Tregs, the key to new treatment for systemic lupus erythematosus

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Abstract.

Systemic lupus erythematosus (SLE) is an auto-immune disorder that affects connective tissue. It is caused by a break in self tolerance which is caused by hyperactive autoreactive T- and B cells. Properly functioning immune systems are regulated by regulatory T cells (Tregs). The goal of this thesis is explaining why Tregs are important for developing new SLE therapies. This will be done by first describing what is known about normal Treg function and then examining the role of Tregs in SLE. Tregs are a heterogeneous population of leukocytes that are anergic to effector T cell stimuli. They can only be distinguished from the conventional T cell population by comparing the expression levels of several cell surface markers. There are different subsets of Tregs that regulate the immune system via different pathways. The role of Tregs in SLE is controversial; there are different reports as to why Tregs fail to suppress the auto-immune response. This failure is reported to be caused by either a reduction in Treg numbers, a reduction in Treg function or a lack of antigen specific Tregs. However all these causes could possibly be treated by isolating Tregs from patients and selecting them for antigen specificity, or possibly making them antigen specific. These Tregs could then be activated and expanded in vitro and then transferred back into the patients. Therefore a better understanding of Treg function and dysfunction and improvement in the ability to manipulate them is an important step in the treatment of SLE.

Introduction.

Systemic lupus erythematosus, or SLE, is a chronic febrile multisystemic autoimmune disorder of the connective tissue. Autoimmune diseases are diseases where chronic inflammatory processes take place caused by the immune system, which can lead to tissue damage. SLE is mostly characterized by causing damage to the skin, joints, kidneys and serosal membranes. SLE is a relapsing disease with periods where the disease is active (called *flares*) and periods of remission. It occurs nine times more often in women than in men. Currently the disease can be managed through treatment such as corticosteroids, but it cannot be cured. SLE is caused by a break in self tolerance which in turn is caused by immune dysregulation. In SLE auto-antigens are presented by hyperactive antigen presenting cells (APC), such as dendretic cells. At present it is not known whether autoreactivity develops in SLE through the presentation of a single or a limited set of auto-antigens, but in active SLE reactions occur with a wide range of self-antigens. The antigens are presented to autoreactive T cells which are also hyperactive and also display several other abnormalities; increased spontaneous signaling and decreased threshold of activation,[1] resistance to the induction of anergy[2], decreased apoptosis and impaired clonal deletion[3]. The autoreactive T cells help cause tissue damage through an overproduction of proinflammatory cytokines or an increase in cell to cell adhesion. These T cells also provide increased help to B cells secreting autoreactive antibodies, which can bind as well as become trapped in tissue causing inflammation and organ damage.[4] The B cells in turn can affect the T cells by acting as APCs and producing antibodies that affect T cell function.

T cells appear to play a central role in the immune dysregulation in SLE which is also confirmed by findings that the disease is blocked in lupus prone mice after T cell depletion[5] and the fact that SLE does not occur in atymic mice.[6] Important regulators of the immune system as a whole, but especially the T cells are regulatory T cells (Tregs). It has been found that Treg deficient mice develop massive lymphoproliferation and fatal multisystem autoimmunity.[7] These Tregs could therefore play an important role in the pathogenesis of SLE. In this thesis the importance of Tregs for future novel therapies will be described by first describing how Tregs are thought to function normally and how they are thought to function in SLE.

Characteristics of normal regulatory T cells.

A properly functioning immune system protects the organism from the threat of non-self posed by pathogens whilst recognizing and not attacking self. This ability to discriminate between self versus non-self antigens, arises from the central tolerance mechanism. These mechanisms include the deletion of thymocytes with high reactivity to self antigens and also the induction of unresponsive autoreactive T cells in the periphery. In recent years it has become clear that the so called regulatory T cells (Tregs) play an important role in inhibiting self reactive responses off the immune system. Tregs are a heterogeneous population of leukocytes that are anergic in vitro in the presence of normal T cell stimuli and that show effector T cell suppressive activity (suppressive activity has been described for other parts of the immune system also). The Treg population consists of natural occurring $CD4^+CD25^+$ Tregs (nTregs) and $CD8^+CD25^+$ T cells which are both thymically derived, induced naïve $CD4^+$ T cells, NKT cells, $CD8^+CD28^-$ and γδ T cells (see fig. 1).[7]

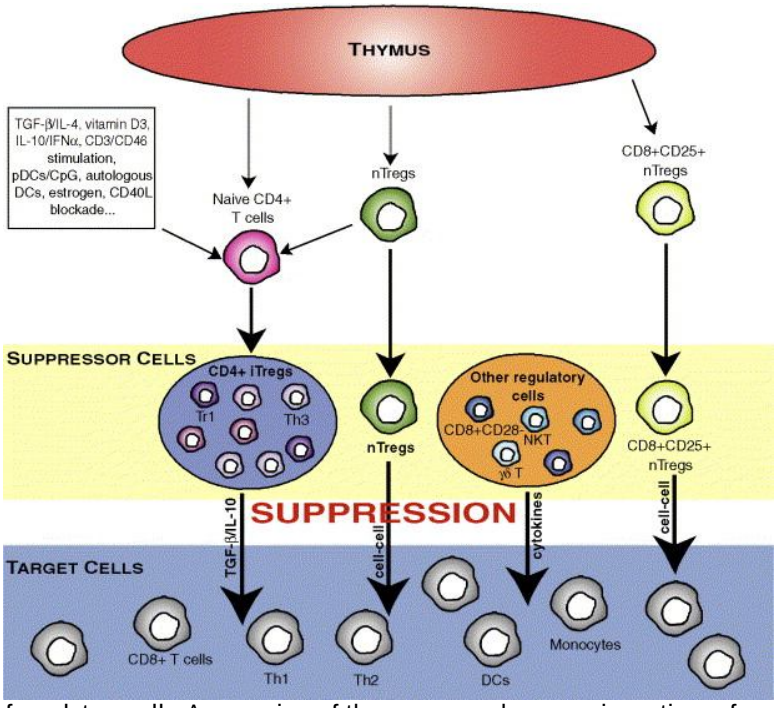

Fig. 1. Overview of regulatory cells. An overview of the sources and suppressive actions of several subsets of regulatory cells. nTregs, $CD8^+CD25^+$ T cells, and naïve T cells are produced in the thymus. Naïve $CD4^+$ cells can be induced in several ways to become regulatory T cells which mediate the suppression in a cytokine dependent manner. nTreg and $CD8^+CD25^+$ T cell suppression require cell-cell interactions. Other regulatory cells include NKT, $CD8^+CD28^-$, and γδ T cells. (Lan R.Y., Ansari A.A., Lian Z.X., Gershwin M.E. Regulatory T cells: Development, function and role in autoimmunity. Autoimmun Rev. 2005. 4:351-363.)

Off all these different regulatory cells the most is known of the natural occurring $CD4^+CD25^+$ regulatory T cells (nTregs), nTregs constitute about 5-10 % of the peripheral $CD4^+$ T cells in humans and mice. nTregs are hard to distinguish from other T cells, they appear similar to memory T cells and their T cell receptor (TCR) assemblage is as diverse as that of the normal T cell population.[8] As stated before nTregs are anergic in vitro in the presence of normal T cell stimuli. However this anergy is not a stable state, when nTregs are sufficiently stimulated by cytokines such as IL-2 and IL-15 the anergy is abolished and the suppressive abilities are no longer functional.[9, 10]

To be better able to identify nTregs efforts have been made to find unique marker for Tregs, however no unique markers have been found. nTregs can only be identified by looking at several cell surface markers and comparing how these are expressed on nTregs as opposed to the conventional T cell population. The most widely used marker is the Il-2Rα receptor molecule or CD25.[11] CD25 is expressed at higher levels on nTregs than on conventional T cells. Cell surface molecules that are also used as markers are among others the glucocorticoid-induced tumournecrosis factor receptor (GITR), CTLA-4 (CD125), galectin-1, CD38, CD62L, OX-40L, CD103, TNFR2, TGF-βR1, Ly6, 4-4-1BB, programmed cell death 1(PD1), CD5, L-selectin, CD45RO, CD45RC, Neuropilin-1, CD103, lymphocyte activition gene 3 (LAG-3) and the forkhead transcription factor FoxP3.[7] Off all of these FoxP3 is probably the most important functional marker because of the role it plays in regulatory T cell development and in mediating nTregs regulatory function.

nTregs grow to a functionally mature state in the thymus. Here they gather in the fibrous septa and medullary areas. They require high avidity interactions between their TCR and the thymic stroma expressing class II MHC-self-peptide complexes for development.[7] FoxP3 is also thought to play an important role in the development of nTregs, because FoxP3 deficient mice develop massive

lymphoproliferation and fatal multisystem autoimmunity due to lack of nTregs. Other molecules thought to play a role are CD28, CD40 and IL-2.[12]

The adaptive as well as the innate immune system has cells that can be suppressed by nTreg, although the mechanism behind this suppression still remains unclear.[9] It is likely that the immunological response is suppressed by nTregs in a variety of ways including negative signals produced by inhibitory nTreg surface molecules, cytotoxic killing, antigen presenting cell (APC) function down regulation, activation of other regulatory cells, as well as a number of cell-cell interactions.[1] One finding is that FoxP3 is also a negative regulator of T cell activation in both human and mice. [13]

The nTregs play an important role in suppressing self reactions of the immune system, but they only represent 5-10% of the CD4+ cells. Subsets of naïve CD4+ T cells can also be induced to become regulatory T cells (iTregs). One subset of iTregs are Il-10 producing Trl iTregs, which can be mostly found in the intestinal muscosa.[14, 15] Trl iTregs in vivo induction is probably due to priming by specialized APC's such as dendritic cells (DCs). [15, 16] Unlike nTregs the suppression by Trl iTregs is mediated in a cell-cell contact independent manner.[8] They can perform cytotoxic function by inducing apoptosis in target cells via the synthesis of granzyme B and perforin in a CD18 dependent manner. The suppression of cytokine production and proliferation of naïve CD4+CD25- T, Th1 and Th2 cells by Trl iTregs is mediated by their high il-10 levels and low TGF-β levels. [15, 17, 18] Trl iTregs are also thought to suppress the production of immunoglobulin by B cells and modulate the antigen-presenting capacity of monocytes and DCs. [15]

Th3 iTregs are a different subset of iTregs characterized by their production of TGF-β. These can be induced in a variety of ways, by the presence of IL-4, by the presence of Il-10 while Il-12 is inhibited, or by the presence of activated DCs.[18, 19, 20] The main suppressive mechanism of TH3 iTregs depends on the production of TGF-β, which suppresses the proliferation of TH1 and TH2 cells. [19, 21]
Besides the Tregs described above other cells also play an important role in the immunoregulation such as CD8+28- regulatory T cells, further research is required to illuminate their role in regulating the immune system.

Characteristics of regulatory T cells in SLE.

In recent years several studies have been published concerning the phenotype and the possible role of Tregs in SLE. As mentioned before Tregs play an important role in suppressing autoimmune reactions and dysregulation of Tregs was thought to contribute to the T-cell and B-cell hyperactivity in SLE. However studies that have examined Tregs are not all in accordance with each other as to how Tregs contribute to SLE. Studies have been published reporting a decrease in Tregs, but these Tregs were still found to be functional. However there are also reports stating that Tregs in SLE are not fully functional and have lost their suppressive ability. Surprisingly there are also a number of studies that report an increase in the number of Tregs and no significant loss of Tregs suppressive ability. First we will look at a number of studies in more detail to identify possible mechanisms of how a decrease, loss of function or even an increase of Tregs leads to the development of SLE. This is important to explain in which ways Tregs could be targeted or used for future treatment of SLE.

Most studies found a significant decrease in $CD4^+CD25^+$ Treg, which appear to be correlated to SLE.[22] Sle1, a SLE susceptibility loci that has been linked to SLE both in mice models as well as in human patients, was found to be associated with a reduction in the level $CD4^+CD25^+$ Treg cells. This was examined in the mouse model NZM2410 (New Zealand black (NZB) x New Zealand white (NZW) F_1 mice). This reduction was not secondary to the disease process, because the levels of $CD4^+CD25^+$ Treg cells were already reduced in very young mice before the appearance of autoantibodies. This

means that the reduction of Tregs in SLE might be causative to the immune dysregulation in the disease process. Concurrent with the decrease in Tregs there was also a decrease in Foxp3 levels, in mice Foxp3 is only expressed in Tregs and not in other T cells. The reduction of Foxp3 was not only found in the periphery, but also in the thymus. Therefore the Sle1 loci may have an effect on the maintenance of Tregs in the periphery as well as the production of Tregs in the thymus.[23]

In another study, which also used the NZM2410 mouse model with the Sle1 loci, the suppressive ability of Tregs was examined. This was done by isolating $CD4^+CD25^+$ fractions; this fraction contains Tregs as well as effector T cells. The Tregs in these fractions showed normal levels of CTLA-4, CD103 and GITR. These molecules are marker usually associated with a normal Treg function. The Tregs were indeed found to be able to suppress effector T cells, though a higher concentration of the $CD4^+CD25^+$ fraction was needed to obtain the same level of suppression as was achieved by the control. This was attributed to the reduced levels of Tregs which caused the fraction to contain less Tregs as compared to the control.[24] although the possibility that the higher fraction of $CD4^+CD25^+$ was needed because the Tregs had a reduced suppressive ability cannot be discounted. This is because the presence of markers at levels normally indicating a functional phenotype does not automatically mean that this is indeed the case. It has been demonstrated that the expression of these marker can also occur in cells that completely lack inhibitory function.[25] The Sle1 loci also affects several other cells of the immune system besides Tregs, including dendretic cells. These dendritic cells appear to inhibit the suppressive function of Tregs. The dendritic cells were found to overproduce Il-6, this overproduction was proven to be necessary for the inhibition of Tregs by a suppression assay using an anti-Il-6 neutralizing antibody. [26]

There are also studies published in which human patients were examined and reduced Treg levels were reported. One such study looked at human pediatric patients with active as well as patients with inactive SLE and these were compared to normal controls. It was found that the numbers of Tregs were significantly decreased in patients with active SLE. An interesting find was that the numbers of Tregs in patients with inactive SLE were not significantly decreased when compared to controls. The numbers of Tregs present were found to be inversely correlated to the disease activity as well as to the levels of auto-antibodies present.[27] Autoreactive B cells require help from $CD4^+$ T cells to produce autoantibodies. The T cells help self-reactive B cells by rescuing them from apoptosis as well as inducing them to produce antibodies. In order for this process to take place the suppressive function of Tregs must be overcome.

When you integrate the above described results a possible mechanism is provided for dysregulation of the immune system in SLE. A genetic defect in the Sle1 loci causes a reduction in the number of regulatory T cells mediated by dendritic cells overproducing Il-6. The reduced numbers of regulatory T cells are presumably no longer able to suppress the self reactive T cells, allowing these T cells to help activate self reactive B cells. These B cells upon activation produce auto-antibodies, which will form immunocomplexes and damage target tissue. However in humans spontaneous remission or improvement of SLE is common, causing active disease to become inactive. Therefore either through medication or a reaction of the body itself the numbers of Tregs are increased again, because in patients with inactive disease the levels of Tregs are not significantly different than those of normal controls. Examining the mechanism behind remissions as well as which genes on the Sle1 loci that contribute to the disease should be a subject of further research.

A study that reported a defect in the suppressive abilities of Tregs also investigated Tregs in human patients with both active and inactive SLE. They examined the level of Foxp3 expression, this is an important molecule for the development of Tregs and also plays an important role in the ability of Tregs to suppress auto-immune responses. The Tregs were defined as $CD4^+CD25^+$ T cells and only the cells with the highest levels of CD25 were looked at. Interestingly enough no difference in the level

of Foxp3 expression was found in patients with inactive disease as compared to normal controls, whilst patients with active disease had significantly less Foxp3 expression. Thus patients with SLE in the active stage of the disease had $CD25^{high/verhigh}$ deficient cells. The suppressive activity of the Tregs was also looked at. $CD4^{+}CD25^{high}$ cells from normal donors showed a direct suppressive effect on T cells which was independent from APC. The Tregs from patients with active SLE exhibited significantly less suppressive activity compared to the normal donors. The $CD4^{+}CD25^{high}$ cells that were isolated from patients with inactive SLE showed no significant difference in their suppressive action compared to the normal donors. When the $CD4^{+}CD25^{high}$ cells isolated from patients with active SLE were activated in vitro the levels of Foxp3 expression increased and the cells regained their suppressive ability. This result would indicate that patients with active SLE have deficient Tregs that have lost their suppressive ability and that this is associated with a reduced Foxp3 expression. The loss of function and reduced Foxp3 expression is reversible however.[28] This could be another mechanism that explains why SLE is a relapsing disease in humans. During inactive stages of the disease a loss of Foxp3 expression is somehow triggered in Tregs which causes them to lose their suppressive abilities. This would cause them to no longer be able to suppress the auto-immune response and the SLE would become symptomatic. Patients are now in the active stages of the disease but since this action is reversible the Tregs could regain their suppressive abilities and patients would once again be in the inactive stage. However it is not known whether this actually happens in the disease process or how the mechanism behind this could work, further research would be required.

Besides studies that describe a reduction in levels of Tregs or a reduction in Treg function there are also those that indicate that higher levels of Tregs are present in SLE and that these appear to be functional. In one such study a characterization of Treg cells was made in BWF1 mice using Foxp3 as a marker. They found that in this model the number of Tregs was substantially higher as compared to controls. Surprisingly the in vivo suppressive activity of the Tregs in lymphoid organs was intact and the migration of Tregs to sites of inflammation was also functional. However, the Treg showed altered distribution in secondary lymphoid organs and they also showed a phenotype suggesting a highly activated state. Interestingly it was also found that the Treg cells isolated from the mouse model were not able to suppress IgG antibody production in vitro even though they did show suppressive ability in vivo. It was assumed that Tregs could suppress IgG antibody production, because this is dependent on the presence of active $CD4^{+}$ cells which can be suppressed by Tregs.[29] A possible mechanism to explain why Tregs could not suppress the IgG antibody production or the occurrence of SLE is that Treg with the correct antigen specificity are not present or that not enough of these are present. It has been shown that Treg cells induced with peptides obtained from anti-DNA antibody can reduce SLE disease activity.[30] It is also possible that even though there is an increased Treg expansion it is not enough for the degree of autoreactivity present. Another possibility is that there isn't a defect in the Tregs but the effector T cells that are influenced by Tregs have become resistant to suppression. There have been studies reporting such an increase in resistance to supression.[31] This resistance was associated with an increase in disease activity. However it is also possible that the resistance found was not innate to the effector T cells isolated from patients with SLE, but that the resistance resulted from accessory cells autologus to SLE which might have been present in the suppression assay.

There are several possibilities as to why there are discrepancies in the role of Treg in SLE. In order to examine the Tregs they must first be identified, markers are used to identify the Tregs. There are different markers that can be used to identify Tregs. One reason why different results are reported could be that different markers were used and subsequently different populations of Tregs were studied. It is also possible that in some studies, besides Tregs themselves, effector T cells were defined as Tregs also owing to the fact that these are difficult to distinguish from one and other. The opposite is also possible, some studies might not include all Tregs discounting them is effector T

cells. Other reasons for the differences are that not all disease models might be completely comparable. There are different mouse model for SLE and it is possible that different dysregulations in the immune system give rise to SLE like syndromes in these models. A difference can also be expected when mouse models are compared to humans. Even though there is a great overlap between human and murine SLE there are differences. Human SLE is a chronic disease where remission frequently occurs as well as relapses into activity of the disease, called flares, and SLE in humans is rarely fatal. Murine SLE on the other hand is a progressive and fatal disease. This difference probably occurs from an underlying difference in the disease as well as the fact that humans are treated for SLE. Differences between patients are also conceivable, not only because there could be a difference in the treatment or the stage the disease is in, but also because the disease might have occurred differently. SLE occurs in humans through a variety of genetic risk factors as well as environmental factors. SLE is also a complex disease, it is therefore possible that there are several mechanisms which could lead to SLE. This should be held in to account when treatments are considered, it could be possible that there are subsets of patients that require different treatment.

Treatment based on the characteristics of regulatory T cells in SLE.

Drugs that are currently used in the treatment of SLE are immunosuppressors. The standard immunosuppressive therapies, such as corticosteroids and cyclophosphamide, target the entire immune system. These therapies increase the risk of infections some severe enough to warrant hospital stay or even severe enough to cause fatality. Newer immunosuppressors such as mycophenolate mofetil and rituximab specifically target certain parts of the immune system and are therefore often less toxic which also means a smaller risk of infection, though severe infections are still possible. Interestingly, treatment with corticosteroids, cyclophosphamide and other immunosuppressors restores the decreased numbers of $CD4^{+}CD25^{+}$ Tregs to normal levels. In treatment with rituximab a significant increase in Tregs is also described at the time of B cell repopulation. This suggests that these therapies result in increased number of Tregs in SLE and that part of their therapeutic effect may be mediated by Tregs. It is therefore interesting to look at the potential of Tregs as a less toxic treatment for SLE.

As discussed earlier there are reports that the numbers of Tregs are decreased in patients with active SLE. These reduced numbers might lead to insufficient suppression of the auto-immune reactions which causes SLE. In the case were increased Tregs are reported in SLE it is also possible that even though the number of Tregs are increased this is not sufficient to suppress the auto-immune reactions in SLE. In both these instances it increasing the number of Tregs present could be beneficial for the disease outcome. In a study be scaplapino et al, Tregs were isolated from New Zealand black/New Zealand white lupus-prone mice and expanded ex vivo. These Tregs were transferred to the mice before the onset of significant clinical disease to examine whether additional Tregs could prevent disease progress in SLE. The development of renal disease was used as a marker of disease progress, renal disease is characterized by the presence of proteinuria. Mice that were injected once with additional Tregs showed a decreased progression in the development of renal disease and there was a significant decrease in mortality. Several weeks after treatment mice did show proteinuria, when these mice were injected again with Tregs the disease progress was once again slowed. Interestingly the presence of proteinuria disappeared in two mice after the second transference of Tregs for six to eight weeks.[32] These results indicate that the transfer of Tregs can not only slow the onset of SLE but can also slow the progress of active SLE as well as possibly cause a remission. Therefore people suffering from SLE could possibly be treated with their own Tregs by isolating some of their Tregs and expanding them ex vivo.

Another possible mechanism behind the insufficient suppression of Tregs in SLE is that the Tregs have lost their suppressive capabilities through a loss of Foxp3. $CD4^+CD25^+$ Tregs deficient in Foxp3 isolated from patients with active SLE can be activated in vitro. After this in vitro activation level of Foxp3 expression was upregulated and the suppressive function of the Tregs in vivo was restored.[28] These activated Tregs could be transferred back into patients and might help in sufficiently suppressing SLE.

The possibility that Treg with the correct antigen specificity are not present or not present in high enough amounts has also been discussed. In SLE the reaction to self-antigens lead to tissue damage, a well established method for inducing tolerance to these self-antigens in mice is injecting them with high doses of antigens.[33] New Zealand black/New Zealand white lupus prone mice can be protected from developing SLE when they are injected with high doses of a synthetic peptide (pCon) which amino peptide sequence is like several murine anti-dsDNA Igs. This protection was shown to be mediated by Tregs.[34] $SDM_{83\text{-}119}$ is a C terminal peptide of SmD1 protein. In 70% of the SLE patients antibodies to this peptide can be found. Therefore inducing tolerance to this peptide is of particular interest. In a study using New Zealand black/New Zealand white lupus prone mice tolerance to this peptide has been induced be injecting the mice with high doses of this peptide. The tolerance to this peptide prolonged survival. It was shown that injection with this peptide induced Tregs specific to this peptide.[35] This method however only works as a protection for developing SLE. Patients should therefore be treated with antigen specific Tregs, this has the added advantage that in transfer studies done for other auto-immune diseases antigen specific Tregs were superior to unselected polyclonal Tregs.[32] To treat SLE with antigen specific Tregs these must be isolated from the patients and expanded if they are present. In the case that antigen specific Tregs are not present they should be made antigen specific in vitro, however no such method exists yet. Another problem is that in SLE several self-antigen are targeted by T and B cells and not all of these are known. Therefore efforts should be made in elucidating which antigens play an important role in SLE.

When Tregs are to be used clinically the treatment possibilities that are described above are probably integrated. The Tregs would be isolated from patients and selected for antigen specificity, or possibly be made antigen specific. These Tregs could then be activated and expanded in vitro and then transferred back into the patients. This will lead to effective treatment with all possible Treg dysregulations which might lie at the root of SLE. When Tregs are expanded and then transfected into the patient this will lead to higher levels of suppression, because there are more Tregs present in the body. Likewise when Tregs are activated in vitro and when Tregs are made antigen specific before transfection this will also lead to a higher suppressive activity. In future it is possible that normal immunosuppressive therapy is no longer necessary if Treg treatment could be made effective enough. This would be a huge advancement, because the auto-immunity could be targeted specifically. This is unlike current therapies where either the whole immune system or parts of the immune system is suppressed. The advantage of only suppressing the auto-immunity is obvious; the immune system will still be able to defend the organism against pathogens. When Treg treatment is clinically useable it will of course not only be used for the treatment of SLE but other auto-immune diseases as well. In fact it is probable that if Treg treatment is ever introduced it will be first used for other auto-immune disease where the antigen that triggers the auto-immunity is known. Tregs can have other clinical uses as well, for instance in suppressing an immune reaction against transplants. If this becomes a possibility less closely matched transplants might also have better outcomes. Another possible use for Tregs is in treating allergies, they could be made specific for the antigens that cause the allergies and thereby suppress the immune response to these antigens. This could prevent the allergy reaction in asthma for instance. In conclusion, a better understanding of Treg function and dysfunction and improvement in the ability to manipulate them is an important step in the treatment of SLE as well as other auto-immune diseases, transplantations and allergies.

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