REGULATORY LYMPHOCYTE CLUB: MECHANISMS OF ACTION, ROLE IN AUTOIMMUNITY AND ALLERGY, PROMISING THERAPEUTIC APPLICATIONS

Klub regulačných lymfocytov: mechanizmy účinku, úloha v autoimunite a alergii, perspektívne terapeutické využitie

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Abstract
The populations of B and T lymphocytes are remarkably diverse, housing numerous subpopulations within them. They serve both effector and regulatory functions. This delicate and complex balance ensures the immune system executes its protective functions without compromising the host organism. The emergence of subpopulations of regulatory lymphocytes in recent years, including Treg, Tr-1, Tr-35, Th17reg, $CD8^+$ and B-regulatory cells, has significantly expanded our understanding. Each group of cells exerts its immunosuppressive activity, even if several share it. The physiological role of regulatory cells is pivotal, as they are the guardians, preventing autoimmune processes, maintaining immune reactions within physiological limits, and fostering the development of tolerance. If there is insufficient activity or numbers of these cells, it can lead to the development of autoimmune and allergic diseases. The immunosuppressive effects of some regulatory lymphocytes can be utilised to treat some autoimmune and potentially allergic diseases *(Tab. 1, Fig. 2, Ref. 41). Text in PDF www.lekarskyobzor.sk.*
KEY WORDS: characteristics of regulatory immune cells, regulatory lymphocytes in autoimmunity and allergy, treatment with Treg cells.
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Abstrakt
Populácie B- a T-lymfocytov sú rôznorodé, zahrňujú viaceré subpopulácie. Plnia efektorové, ako aj regulačné funkcie, zabezpečujú jemnú a zložitú rovnováhu, ktorá umožňuje, že imunitný systém zabezpečuje svoje obranné funkcie bez ohrozenia vlastného organizmu. Identifikácia subpopulácií regulačných lymfocytov v posledných rokoch, najmä Treg, Tr-1, Tr-35, Th17reg, $CD8^+$ a B-regulačných lymfocytov, výrazne rozšíril naše chápanie. Každá z uvedených skupín buniek má svoju imunosupresívnu aktivitu, aj keď ju zdieľajú viaceré. Fyziologická úloha regulačných lymfocytov je kľúčová, pretože zabraňujú autoimunitným procesom, udržiavajú imunitné reakcie vo fyziologických medziach a podporujú rozvoj imunitnej tolerancie. Ak je nedostatočná aktivita alebo počet týchto buniek, môže to spôsobiť rozvoj autoimunitných a alergických chorôb. Imunosupresívne účinky niektorých regulačných lymfocytov možno využiť na liečbu niektorých autoimunitných a potencionálne aj alergických chorôb *(tab. 1, obr. 2, lit. 41). Text v PDF www.lekarskyobzor.sk.*
KĽÚČOVÉ SLOVÁ: charakteristika regulačných imunitných buniek, úloha regulačných lymfocytov pri autoimunite a alergii, liečba Treg-lymfocytmi.

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The immune system (IS) operates under two fundamental principles. Firstly, it must identify and eliminate foreign antigens while tolerating its own antigens. Secondly, it must regulate immune and inflammatory reactions within an appropriate range, removing threats to the organism's integrity without damaging its own organs or tissues. The IS achieves this through two primary mechanisms: physically eliminating autoreactive lymphocytes in primary lymphoid organs and, if they manage to escape, preventing their activation in the periphery. This second function of the immune system is primarily carried out by regulatory cells, which come in several types and play a pivotal and crucial role in maintaining immune balance and preventing autoimmune diseases.

Among the oldest known are **regulatory T cells** (Treg), which are either natural (nTreg) or induced (iTreg). Natural regulatory cells arise in the thymus as a separate, independent population. Their differentiation requires the activation of a specific gene, *FOXP3* (forkhead box P3; Xp11.23-q13.3), which encodes the transcription factor of the same name. Its critical biological importance is evidenced by the fact that mutations of this gene result in a disease incompatible with life -

IPEX (immune dysregulation, polyendocrinopathy, enteropathy) (1, 2, 3, 4). nTregs represent 5 - 10% of $CD4^+CD8^-$ thymocytes (CD = cluster of differentiation = membrane molecules of cells of the immune system); in the periphery, they represent approximately 10% of the entire population of $CD4^+$ cells. These long-lived cells are characterised by CD4, CD25 and reduced expression of CD127 (1).

During an immune response, **induced regulatory T cells** (iTreg) differentiate from naive $CD4^+$ T cells. They arise mainly in mucosal spaces, with the gut being the most well-studied site. When naive $CD4^+$ T cells are exposed to cytokines TGF-β, IL-2 (cytokines = hormones of the immune system) and retinoic acid produced by dendritic cells through the processing of vitamin A, they differentiate into iTreg cells (5).

Most recently, it has been discovered that iTreg cells can also be formed under the influence of IL-33, which is constitutively produced by intestinal epithelial cells and is released into the environment when they are damaged, thus acting as an alarmin. After binding to its receptor, ST2, in the membranes of $CD4^+$ T cells, together with signalling triggered by TGF-β, it promotes the transcriptional activation of the GATA3 factor, a transcriptional regulator of the *FOXP3* gene. In addition, IL-33 also increases the synthesis of its receptors, increasing the persistence of iTreg cells in the given microenvironment (6).

The mechanisms of the inhibitory action of both nTreg and iTreg cells are multiple and include the synthesis of immunosuppressive cytokines (e.g. IL-10, IL-35, TGF-β etc.), a direct killing of effector T cells, inhibition of dendritic cell (DC) maturation, and increased consumption of IL-2, which is thus not available for effector Th1 or cytotoxic T cells; we refer to this as metabolic deprivation. What is the difference in the action of nTreg- and iTreg-cells? The role of the former is mainly to prevent the onset of autoimmune processes, while the latter primarily regulates the extent of ongoing immune and inflammatory reactions (7, 8).

Another essential regulatory T cell group is **Type 1 regulatory T cells (Tr1)**. This subpopulation of $CD4^+$ cells arises from naive T cells under the influence of IL-27 and TGF-β. These cytokines induce the formation of several transcription factors, though not FOXP3, which is necessary to differentiate Treg cells. The most important for their immunosuppressive activity are the membrane molecules PD-1 (CD279), CTLA-4 (CD152), and LAG-3 (CD223), along with the synthesis of IL-10 and TGF-β. They also have cytotoxic effects and cause metabolic deprivation, making their immunosuppressive ability comparable to those of nTreg cells (9, 10). By synthesising IL-10, they also support forming regulatory B cells (Breg) (see below).

Interleukin-35-induced regulatory T cells (Tr35) were recently added to the regulatory lymphocyte club, resembling Tr1 cells. However, they differ in their key immunosuppressive mechanisms. While Tr1 cells mediate their inhibitory activities by producing IL-10, Tr35 cells primarily function via IL-35 secretion. Moreover, Tr1 cells express higher levels of chemokine receptors CCR5 and CCR4, along with lower levels of CCR7. This suggests that Tr1 cells are more likely to migrate out of lymphoid organs to the periphery to exert inhibitory functions. However, Tr35 cells have low expression of CCR5 and CCR4, but high expression of CCR7, suggesting that Tr35 cells mainly remain in the lymphoid organs, where they can exert their regulatory functions (11). Transcription factor FOXP3 is a principal molecule for differentiation on nTreg cells and is not required for IL-35 expression. However, mutations in *FOXP3* significantly reduce IL-35 expression in Tregs. Therefore, the *FOXP3* gene may be critical for stable IL-35 expression in both Treg and Tr35 cells (11, 12).

Naive T helper lymphocytes (Th0) differentiate into seven subsets depending on their particular environment: Th1, Th2, Th9, Th17, Th22, T_{FH} and iTreg cells. By releasing their characteristic cytokines, they perform their specific effector functions (Table 1) (for review, see 13, 14). Th17-cells are characterised by synthesising cytokines IL-17A and IL-17F, which perform significant pro-inflammatory activities. They arise as a subpopulation of naive T cells under the influence of IL-23 and IL-6. However, in an environment containing IL-6 and TGF-β, rather than these pro-inflammatory cytokines, an immunosuppressive population of **Treg17 cells** is formed. These produce IL-21 and IL-10 to an increased extent. The differentiation of Treg17 cells is controlled by the transcription factor, RORγt, although these cells also express variable levels of the transcription factors T-BET and FOXP3 (15).

Table 1. Cytokines characteristic for subsets of T helper cells.

Th1	Th2	Th9	Th17	Th22	T_{FH}	iTreg
IFN-γ	IL-4	IL-9	IL-17A	IL-22	IL-4	IL-10
TNF	IL-5	IL-10	IL-17F		IL-21	TGF-β
LT	IL-6	IL-21	IL-21		IFN-γ	
IL-2	IL-10		IL-22			
	IL-13					
	IL-24					

IFN - interferon, IL - interleukin, iTreg - induced T regulatory cells, LT - lymphotoxin, TGF - transforming growth factor, TNF - tumour necrosis factor

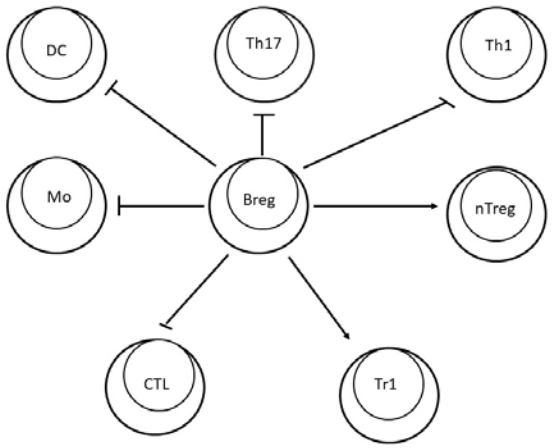
$CD8^+$ lymphocytes are mostly perceived as cells endowed with cytotoxic properties, killing virus-infected and malignant cells. However, recent evidence has demonstrated that they also possess important regulatory activities in humans. Except for CD8, their principal receptors represent killer inhibitory receptors (KIRs). **KIR^+CD8^+ T cells** are terminally differentiated cells likely to target pathogenic $CD4^+$ T cells directly (16).

An increased frequency of KIR^+CD8^+ T cells were observed in both the blood and inflamed tissues of patients with autoimmune diseases. The expansion of KIR^+CD8^+ T cells in the context of autoimmune diseases

may act as a negative feedback mechanism to ameliorate pathogenesis by killing autoreactive T cells. Moreover, elevated levels of KIR^+CD8^+ T cells were found in SARS-CoV-2 or influenza-infected patients and were associated with autoimmune-related complications in COVID-19 patients (16). This suggests that elevated levels of KIR^+CD8^+ T cells are a general mechanism induced during an infection.

B regulatory lymphocytes (Breg) are the most recently identified population with immunosuppressive properties. They act through the production of their cytokines IL-10, IL-35, and TGF-β and inhibit the activity of Th1 and Th17 cells, dendritic cells, monocytes, and cytotoxic T-cells. Conversely, they support the activity of iTreg-, Tr1-, and iNKT cells. Additionally, $Breg_s$ mediate their immunosuppression via CD73 (ecto-5′-nucleotidase) expression and adenosine production (17, 18, 19) (Fig. 1).

Figure 1. Mechanism of immunosuppressive action of regulatory B cells. B cells (Breg), by synthesising IL-10, TGF-β and IL-35, can suppress the differentiation of pro-inflammatory cells, such as monocytes (Mo) producing tumour necrosis factor, dendritic cells (DC) synthesising IL-12, Th1, Th17 and cytotoxic $CD8^+$ lymphocytes (CTL). Additionally, Breg cells can induce the differentiation of both $FOXP3^+$ and Tr1 regulatory cells.



The significant immunosuppressive ability to suppress ongoing immune processes led scientists to use this ability in treating autoimmune or allergic diseases. Since we know the specific membrane molecules of Treg cells, we can currently isolate them from peripheral blood, expand them *in vitro*, and then apply them to patients (20, 21).

In addition, Treg cells can also be isolated from umbilical blood, and the thymuses of newborns can be removed during cardiac surgery (20, 22). Two methods are used when collecting from peripheral blood: FACS (Fluorescence Activated Cell Separation) or MACS (Magnetic Activated Cell Separation). However, the yield of Treg cells from these techniques is typically insufficient for effective therapeutic use, necessitating their expansion *in vitro*. This is achieved by activating them with anti-CD3 and anti-CD28 monoclonal antibodies in the presence of IL-2, which ensures the proliferation of activated cells and the creation of a clone. The cultivation process lasts approximately two weeks, after which the expanded Treg cells are administered intravenously to the patient. Fully functional Treg cells survive and function in the recipient's organism for approximately one year (20, 23).

However, the use of *in vitro* expanded nTreg cells for treatment also has disadvantages. The problem is that regulatory T cells are created during reproduction and have different specificities. This means that after being introduced into the patient's body, these cells suppress the activity of effector cells responsible for the immunopathological process we want to suppress. Simultaneously, another subset can also suppress effector cells that eliminate unwanted antigens, e.g., pathogenic microorganisms. Consequently, there is a need to generate monospecific Treg cells. This is possible by creating Treg cells with a specific **chimeric antigen receptor** (**CAR**).

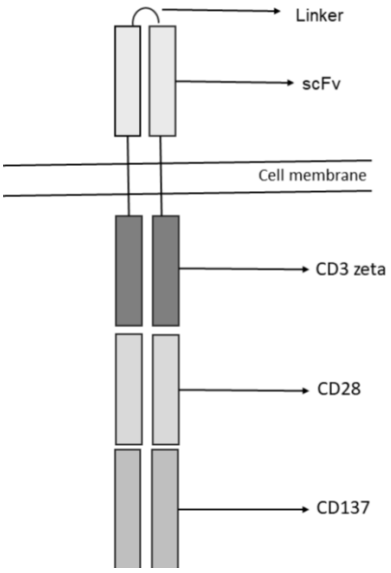
CARs are artificially created receptors that enable T cells to activate immediately upon recognizing a specific antigen, bypassing the need for additional costimulatory signals required in a physiological immune response. CAR is a synthetic structure, which, at the gene level, has variable genes for the heavy (V_H) and light chain (V_L) of monoclonal antibodies specific for the given antigen (ScFv - single chain variable fragment), along with gene segments encoding the activation sections of the CD3 zeta chain (ITAM - immunoreceptor tyrosine-based activation motif). CAR may include gene segments encoding costimulatory molecule CD28 or 4-1BB (CD137); or both, in the case of more effective constructs (the 4^{th} generation - Fig. 2). This artificially created CAR gene is transferred with the help of vectors to previously isolated and multiplied Treg cells, which are then applied to the recipient. CAR Treg cells specifically recognise antigens, become activated, and exert their immunosuppressive actions (24, 25). A modification of CAR is the **chimeric autoantibody receptor (CAAR)**. Structurally similar to CARs, CAARs contain a specific autoantigen, such as desmoglein (Dsg), in place of ScFv. For example, in psoriasis vulgaris, CAARs can be designed to target and eliminate Dsg3-specific B cells *in vivo* (26).

Therapeutic effects of antigen-specific $Treg_s$ have been demonstrated in several autoimmune diseases. Positive results were reported in the treatment of type 1 *diabetes mellitus* (27), ulcerative colitis (28), graft rejection (29), haemophilia (30), and other diseases (for review see 31, 32, 33). However, applying this approach to the treatment of allergies may present greater challenges, as it requires precise identification of the causal allergen and the development of specific monoclonal antibodies targeting it.

What is the relationship of T-regulatory cells to the development of allergic processes? We usually blame the second type of immune response for their induction, represented mainly by Th2 and ILC2 cells and cytokines, typically IL-4, -5, -13, and more recently also

epithelial ones, i.e., IL-25 and TSLP (34). One of the causes of the development of allergic processes is also a decrease in the number of regulatory T cells, both Treg and Tr1. This has been proven in bronchial asthma, where significantly fewer regulatory T cells are found in both peripheral blood and bronchoalveolar lavage (BAL) compared to healthy individuals (35). Regulatory T cells, especially Tr1, are essential in allergen immunotherapy (AIT). It has been proven that Tr1 cells are also formed during AIT by synthesising their IL-10, which supports the differentiation of B lymphocytes into plasma cells. These plasma cells begin to synthesise IgG4 antibodies that compete with IgE antibodies for allergens, thus contributing to the success of AIT (36, 37).

Figure 2. Structure of a chimeric antigenic receptor.



A chimeric antigen receptor (CAR) at the gene level consists of variable genes for the heavy (V_H) and light chain (V_L) of monoclonal antibodies that recognise a given antigen (scFv). This is followed by gene segments for the hinge region and the transmembrane segment of the receptor. Additionally, gene segments for intracellular signalling domains are incorporated, i.e. ITAM domains of the CD3-molecule and those encoding costimulatory molecules such as CD28 or 4-1BB (CD137), or both in more advanced constructs, such as the fourth--generation CARs.

The hygiene hypothesis assumes that a balance must be maintained between hygiene and exposure to microorganisms so that the immune system is adequately stimulated, thereby preventing the induction of allergic reactions. While this hypothesis has been the subject of extensive debate and research, there is still insufficient evidence to fully confirm it (38). One of the pieces of evidence proving the correctness of the hygiene hypothesis was provided by a recent study, which, however, pointed out that the exposure of an individual to microorganisms is not enough. Instead, the quality of an individual's microbiome plays a critical role; specifically, the microbiome must include bacteria capable of inducing the formation of regulatory T cells during the neonatal period, while maintaining their formation throughout life. The authors of the mentioned study focused on the incidence of allergy in the neighbouring Nordic countries, Finland and Estonia. Given approximately the same environmental conditions, the incidence of allergy was expected to be the same in both. However, the reality was quite different; the prevalence of allergic diseases, e.g., *asthma bronchiale*, was 9.3% in Estonia, while in Finland, it was 19%. The authors found that these differences cause different maturation of Treg cells and colonisation of the intestine. In a comparison of children aged 1 to 3 years, it was found that those from Estonia had a higher number of nTreg cells in peripheral blood and the presence of beneficial gut bacteria such as *Bifidobacterium longum* and butyrate--producing bacteria, which are critical for the maturation of epithelial cells. In Finnish children, these levels were lower, with nTreg cells and bacteria appearing later, correlating with an increased risk of IgE sensitisation (39). The above data confirm the positive effect of probiotics on treating allergies. Probiotics (*Lactobacilluas acidophilus*, L. *casei*, *L. reuteri*, *Bifidobacterium bifidium,* and *Streptococcus thermophilus*) have been found to support the suppressive activity of nTreg cells. Therefore, it is recommended that allergy sufferers also incorporate probiotics into their standard treatment regimen (40). Additionally, it has been proven that during sublingual allergen immunotherapy, iTreg cells (also Tr35 producing immunosuppressive IL-35) are formed in the tonsils, which inhibit allergic processes in hay fever. This effect was further enhanced by the addition of probiotics containing *Lactobacillus rhamnosus* (41).

Regulatory T cells are also involved in the induction of oral tolerance. Dietary proteins are processed in the duodenum and jejunum. They are absorbed and processed by dendritic cells in the proximal lymph nodes, which are abundant here. However, by their nature, they are tolerogenic and induce the formation of iTreg cells to prevent immune responses against food proteins and instead foster tolerance. However, along with food, potentially pathogenic bacteria may also enter the intestine, requiring elimination. This occurs in the ileum and colon, where microorganisms reach lymph nodes containing pro-inflammatory dendritic cells. These induce the formation of Th17 cells, which produce IL-17. It is a pro-inflammatory cytokine that induces mild local inflammation, making it impossible for pathogens to reproduce and exert their pathogenic properties (42).

In summary, regulatory cells are essential for maintaining immune system homeostasis and preventing excessive immune responses, including those seen in autoimmune diseases and allergies. Moreover, some of them will be able to be used soon, especially in treating some autoimmune diseases. CAR-T cells also entered clinical practice. However, although pre-clinical studies have been performed with positive results, there is still a long way to go before we can apply them to routine clinical practice.*

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