treatment of T cells with VDR agonists augmented the expression of this receptor in Th2 cells and further promoted

their conversion to Tregs (iTregs), upregulating TGF- β mRNA levels.¹⁰² In mouse models for autoimmune diseases, such as RA and T1D, vitamin D₃ was also observed to prevent disease by elevating Treg levels.^{103,104} In healthy individuals, administration of a high dose of vitamin D₃ (140,000 IU per month for 3 months) enhanced Treg frequency accompanied by a significant correlation with increasing 25(OH)D₃ serum levels.^{105,106} When activated human T cells from healthy individuals in culture are examined, treatment with 1 α ,25(OH)₂D₃ promoted iTreg development with significant upregulation of *CTLA-4* and *FOXP3* expression; the latter required IL-2 in the culture.⁹⁴ Furthermore, 1 α ,25(OH)₂D₃ enhanced the selective expansion of FOXP3⁺Tregs over conventional T cells (FOXP3⁻) *in vitro*, and the former population had increased key Treg molecules (ie, *CTLA-4*, *PD-1*, and *CD25*), suppressive function, and a migratory signature specific for homing to sites of inflammation.^{107,108} Importantly, these results were also detected using *ex vivo* T cells from patients with autoimmune diseases, including T1D and MS.^{27,109}

Although further research is needed to clarify the potential benefits of vitamin D supplementation, its long-term use may influence the Treg compartment indirectly through antigen-presenting cells and/or directly via VDR signals in CD4⁺FOXP3⁺ T cells. Accordingly, VDREs were found in a conserved noncoding sequence region of the human *FOXP3* gene, and in response to 1 α ,25(OH)₂D₃, such VDREs augmented the *FOXP3* promotor activity. Therefore, through direct VDR binding to the *FOXP3* gene, 1 α ,25(OH)₂D₃ may affect human immune responses by regulating *FOXP3* expression.¹¹⁰ Although many individuals take vitamin D to support bone health, if elevation of Treg levels occurs in this population, it could result in an off-target benefit by preventing or diminishing inflammatory associated disease or alternatively could have a negative effect on cancer development/progression.

TNF Inhibitor Family: Modulation of the Immune System to Control Inflammation and Unintentional Regulation of the Patient's Treg Compartment

TNF antagonists are medications that are not being prescribed with the intent to augment the patient's Treg compartment to treat inflammatory diseases. These inhibitors, which block signaling via TNF receptors (TNFRs) 1 and 2, are currently being used to treat millions of patients for such diseases. TNFR2 reduces the severity of arthritis in preclinical animal studies, and it is important in the maintenance of Treg stability and function.¹¹¹ Since 1998, five anti-TNF- α compounds have received FDA approval (Table 2¹¹²) and are clinically available to treat an increasing number of autoimmune inflammatory disorders. These drugs, which include monoclonal antibodies and a fusion protein, effectively impede TNFR signaling via binding to the TNF- α ligand. An estimated 7% to 10% of

the world's population is affected by autoimmune diseases.¹¹³ Chronic inflammation and many disease symptoms in RA, ulcerative colitis, Crohn disease, psoriasis, psoriatic arthritis, ankylosing spondylitis, and juvenile idiopathic arthritis have been diminished in patients after biologic TNF- α inhibitors have been used for treatment.^{7,28,114} Currently, the underlying cause of these beneficial effects remains to be understood, but it has been assumed to be reduced Th1/Tc1 inflammation by direct inhibition of TNF signaling in these T cells.

TNF- α is the first cytokine to appear in the blood after injury or stress and is typically overproduced in many autoimmune diseases.^{7,28,114} Two TNFRs can bind and signal via nonoverlapping pathways. TNFR1 (p55) and TNFR2 (p75), although structurally related, mediate different biological function after binding of TNF- α .²⁹ Notably, in contrast to TNFR1, which is ubiquitously expressed, TNFR2 is limited to lymphocyte populations. This receptor is reportedly activated more effectively by transmembrane TNF compared with the soluble form.¹¹⁵ TNFR1 contains a death domain and, as the primary signaling receptor on most cell types, is responsible for most of the proinflammatory, cytotoxic, and apoptotic effects typically attributed to TNF.¹¹⁵ In contrast, TNFR2 lacks a death domain but is principally responsible for signals that promote lymphocyte activation and proliferation via NF- κ B, activator protein 1, and the mitogen-activated protein kinase pathways.^{115–117} Interestingly, TNF family members, including TNFR2, expressed on Treg progenitors in the thymus contribute to signals that drive their differentiation.¹¹⁸ Notably, in the context of the present review, TNFR2 can provide co-stimulatory function in mature T cells and augment responses to antigen-driven T-cell receptor signaling.^{30,119} More specifically, TNFR2 is predominantly expressed by a subset of human and mouse CD4⁺CD25⁺FOXP3⁺ Tregs, estimated to be approximately 30% to 40% of activated memory populations, but not naive Tregs, which reportedly lack TNFR2 expression.¹²⁰ TNFR2 expression on Tregs is associated with functional ability, and several experimental studies have found that activation of TNFR2 can augment host Treg cell expansion and protect against acute GVHD.^{30,119,121,122}

Although controlling symptoms and ameliorating disease evidence a pathogenic role for TNF- α in multiple autoimmune and inflammatory disorders, up to 40% of individuals do not respond to anti-TNF treatment, and disease augmentation has also been observed.^{7,123} The explanation for such diverse outcomes likely resides in different signaling pathways associated with various diseases together with how an individual's immune system responds, including which cell types and the biological reagent prescribed. For example, in studies of patients with RA, the TNFR2 fusion protein etanercept did not affect Tregs, whereas patients treated with adalimumab or infliximab exhibited increased numbers and functions by these cells.^{112,124,125} In addition, it is likely that the timing of treatment and period of exposure to the biologicals are

important, as Bystrom et al⁷ reported in RA; although TNF- α can enhance T-cell responses, sustained exposure renders them hyporesponsive, so TNF blockers may have different effects administered early in disease.

Multiple cell populations can be modulated in patients after even brief TNF inhibitor exposure. Some populations of Tregs and Th17 cells derive from similar progenitors, and Tregs can promote differentiation of Th17 cells, whereas Th17 can promote expansion of Tregs.¹²⁶ It may therefore not be surprising that interfering with TNF signaling affects both regulatory and effector T-cell populations. A number of studies support the notion that TNF inhibitor treatment of patients with RA affects Treg numbers and function and that these Treg outcomes are associated with improvement in RA symptoms.^{31,124,127,128} However, in one RA study, patients receiving adalimumab exhibited a decrease in Th1 cells, Th17 cells, and IFN- γ producing CD8⁺ T cells and an increase in the proportion of Tregs, but differences in Treg levels were not found between patients responding or not responding to therapy.¹²⁵ The authors speculated that an increase in IFN- γ production caused by recovery of natural killer cell function, together with expanded Tregs, contributed to decreasing the Th17 response in anti-TNF-treated patients with RA. Thus, in this study, blocking TNF and likely further lowering free TNF levels because of ameliorating disease led to an increased proportion of Tregs. These results may represent compensatory effects that result from control of the patient's immune system by the TNF antagonists.

Before treatment, despite elevated levels of circulating TNF- α , both diminished and no change in Treg levels have been reported in patients with ankylosing spondylitis versus healthy controls.^{32,129} In a fairly large study, patients with ankylosing spondylitis (>200) treated for 6 months with etanercept, adalimumab, or intravenous infliximab resulted in approximately 75% of clinical responders with significantly decreased levels of Th17 and increased levels of circulating Treg cells, whereas nonresponders whose disease was not diminished failed to have these changes.³² Significantly, lower frequencies of Tregs found in both responders and nonresponders compared with healthy controls support the notion that this compartment is important in disease outcome. Treg manipulation, although unintentional in this study, warrants more direct and specific targeting to further improve AS treatment.³² Moreover, it is apparent that elevation of TNF levels in some autoimmune patients may not translate to higher levels of Tregs, a result that is not surprising in that multiple cytokines are dysregulated and thus multiple signals are likely involved in determining the outcome on Treg cell counts and function. Results regarding Tregs have also varied in patients with psoriasis. In one study ($n = 21$), adalimumab decreased Th17, Th1, and other effector cells, and the number of CD4⁺CD25⁺FOXP3⁺ cells increased in the peripheral blood.¹³⁰ The Treg increase was associated with a good clinical response (reduction in the psoriasis area severity index). Nonetheless, some patients had decreased numbers of Tregs, although this decrease was associated with

aggravation of disease.¹³⁰ In a recent study ($n = 32$) of patients severely affected with psoriasis 1 week after a single pulse treatment with adalimumab, Tregs from peripheral blood exhibited decreased anti-CD3 and anti-CD28 *ex vivo* stimulation and diminished cytokine (eg, IL-10) production.¹³¹ The authors also conclude that the number of Treg cells was reduced after pulse therapy; however, it is not known whether such results would have been maintained after continued treatment and longer periods after therapy.¹³¹

Recently, the risk of ulcerative colitis in a Chinese population was proposed to be associated with single-nucleotide polymorphisms in the human *FOXP3* locus, which could be associated with diminished numbers or function.¹³² Studies have found lower levels of Treg cells in the lamina propria in IBD and increased apoptosis of these cells.^{133,134} Infliximab treatment of patients with active IBD diminished Treg apoptosis.¹³⁴ Several studies of patients with IBD also reported increases in peripheral Treg cells after anti-TNF- α treatment. Guidi et al¹³⁵ reported elevation in blood but not the lamina propria of patients ($n = 32$) after several weeks of infliximab, certolizumab, or adalimumab treatment. A recent study with a small number of patients with CD ($n = 8$) reported diminished disease index scores after a single treatment with adalimumab or infliximab treatment, which was associated with increased CD127⁺ Treg levels.¹³⁶

In total, use of TNF- α biological blocking agents in a number of autoimmune and inflammatory disorders clearly via direct and/or indirect signaling affected the patients' Treg cell compartment. Reported effects have been observed after both single and more prolonged treatment protocols, and both increases and decreases in Tregs have been identified. It may be worthwhile to consider that because TNF inhibitors promote demargination, such changes in their trafficking could differentially alter the levels of Tregs in the peripheral blood and tissue compartments. Currently, it remains unclear whether TNF stimulates or inhibits functional Treg suppression. In addition, whether such Treg compartment changes may alter not only the disease extant but also other responses in the patient remains to be understood.

Conclusion

CD4⁺CD25⁺FOXP3⁺CD127^{lo} Treg cells are nonredundant mediators of immune tolerance, critical to prevent autoimmune disease and promote an anti-inflammatory tissue environment. A number of frequently prescribed medications, including estrogen, metformin, statins, vitamin D, and TNF blockers, can unintentionally alter the Treg compartment in patients. Other unidentified drugs may also affect patient Treg cells. For example, aspirin, a broadly used nonsteroidal anti-inflammatory drug to treat pain and inflammation, possesses antithrombotic properties. Low-dose daily aspirin is commonly prescribed in patients who have cardiovascular disease or who have already had a

myocardial infarction or stroke to decrease the risk of recurrence and other blood flow problems.¹³⁷ Preclinical studies have found that aspirin treatment can augment Treg numbers and their development in animal models of inflammatory disease, such as MS; however, nonsteroidal anti-inflammatory drug alterations of Tregs in humans await clinical evaluation.¹³⁸ Clearly, inflammation may underlie and/or complicate nearly each of the chronic conditions discussed in the present review. It is therefore important to note that inflammation can directly and/or indirectly regulate Tregs via affecting infiltrating and parenchymal cell populations. Chemokines, cytokines, prostaglandins, and eicosanoids produced by monocytes and epithelial cells can have a multitude of effects on Tregs, including affecting their stability, trafficking, and function. One pathway through which inflammatory environments could affect Treg cells is via stability of FOXP3 expression after epigenetic modifications (ie, as a consequence of the methylation of the *FOXP3* locus). For example, the proinflammatory cytokine IL-6 contributes to the methylation of this gene via the methyltransferase Dnmt3a, leading to the loss of Foxp3 in inflamed tissues.^{139,140} Within the context of this review, it is interesting that signaling through TNFR2 was recently demonstrated to inhibit DNA methylation at the *FOXP3* promoter, thereby maintaining the expression of this master transcription factor in Tregs.¹¹¹

Varying and sometimes opposing effects on the Treg compartment have been observed using the same medication. The length of treatment, dosing regimen, and patient age, ethnicity, and sex may account for such findings. In addition, the specific disorder, the tissue compartment, and the stage of disease likely affect the differentiated state of the Treg populations and therefore the specific signaling pathways affected by the medication administered. Enhancing the Treg compartment can skew the patient's immune system toward an anti-inflammatory phenotype, which, dependent on the condition being treated, could provide unanticipated benefit. In some circumstances, for example, patients with cancerous or precancerous conditions, such effects might theoretically promote tumor development or progression, but this requires further study. Currently, it is accurate to state that multiple medications prescribed to large numbers of patients influence the Treg compartment; however, it remains to be understood how such effects affect their disease outcome and long-term health.

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