

Review article

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CD4⁺ T Cell Profiles in Autoimmune Hemolytic Anemia

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Abstract: Autoimmune hemolytic anemia (AIHA) is an immune mediated disorder characterized by the reduced lifespan of red blood cells (RBCs) due to enhanced intravascular and extravascular destruction. Traditionally, the immunopathogenesis of AIHA has been considered in the context of the immunological tolerance breakdown of B cells, since the autoantibodies are the main disease mediators. However, more recent data suggest that the production of anti-RBC antibodies by B cell is only an epiphenomenon and that the tolerance breakdown in the CD4⁺ T cell compartment is actually a key point in early AIHA development. In AIHA, there are both numerical and functional alterations of the essential CD4⁺ T cell subpopulations, including Th1, Th2, Th17, regulatory T cells and follicular helper T cells. In this review, the main characteristics of the cellular immune response during the development of AIHA, as well as the potential mechanisms by which CD4⁺ T cells promote the initiation and maintenance of the autoimmune process, are summarized. Identification of these characteristics and mechanisms would be of practical importance in the therapeutic sense because it opens up the possibility of designing more specific immunotherapy that is still not available for AIHA patients.

Key words: autoimmune hemolytic anemia; red blood cells; Th1 cells; Th2 cells; Th17 cells; Treg cells; Tfh cells.

CD4⁺ T ćelijski profili u autoimunskoj hemoliznoj anemiji

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CD4⁺ T ćelijski profili u autoimunske hemolizne anemije

Apstrakt: Autoimunska hemolizna anemija (AIHA) je imunski posredovana bolest koja se karakteriše skraćenjem životnog veka eritrocita usled pojačane intravaskularne i ekstravaskularne destrukcije. Tradicionalno, imunopatogeneza AIHA je sagledavana u kontekstu prekida imunske tolerancije B ćelija, uzimajući u obzir da su autoantitela osnovni medijatori bolesti. Međutim, skorašnji podaci sugerišu da je produkcija anti eritrocitnih antitela od strane B ćelija samo epifenomen i da je prekid tolerancije u kompartmentu CD4⁺ T ćelija zapravo centralni događaj u ranom razvoju AIHA. U AIHA, postoje i numeričke i funkcionalne alteracije osnovnih subpopulacija CD4⁺ T ćelija uključujući Th1, Th2, Th17, regulatorne T ćelije kao i folikularne pomoćne T ćelije. U ovom preglednom radu sagledane su osnovne karakteristike celularnog imunskog odgovora tokom razvoja AIHA kao i potencijalni mehanizmi kojima CD4⁺ T ćelije promovišu inicijaciju i održanje autoimunskog procesa. Identifikacija ovih karakteristika i mehanizama bila bi od praktičnog značaja u terapijskom smislu jer otvara mogućnost dizajniranja specifičnije imunoterapije koja još uvek nije dostupna AIHA pacijentima.

Ključne reči: autoimunska hemolizna anemija; eritrociti; Th1 ćelija; Th2 ćelija; Th17 ćelija; Treg ćelija; Tfh ćelija.

1. AIHA pathophysiology

Autoimmune hemolytic anemia (AIHA) is an immune mediated disorder characterized by the reduced lifespan of red blood cells (RBCs) due to enhanced intravascular and extravascular destruction. The main pathological feature of AIHA is the production of autoantibodies directed against different RBC antigens, which are only the reflection of much profound dysregulation of immune mechanisms involving multiple cell types.

Based on the clinical features and laboratory findings, there are three basic types of AIHA: warm AIHA, cold AIHA, and mixed type AIHA [1]. These different types of AIHA are usually distinguished by the use of direct antiglobulin test (DAT), also known as direct Coombs test, which detects autoantibodies and/or components of the complement system (C3d most commonly) on the surface of the RBCs.

Warm AIHA (wAIHA) is the most common type, comprising 70% - 80% of all adult cases and 50% of childhood cases [2]. It is named after the autoantibodies that optimally react with RBCs at 37°C, warm agglutinins. These antibodies are mainly of IgG class and polyclonal by nature - directed against different RBC membrane proteins, such as Rh antigen system but also the antigens of Kell, Kidd, Duffy, and Diego blood group systems [3]. When attached, these antibodies facilitate and accelerate RBC phagocytosis by spleen macrophages that express multiple receptors for Fc fragment of IgG antibodies (FcγR), thus leading to extravascular hemolysis. However, more often, only the parts of RBC membrane are phagocytized but not their cytoplasm, which disrupts their typical, discoid biconcave appearance, i.e. RBCs become smaller in size and assume more spherical shape, which is designated as spherocytosis. Spherocytes are subjected to further degradation during their re-passage through the spleen. Antibody-dependent cellular cytotoxicity (ADCC) involving NK cells and CD8⁺ T cells is also proposed as a possible mechanism of extravascular RBC destruction in the spleen and other secondary lymphoid organs. IgG antibodies are able to activate the complement cascade by classical pathway; however, to a much lesser extent compared to IgM antibodies. During this process, C3b component of the complement is formed and deposited at RBC surface, rendering them as a target for phagocytosis by complement receptor (CR) expressing Kupffer cells in the liver. Besides extravascular hemolysis, which is the dominant form of hemolysis in wAIHA, intravascular hemolysis can also occur due to formation of membrane attack complex, the end product of the complement system activation, which perforates cell membrane of circulating RBCs [4]. Based on etiology, wAIHA can be classified as

primary, idiopathic, when it is presented as an isolated clinical syndrome (no underlying disorder can be identified) or as secondary, when it is associated with various other disorders including lymphoid malignancies (chronic lymphocytic leukemia, non-Hodgkin's lymphoma), autoimmune disorders (systemic lupus erythematosus and other systemic autoimmune disorders of connective tissue), immunodeficiencies, viral infections, drugs etc. Considering these pathological features of wAIHA, DAT is positive for IgG only or for IgG and C3d complement component [1,4,5].

Cold AIHA (cAIHA) encompasses three basic clinical entities: cold agglutinin disease (CAD), secondary cold agglutinin syndrome (CAS), and paroxysmal cold hemoglobinuria (PCH). Generally, these entities are characterized by the presence of autoantibodies that react with RBCs at lower temperatures, which is why they are designated as cold agglutinins [4].

CAD is the second most common form of AIHA (20-25%), characterized by the production of autoantibodies that optimally react with RBCs at 4°C; however, these cold agglutinins can be also functional at higher temperatures, exceeding 28°C to 30°C, what actually makes them pathogenic. They are mainly of IgM class, in the form of pentameric or hexameric macromolecules, and monospecific, directed against the I/i antigen system of the RBCs [4]. Monoclonality of these antibodies suggests underlying clonal B-cell lymphoproliferative disorder, which is in 2022 recognized by the World Health Organization (WHO) as a distinct lymphoid neoplasm and a special type of monoclonal gammopathy [6]. In the distal, acral areas of the body, such as fingers, toes, nose, and ears, the temperature of the blood is lower than in the central parts, thus allowing IgM molecules to attach to the RBC surface and cause their agglutination. Being the most potent complement activator, IgM leads to the excessive formation of C3b component which also deposits on the RBC surface. When the blood temperature rises again, in the central parts of the body, IgM detaches from the RBCs allowing them to separate from each other, but C3b molecules remain bound to their surface. A portion of C3b coated RBCs is recognized by the cells of the mononuclear phagocytic system, predominantly by the Kupffer cells of the liver, and removed from the circulation. In this case, the entire RBCs are phagocytized rather than the parts of their membrane, which is why the spherocytosis is rarely seen in CAD. On the other hand, C3b can be enzymatically cleaved to C3d, which allows RBCs to survive. In the circulation, complement activation at RBC surface can proceed to the final phase, resulting in the formation of membrane attack complex and intravascular hemolysis. In CAD, DAT is IgG negative but C3d positive and typically, a spontaneous RBC agglutination occurs at room temperature [4,7].

In contrast to CAD, CAS develops in the context of various other diseases. It is also characterized by the presence of cold reacting autoantibodies mainly of IgM class, however these antibodies can be both monoclonal, if CAS accompanies lymphoid malignancy (non-Hodgkin lymphoma, Waldenström macroglobulinemia) and polyclonal if it is associated with infections (*M. pneumoniae*, Epstein-Barr virus) or autoimmune disorders (systemic lupus erythematosus, rheumatoid arthritis) [4,7].

PCH is rare form of cAIHA mainly affecting children, which almost always arises as a postinfectious complication [8]. It is mediated by Donath-Landsteiner hemolysins which are cold reacting, polyclonal IgG antibodies directed against the P antigen of RBCs. These antibodies are biphasic by nature, meaning that they attach to the RBC surface at lower temperatures and fix the components of the complement system; however, at high temperatures (37°C) IgG detaches, but complement components then become active, causing massive intravascular hemolysis. In contrast to CAD and CAS, in PCH there is no RBC agglutination and the hemolysis is completely complement dependent [4].

Mixed type AIHA (mAIHA) is defined by the presence of both warm and cold agglutinins. In this case, DAT is positive for IgG and C3d; however, cold IgM agglutinins with high thermal amplitude are also present in high titer. They cause spontaneous agglutination at 20°C which diagnostically differentiate this condition from IgG⁺C3d⁺ wAIHA [9]. Due to the difficulties in the diagnosis, particularly, clinically irrelevant cold agglutinins with low thermal amplitude that can also be present in patients with wAIHA as well as in healthy persons; mAIHA is probably much less common than it was previously believed [10].

In some patients with wAIHA, autoantibodies of the IgA class can be found in addition to IgG and IgM molecules; however, there are also very rare cases of IgA exclusively mediated wAIHA. They are characterized by the severe clinical presentation, refractoriness to glucocorticosteroid therapy and interestingly, very little free autoantibodies detectable in the serum, probably due to their high RBC binding affinity [11,12]. The precise mechanism of hemolysis in this type of wAIHA is still not clear; however, massive hemagglutination and subsequent RBC sequestration in spleen is often seen. It appears that complement activation, FcγRI mediated phagocytosis, ADCC, and RBC apoptosis due to membrane alterations do not play substantial role in RBC destruction [12].

2. CD4⁺ T cells in AIHA pathogenesis

Traditionally, the immunopathogenesis of AIHA has been considered in the context of the immunological tolerance breakdown of B cells, since the autoantibodies are the main disease mediators. However, the production of RBC specific antibodies by B cells is only an epiphenomenon, caused by much profound immune dysregulation involving multiple other cell types. From immunological point of view, AIHA can be classified as either IgG or IgM mediated, taking into account the different immune mechanisms that mediate the production of these classes of antibodies as well as the mechanisms of RBC destruction. Namely, B cells in order to produce antibodies of the IgG class need to undergo the process of immunoglobulin class switching, during which the cooperation of B cells with antigen specific CD4⁺ T cells is necessary. IgG antibodies mainly induce hemolysis by FcγR mediated phagocytosis or cytotoxicity. On the other hand, although IgM antibody production is a T cell independent process, CD4⁺ T cells also have the ability to facilitate B cells production of this antibody class [13]. In this case, hemolysis is mainly complement dependent. Therefore, RBC specific CD4⁺ T cells are of particular importance during AIHA pathogenesis, especially wAIHA which is the most common disease type. Accordingly, it has been demonstrated on a murine model, that RBC specific B cells are constantly present in healthy animals and are able to respond to CD4⁺ T cell stimulation by autoantibody production. On the contrary, RBC specific CD4⁺ T cells, although also present in healthy animals, following antigen stimulation become functionally non-responsive, anergic. Taken together, these findings suggest that breakdown of immunological tolerance in T, but not B cell compartment is actually a key point in early AIHA development [14]. Also, splenic T cells isolated from New Zealand Black (NZB) mice which spontaneously develop AIHA, underwent an extensive clonal expansion *in vitro* after RBC antigen challenging, predominantly in the CD4⁺ T cell compartment [15]. In the same model, anti-CD4 monoclonal antibody treatment was able to prevent and abrogate the production of RBC specific antibodies, but not the development of anemia, indicating that CD4⁺ T cells are crucial for the autoantibody production but also normal erythropoiesis [16-18]. Similar findings were reported in humans. In contrast to healthy donors, T cells of AIHA patients were able to mount recall response to Rh protein epitopes *in vitro*. This effect was abrogated by anti-HLA-DR antibodies, specifying that CD4⁺ T cells were the main responding cell population, since their process of antigen recognition is HLA class II restricted [19]. Recently, the expansion of a special subset of CD4⁺ T cells (CD3⁺CD4⁺CD28⁻) was documented in the patients with idiopathic wAIHA [20]. This T

cell subset is highly pro-inflammatory profiled and interestingly has a cytotoxic capacities due to the secretion of cytolytic enzymes, perforin, granzyme A and B; therefore, besides the supportive role, CD4⁺ T cells could be also directly involved in RBC destruction [20,21].

Phenotypically, CD4⁺ T cells are very heterogeneous cell population and depending on the microenvironment conditions, they can differentiate into diverse polarization profiles, classified as Th1, Th2, Th17, Th9, Th22, regulatory T cells and follicular helper T cells. Each of these CD4⁺ T cell subsets has unique characteristics including the master transcription factor, cytokine profile and specific functions.

While the importance of CD4⁺ T cells in the pathogenesis of AIHA is currently being intensively studied, there are still many unknowns related to the precise phenotypic characteristics that determine the pathogenicity of these cells during the disease development. Identification of these characteristics as well as the mechanisms by which CD4⁺ T cells promote the disease onset and maintenance would be of practical importance in the therapeutic sense because it opens up the possibility of designing more specific immunotherapy that is still not available for AIHA patients.

3. Th1 and Th2 cells in AIHA

Historically, the pathogenesis of most autoimmune disorders has been discussed in the context of a disturbed balance between Th1 and Th2 cellular immune responses, bearing in mind that these are the first two identified subtypes of CD4⁺ T cells. Th1 differentiation program is initiated by IL-12 and/or IFN- γ , and it involves the activation of T-bet as the master transcription factor. Fully differentiated Th1 cells produce large quantities of IFN- γ , but also IL-2, TNF- α and lymphotoxin (TNF- β), thus promote cell-mediated immunity primarily by inducing phenotype switch of phagocytes towards pro-inflammatory M1 phenotype. On the other hand, Th2 cells require IL-4 and the expression of GATA-3 as the master transcription factor for complete differentiation and produce cytokines IL-4, IL-5, IL-9, IL-10, and IL-13. Generally, this T cell lineage promotes antibody-mediated immunity by stimulating B cell antibody production, but also promote the development of M2 phenotype of macrophages that is associated with tissue regeneration and fibrosis [22,23].

Pioneering studies investigating Th profiles in the pathogenesis of AIHA supported the notion that AIHA is a predominantly Th2 mediated disease. Namely, in basal conditions, peripheral

blood mononuclear cells (PBMCs) isolated from AIHA patients produced significantly higher amount of IL-4, signature cytokine of Th2 cells, and less IFN- γ , signature cytokine of Th1 cells, compared with healthy controls. When artificially stimulated, AIHA PBMC cultures also behaved differently in the sense of higher IL-10 production and lower production of IL-12, which further favors Th2 polarization and the inhibition of IFN- γ production [24]. Similar results were obtained on the whole blood cultures of AIHA patients, which following mitogen stimulation shown an increase in the production of Th2 profiled cytokines (IL-4, IL-10 and IL-13) and reduced IFN- γ production, that was more pronounced in AIHA patients with active hemolysis. Interestingly, the addition of Th2 cytokines into the cell cultures increased both autoantibody production and their binding to autologous RBCs [25]. In order to minimize individual variation in cytokine production, *Kruizinga et al.* (2018) calculated Th1/Th2 cytokine ratio in the serum of hematopoietic stem cell transplanted patients with autoimmune cytopenias, including AIHA, and also found a more pronounced Th2 cytokine profile compared with control subjects without such complications [26]. Moreover, some therapeutic strategies that proved efficient, especially in wAIHA treatment, such as low doses of rituximab together with a short course of corticosteroids, were able to partially restore Th1/Th2 balance by enhancing the levels of IFN- γ , IL-12, and TNF- α and transitory reduction of IL-4 production [27].

The very origin of the Th2 immunity dominance in the pathogenesis of AIHA is still unclear, however recently increased levels of the cytokine IL-33 have been reported in the patients with wAIHA [28]. Initially, this cytokine was identified as a strong inducer of Th2 cell differentiation; although more recent studies have shown that IL-33 also has stimulatory effects on a number of other cells, including Treg and Th1 cells [29]. The levels of IL-33 were positively correlated with disease activity (measured by the number of reticulocytes and the levels of hemoglobin and lactate dehydrogenase (LDH)) but also with the production of anti-RBC antibodies. In PBMC cultures isolated from the patients with active AIHA, IL-33 promoted anti-RBC antibody production in a dose dependent manner, mainly by increasing the production of the Th2 cytokines IL-4, IL-6, and IL-13 [28]. RBCs contain substantial amount of IL-33, and due to hemolysis, they can be a significant source of this cytokine during AIHA development [30].

Considering physiological functions of Th2 cells in promoting humoral immunity, data supporting the importance of Th2 polarization during AIHA pathogenesis appear to be expected

and logical. However, there are certain facts that do not fit into the concept of AIHA as a classical Th2-mediated disease.

Firstly, signature cytokine of Th2 cells, IL-4 as well as IL-13 preferably stimulate the production of antibodies of the IgE and IgG4 class, which do not fix the complement [31], whereas the complement is an important factor predominantly during cAIHA, but also at a lesser extent wAIHA pathogenesis [4,32]. In fact, in wAIHA, anti-RBC antibodies are most often of the IgG1 and IgG3 subclass, latter being the most effective in RBC destruction [33,34]. In humans, such antibody isotype switching pattern is primarily associated with biological functions of IL-10 [35], which levels were indeed found to be elevated in patients with AIHA [24,36,37]. Based on the mice studies, IL-10 was originally included in the Th2 cytokine palette; however in humans this cytokine can be produced by different types of immune cells including monocytes, macrophages, DCs, NK cells, B cells, T regulatory (Treg) cells, even Th1 and Th17 cells [38]. Consistent with this, the higher basal production of IL-10 was observed in the monocyte cultures obtained from AIHA patients [39]. Additionally, immune complexes (including IgG-coated RBCs) have the ability to polarize macrophages towards a specific M2b phenotype, which differs from the M2a phenotype typically induced by Th2 cells [40]. M2b macrophages are characterized by extensive IL-10 production and the absence of IL-12 secretion, and their pathogenicity is well documented in the settings of systemic lupus erythematosus (SLE) [40,41]. Therefore, the importance of IL-10 in the pathogenesis of AIHA, as suggested by some Authors [24,25,42], cannot be strictly attributed to Th2 cell dominance.

Secondly, FcγR mediated phagocytosis is the most important mechanism of RBC destruction in wAIHA [43]. In monocytes, IL-4 down-regulated the expression of stimulatory FcγRs (FcγRI, FcγRIIa, and FcγRIIIa), while stimulated the expression of inhibitory FcγRIIb and thus actually compromised their ability to internalize IgG coated particles [44,45]. In contrast, peripheral blood monocytes of AIHA patients showed higher expression level of FcγRI compared with healthy controls [46]. On the other hand, in cAIHA, the dominant mechanism of hemolysis is CR mediated phagocytosis of C3b coated RBCs [4]. Th2 cytokines, IL-4 and IL-13 were also found to down-regulate the expression of immunoglobulin superfamily complement receptor (CRIg) and decrease the ability of macrophages to phagocytize complement opsonized particles [47]. Considering these data, one could speculate that Th2 cells actually have a protective function during the development of AIHA as they reduce the degree of RBC hemolysis.

Third, AIHA does not show the typical features of classical Th2-mediated inflammation, such as atopic reactions and parasite infestation, in which, in addition to an increased titer of IgE antibodies, there is also an increased infiltration and activation of eosinophils. By producing IL-5, Th2 cells support eosinophil hematopoiesis in the bone marrow, while IL-4 and IL-13 induce the expression of eotaxin I (CCL11) in stromal cells and thus enhance eosinophilic infiltration. However, the cooperation between Th2 cells and eosinophils appears to be much more complex in nature and of particular importance in initiating and maintaining the Th2 immune response [48, 49]. To our current knowledge, there are only a few reported cases of the coexistence of AIHA and eosinophilia in the literature and their interconnection was not investigated [50-52].

Although, the most studies point to Th2 cell polarization, it is clear that the pathogenesis of AIHA cannot be explained by a simplistic model of Th1/Th2 imbalance. In concordance to that, new subtypes of CD4⁺ T cells were described as well as many different intermediate phenotypes, which have also been shown to play an important role during the AIHA onset and development.

4. Th17 and Treg cells in AIHA

The established Th1/Th2 paradigm was questioned by the discovery of Th17 cells, which were shown to mediate protection against fungi and extracellular bacteria, but at the same time have an important role in the development of various autoimmune diseases. This subpopulation of CD4⁺ T cells is characterized by the production of cytokines IL-17, IL-22, IL-21, and IL-26, expression of ROR γ t as a master transcription factor, while their differentiation program, although still enigmatic, most likely includes IL-6 and TGF- β for initial Th17 development, and IL-21, IL-1 β , and IL-23 for phenotype stabilization [53].

The first evidence to suggest the pathogenicity of Th17 cells in AIHA was based on higher levels of their signature cytokine, IL-17 in the patient's serum, which closely correlated with disease activity [54,55]. Moreover, PBMCs of AIHA patients when co-cultured with autologous RBCs as well as Rh peptides produced significantly higher amount of IL-17 compared with healthy controls [55]. These results were reconfirmed by the work of Xu *et al.* (2012), who also detected elevated frequency of Th17 cells in the patient's blood. In this study, both Th17 cell frequency and serum IL-17 levels were closely correlated with the levels of anti-RBC antibodies, hemoglobin, C3 component, and the activity of LDH [56]. The important role of Th17 cells during disease development was additionally emphasized on the murine models of AIHA. Adoptive transfer of

Th17 cells purified from AIHA mice was able to induce the higher incidence and more severe clinical presentation of AIHA in healthy animals compared with the adoptive transfer of Th0 cells. IL-17 was proposed to be the main mediator of Th17 cell pathogenicity, considering a significantly lower incidence of AIHA induction in the animals pretreated with IL-17 neutralizing antibodies or in *IL-17^{-/-}* animals [56].

Treg cells represent a special subset of CD4⁺ T cells that mediates the suppression of the inflammatory response and the establishment of immunological tolerance. In addition to CD4, these cells express CD25 and FoxP3 as the master transcription factor, and these molecules are most commonly used identification markers of so called conventional Treg cells. However, there is a whole spectrum of phenotypically different cells, some of which do not express FoxP3 but perform immunosuppressive functions, and they are designated as a non-conventional Treg cells. IL-2 is required for TGF- β to induce FoxP3 expression and the differentiation program of conventional Treg cells [57]. When activated, these cells achieve immunosuppression by different mechanisms including the secretion of immunomodulatory cytokines IL-10, IL-35 and TGF- β , the expression of inhibitory molecules PD-1L and CTLA-4, IL-2 deprivation and the generation of cAMP and adenosine [58].

Significant role of Treg cells during AIHA pathogenesis was suggested on Marshall-Clarke and Playfair model of murine AIHA. Specifically, mice pretreated with anti CD25 antibody, that depletes Treg cells, had much higher incidence of AIHA following rat RBC immunization. Additionally, the adopted transfer of splenic CD4⁺CD25⁺ cells from AIHA mice was able to prevent the production of anti-RBC antibody in healthy mice following immunization with rat RBCs. This effect was not observed when population of splenic CD4⁺CD25⁻ cells was adoptively transferred [59]. In IL-2 α R deficient mice which develop systemic autoimmune disease and the lethal form of AIHA, decreased number and impaired function of CD8⁺ Treg cells was found to be a decisive factor for the early appearance of a more severe form of the disease [60]. In humans, reduced percentage of conventional Treg cells in the blood of AIHA patient was detected, and their number was closely associated with the parameters of hemolysis: reticulocytes and haptoglobin [36]. Another indirect support of Treg cell role in AIHA control, comes from the frequent occurrence of autoimmune hemolysis after treatment with purine analogs, especially fludarabine [61]. Fludarabine has excessive toxicity to T cells, among which are Treg cells as well. According to some authors, this could be beneficial in immune reconstitution during treatment [62], but the

depletion of Treg cells might consequently lead to immune tolerance disruption and autoimmune hemolysis.

Although perform completely different functions, Th17 and Treg cells share a common differentiation factor, and that is TGF- β , meaning that additional microenvironment factors are crucial in deciding the final fate of naïve CD4⁺ T cell differentiation process. More specifically, TGF- β in combination with IL-2 promotes Treg differentiation; whereas in combination with IL-6 drives Th17 differentiation program [53,57]. Higher serum levels of TGF- β were found in AIHA patients [63,64], as well as higher frequency of TGF- β single nucleotide polymorphisms (SNPs) associated with enhanced production of this cytokine, especially in patients with more severe clinical presentation of the disease [65]. Additionally, in the mitogen-stimulated whole blood cultures, TGF- β production was significantly increased whereas IL-2 secretion was reduced in patients with active form of AIHA compared with non-haemolytic AIHA patients [25]. This cytokine milieu generally favors the generation of Th17 cells and, thus, may be the cause of the disturbed balance between Th17 and Treg cells observed in AIHA patients.

5. T follicular helper cells in AIHA

T follicular helper (Tfh) cells are particularly interesting from the aspect of AIHA pathogenesis, bearing in mind that these cells provide necessary signals for B cell maturation, but also stimulate antibody production, class switching and affinity maturation. Due to specific pattern of chemokine receptor expression (CXCR5⁺CCR7⁻), Tfh cells migrate towards B cell-rich zones; where they regulate B cell maturation and differentiation by the expression of both co-stimulatory (ICOS, CD40L) and inhibitory (PD-1) molecules and the production of IL-21. The differentiation of this unique CD4⁺ T cell subset is dependant on IL-6 and ICOSL signaling, while B cell lymphoma 6 (Bcl-6) is identified as the master transcription factor [66]. Recently, T follicular regulatory (Tfr) cells have been described which, in addition to ICOS, CD40L, PD1, and Bcl-6, express markers of Treg cells, CD25 and FoxP3, and thus are specialized in suppressing Tfh mediated B cell activation and antibody production [67,68].

Disturbed balance between Tfh and Tfr cells was previously implicated in the pathogenesis of various autoimmune disorders including rheumatoid arthritis, SLE, Sjögren's syndrome and others [68]. In the mouse model of AIHA, higher frequency of CD4⁺CXCR5⁺CD25⁻ Tfh cells was documented in autoantibody-positive mice, as well as a high ratio of Tfh/Tfr cells. Moreover,

adoptive transfer of CD4⁺CXCR5⁺CD25⁻ T cells, but not CD4⁺CXCR5⁻CD25⁻ T cells, was able to promote the induction of autoantibody production [69]. In antibody-positive mice, serum levels of Tfh associated cytokines IL-6 and IL-21 were also found to be elevated as well as the T cell mRNA expression of IL-21 and transcription factor Bcl-6 [69]. In IL-2 deficient BALB/c mice which early develop a lethal form of AIHA, higher number of CD4⁺ but also specific CD8⁺ Tfh cells was documented, considering that IL-2 is a negative regulator of the differentiation program of this T cell subset [70,71]. In humans, the expansion of circulating Tfh cell population was reported in the peripheral blood of AIHA patients [72]. Besides germinal center B cells, Tfh and Tfr cells also regulate development and the activity of regulatory B (Breg) cells which perform immunosuppressive functions [73]. Previously, it has been shown that Breg cells have a significant role during autoimmunity development [74]; however, we were unable to find studies investigating the importance of Breg cells in the pathogenesis of AIHA.

6. Conclusion

Today, the importance of CD4⁺ T cells in the pathogenesis of AIHA, especially wAIHA, is indisputable; however, there are many conflicting results regarding the phenotypic characteristics of these cells. The reason for this discrepancy may be the pronounced heterogeneity of the study designs and participant inclusion criteria, as well as the differences in the animal models of AIHA used to study the disease. On the other hand, AIHA *per se* is a very heterogeneous disease, so it is possible that there are several different mechanisms that result in the production of anti-RBC antibodies, depending on the cause of the disease itself. In general, altered CD4⁺ T cell compartment is in the background of pathological B cell activation and the production of autoantibodies, therefore should be considered as potential target of future therapeutic strategies. In order to achieve this goal, additional studies are needed that will determine the precise pathogenic profile of CD4⁺ T cells active during the onset of AIHA.

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References

- [1] Barcellini W. New Insights in the Pathogenesis of Autoimmune Hemolytic Anemia. *Transfus Med Hemother* 2015; 42(5):287-93.
- [2] Kalfa TA. Warm antibody autoimmune hemolytic anemia. *Hematology Am Soc Hematol Educ Program* 2016; 2016(1):690-7.
- [3] Soler NG, Bencomo HA, Aquino RS, Romero DY. Specificities and isotypes of erythrocytes autoantibodies in patients with warm autoimmune hemolytic anemia. *Rev Cubana Hematol Immunol Hemoter* 2020; 36(4):1-23.
- [4] Berentsen S, Sundic T. Red blood cell destruction in autoimmune hemolytic anemia: role of complement and potential new targets for therapy. *Biomed Res Int* 2015; 2015:363278.
- [5] Fattizzo B, Barcellini W. Autoimmune hemolytic anemia: causes and consequences. *Expert Rev Clin Immunol* 2022; 18(7):731-45.
- [6] Alaggio R, Amador C, Anagnostopoulos I, Attygalle AD, de Oliveira Araujo IB, Berti E. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. *Leukemia*. 2022; 36(7):1720-48.
- [7] Brugnara C, Berentsen S. Cold agglutinin disease [Internet]. Waltham, MA: Wolters Kluwer, 2023 [updated 2023 March 21; cited 2023 April 5] Available from: <https://www.uptodate.com/contents/cold-agglutinin-disease>.
- [8] Cooling LL. Kids, colds, and complement: paroxysmal cold hemoglobinuria. *Transfusion* 2017; 57(6):1332-5.
- [9] Fattizzo B, Giannotta JA, Serpenti F, Barcellini W. Difficult Cases of Autoimmune Hemolytic Anemia: A Challenge for the Internal Medicine Specialist. *J Clin Med* 2020; 9(12):3858.
- [10] Mayer B, Yürek S, Kiesewetter H, Salama A. Mixed-type autoimmune hemolytic anemia: differential diagnosis and a critical review of reported cases. *Transfusion* 2008; 48(10):2229-34.
- [11] Sokol RJ, Booker DJ, Stamps R et al. IgA red cell autoantibodies and autoimmune hemolysis. *Transfusion* 1997; 37(2):175-81.
- [12] Chadebech P, Michel M, Janvier D, Yamada K, Copie-Bergman C, Bodivit G et al. IgA-mediated human autoimmune hemolytic anemia as a result of hemagglutination in the spleen, but independent of complement activation and FcγRI. *Blood* 2010; 116(20):4141-7.
- [13] Kim JR, Lim HW, Kang SG, Hillsamer P, Kim CH. Human CD57+ germinal center-T cells are the major helpers for GC-B cells and induce class switch recombination. *BMC Immunol* 2005; 6:3.

- [14] Hudson KE, Hendrickson JE, Cadwell CM, Iwakoshi NN, Zimring JC. Partial tolerance of autoreactive B and T cells to erythrocyte-specific self-antigens in mice. *Haematologica* 2012; 97(12):1836-44.
- [15] Perry FE, Barker RN, Mazza G, Day MJ, Wells AD, Shen CR et al. Autoreactive T cell specificity in autoimmune hemolytic anemia of the NZB mouse. *Eur J Immunol* 1996; 26(1):136-41.
- [16] Oliveira GG, Hutchings PR, Roitt IM, Lydyard PM. Production of erythrocyte autoantibodies in NZB mice is inhibited by CD4 antibodies. *Clin Exp Immunol* 1994; 96(2):297-302.
- [17] Oliveira GG, Hutchings PR, Lydyard PM. Anti-CD4 treatment of NZB mice prevents the development of erythrocyte autoantibodies but hastens the appearance of anaemia. *Immunol Lett* 1994; 39(2):153-6.
- [18] Oliveira GG, Holton J, Lydyard PM. Long-term treatment of NZB mice with anti-CD4 results in wasting disease, lymphoid atrophy and chronic diarrhea. *Gut Microbes* 2010; 1(5):345-55.
- [19] Barker RN, Hall AM, Standen GR, Jones J, Elson CJ. Identification of T-cell epitopes on the Rhesus polypeptides in autoimmune hemolytic anemia. *Blood* 1997; 90(7):2701-15.
- [20] Youssef SR, Elsalakawy WA. First report of expansion of CD4+/CD28 null T-helper lymphocytes in adult patients with idiopathic autoimmune hemolytic anemia. *Hematol Transfus Cell Ther* 2021; 43(4):396-401.
- [21] Maly K, Schirmer M. The story of CD4+ CD28- T cells revisited: solved or still ongoing? *J Immunol Res* 2015; 2015:348746.
- [22] Chatzileontiadou DSM, Sloane H, Nguyen AT, Gras S, Grant EJ. The Many Faces of CD4+ T Cells: Immunological and Structural Characteristics. *Int J Mol Sci* 2020; 22(1):73.
- [23] Romagnani S. Th1 and Th2 in human diseases. *Clin Immunol Immunopathol* 1996; 80(3 Pt 1):225-35.
- [24] Fagiolo E, Toriani-Terenzi C. Th1 and Th2 cytokine modulation by IL-10/IL-12 imbalance in autoimmune haemolytic anaemia (AIHA). *Autoimmunity* 2002; 35(1):39-44.
- [25] Barcellini W, Clerici G, Montesano R, Taioli E, Morelati F, Rebulli P, Zanella A. In vitro quantification of anti-red blood cell antibody production in idiopathic autoimmune haemolytic anaemia: effect of mitogen and cytokine stimulation. *Br J Haematol* 2000; 111(2):452-60.
- [26] Kruizinga MD, van Tol MJD, Bekker V, Netelenbos T, Smiers FJ, Bresters D et al. Risk Factors, Treatment, and Immune Dysregulation in Autoimmune Cytopenia after Allogeneic Hematopoietic Stem Cell Transplantation in Pediatric Patients. *Biol Blood Marrow Transplant* 2018; 24(4):772-8.

- [27] Barcellini W, Zaja F, Zaninoni A, Imperiali FG, Battista ML, Di Bona E et al. Low-dose rituximab in adult patients with idiopathic autoimmune hemolytic anemia: clinical efficacy and biologic studies. *Blood* 2012; 119(16):3691-7.
- [28] Bu X, Zhang T, Wang C, Ren T, Wen Z. IL-33 reflects dynamics of disease activity in patients with autoimmune hemolytic anemia by regulating autoantibody production. *J Transl Med* 2015; 13:381.
- [29] Cayrol C, Girard JP. Interleukin-33 (IL-33): A critical review of its biology and the mechanisms involved in its release as a potent extracellular cytokine. *Cytokine* 2022; 156:155891.
- [30] Wei J, Zhao J, Schrott V, Zhang Y, Gladwin M, Bullock G, Zhao Y. Red Blood Cells Store and Release Interleukin-33. *J Investig Med* 2015; 63(6):806-10.
- [31] Moens L, Tangye SG. Cytokine-Mediated Regulation of Plasma Cell Generation: IL-21 Takes Center Stage. *Front Immunol* 2014; 5:65.
- [32] Teigler J, Low J, Rose S, Cahir-Mcfarland E, Yednock T, Kroon H et al. Evidence of Classical Complement Pathway Involvement in a Subset of Patients with Warm Autoimmune Hemolytic Anemia. *Blood* 2021; 138(Suppl 1):2001.
- [33] Li Z, Shao Z, Xu Y, Shen L, Chen G, Zhang Y, Chu Y. Subclasses of warm autoantibody IgG in patients with autoimmune hemolytic anemia and their clinical implications. *Chin Med J (Engl)* 1999; 112(9):805-8.
- [34] Zhang Y, Chu Y, Shao Z. The clinical implications of IgG subclass in 84 patients with autoimmune hemolytic anemia. *Zhonghua Xue Ye Xue Za Zhi* 1999; 20(10):524-6.
- [35] Malisan F, Brière F, Bridon JM, Harindranath N, Mills FC, Max EE et al. Interleukin-10 induces immunoglobulin G isotype switch recombination in human CD40-activated naive B lymphocytes. *J Exp Med* 1996; 183(3):937-47.
- [36] Ahmad E, Elgohary T, Ibrahim H. Naturally occurring regulatory T cells and interleukins 10 and 12 in the pathogenesis of idiopathic warm autoimmune hemolytic anemia. *J Investig Allergol Clin Immunol* 2011; 21(4):297-304.
- [37] Toriani-Terenzi C, Pozzetto U, Bianchi M, Fagiolo E. Cytokine network in autoimmune haemolytic anaemia: new probable targets for therapy. *Cancer Detect Prev* 2002;26(4):292-8.
- [38] Rasquinha MT, Sur M, Lasrado N, Reddy J. IL-10 as a Th2 Cytokine: Differences Between Mice and Humans. *J Immunol* 2021; 207(9):2205-15.

- [39] Fagiolo E, Terenzi CT. Enhanced IL-10 production in vitro by monocytes in autoimmune haemolytic anaemia. *Immunol Invest* 1999; 28(5-6):347-52.
- [40] Sutterwala FS, Noel GJ, Salgame P, Mosser DM. Reversal of proinflammatory responses by ligating the macrophage Fcγ receptor type I. *J Exp Med* 1998; 188(1):217-22.
- [41] Wang LX, Zhang SX, Wu HJ, Rong XL, Guo J. M2b macrophage polarization and its roles in diseases. *J Leukoc Biol* 2019; 106(2):345-58.
- [42] Toriani-Terenzi C, Fagiolo E. IL-10 and the cytokine network in the pathogenesis of human autoimmune hemolytic anemia. *Ann N Y Acad Sci* 2005; 1051:29-44.
- [43] Clynes R, Ravetch JV. Cytotoxic antibodies trigger inflammation through Fc receptors. *Immunity* 1995; 3(1):21-6.
- [44] Pricop L, Redecha P, Teillaud JL, Frey J, Fridman WH, Sautès-Fridman C, Salmon JE. Differential modulation of stimulatory and inhibitory Fc γ receptors on human monocytes by Th1 and Th2 cytokines. *J Immunol* 2001; 166(1):531-7.
- [45] te Velde AA, Huijbens RJ, de Vries JE, Figdor CG. IL-4 decreases Fc γ R membrane expression and Fc γ R-mediated cytotoxic activity of human monocytes. *J Immunol* 1990; 144(8):3046-51.
- [46] Fries LF, Brickman CM, Frank MM. Monocyte receptors for the Fc portion of IgG increase in number in autoimmune hemolytic anemia and other hemolytic states and are decreased by glucocorticoid therapy. *J Immunol* 1983; 131(3):1240-5.
- [47] Munawara U, Small AG, Quach A, Gorgani NN, Abbott CA, Ferrante A. Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of *Candida albicans* in human macrophages: A control point in anti-microbial immunity. *Sci Rep* 2017; 7(1):4050.
- [48] Spencer LA, Weller PF. Eosinophils and Th2 immunity: contemporary insights. *Immunol Cell Biol* 2010; 88(3):250-6.
- [49] Long H, Liao W, Wang L, Lu Q. A Player and Coordinator: The Versatile Roles of Eosinophils in the Immune System. *Transfus Med Hemother* 2016; 43(2):96-108.
- [50] Tamura J, Jinbo T, Murata N, Itoh K, Murakami H, Take H et al. Autoimmune hemolytic anemia with eosinophilia in elderly patient. *Nihon Ronen Igakkai Zasshi* 1996; 33(8):603-6.
- [51] Sweidan AJ, Brys AK, Sohn DD, Sheth MR. Diagnostic and therapeutic considerations in idiopathic hypereosinophilia with warm autoimmune hemolytic anemia. *J Blood Med* 2015; 6:257-60.

- [52] Kuk JS, Maceachern JA, Soamboonsrup P, McFarlane A, Bengner A, Patterson W et al. Chronic eosinophilic leukemia presenting with autoimmune hemolytic anemia and erythrophagocytosis by eosinophils. *Am J Hematol* 2006; 81(6):458-61.
- [53] Bhaumik S, Basu R. Cellular and Molecular Dynamics of Th17 Differentiation and its Developmental Plasticity in the Intestinal Immune Response. *Front Immunol* 2017; 8:254.
- [54] He HS, Xie YH. Change and significance of Th17 cells and its secretive IL-17 in patients with autoimmune hemolytic anemia. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2011; 27(6):662-3.
- [55] Hall AM, Zamzami OM, Whibley N, Hampsey DP, Haggart AM, Vickers MA, Barker RN. Production of the effector cytokine interleukin-17, rather than interferon- γ , is more strongly associated with autoimmune hemolytic anemia. *Haematologica* 2012; 97(10):1494-500.
- [56] Xu L, Zhang T, Liu Z, Li Q, Xu Z, Ren T. Critical role of Th17 cells in development of autoimmune hemolytic anemia. *Exp Hematol* 2012; 40(12):994-1004.e4.
- [57] Rocamora-Reverte L, Melzer FL, Würzner R, Weinberger B. The Complex Role of Regulatory T Cells in Immunity and Aging. *Front Immunol* 2021; 11:616949.
- [58] Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol* 2012; 3:51.
- [59] Mqadmi A, Zheng X, Yazdanbakhsh K. CD4+CD25+ regulatory T cells control induction of autoimmune hemolytic anemia. *Blood*. 2005;105(9):3746-3748.
- [60] Mullins GN, Valentine KM, Al-Kuhlani M, Davini D, Jensen KDC, Hoyer KK. T cell signaling and Treg dysfunction correlate to disease kinetics in IL-2R α -KO autoimmune mice. *Sci Rep* 2020; 10(1):21994.
- [61] Garratty G. Drug-induced immune hemolytic anemia. *Hematology Am Soc Hematol Educ Program* 2009:73-9.
- [62] Gassner FJ, Weiss L, Geisberger R, Hofbauer JP, Egle A, Hartmann TN et al. Fludarabine modulates composition and function of the T cell pool in patients with chronic lymphocytic leukaemia. *Cancer Immunol Immunother* 2011;60(1):75-85.
- [63] Fattizzo B, Zaninoni A, Gianelli U, Zanella A, Cortelezzi A, Kulasekararaj AG, Barcellini W. Prognostic impact of bone marrow fibrosis and dyserythropoiesis in autoimmune hemolytic anemia. *Am J Hematol* 2018;93(4):E88-E91.

- [64] Fattizzo B, Michel M, Zaninoni A, Giannotta J, Guillet S, Frederiksen H et al. Efficacy of recombinant erythropoietin in autoimmune hemolytic anemia: a multicenter international study. *Haematologica* 2021; 106(2):622-5.
- [65] Zaninoni A, Fattizzo B, Giannotta J, Fermo E, Bianchi P, Cecchi N, Barcellini W. p1526: Autoimmune hemolytic anemias: relationship between single nucleotide polymorphisms of cytokine genes and clinical/hematological parameters. *Hemasphere* 2022; 6(suppl):1407-8.
- [66] Qi H. T follicular helper cells in space-time. *Nat Rev Immunol* 2016; 16(10):612-625.
- [67] Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* 2016; 271(1):246-259.
- [68] Huang Y, Chen Z, Wang H, Ba X, Shen P, Linet W al. Follicular regulatory T cells: a novel target for immunotherapy?. *Clin Transl Immunology* 2020; 9(2):e1106.
- [69] Gao Y, Jin H, Nan D, Yu W, Zhang J, Yang Y et al. The Role of T Follicular Helper Cells and T Follicular Regulatory Cells in the Pathogenesis of Autoimmune Hemolytic Anemia. *Sci Rep* 2019; 9(1):19767.
- [70] Valentine KM, Davini D, Lawrence TJ, Mullins GN, Manansala M, Al-Kuhlani M et al. CD8 Follicular T Cells Promote B Cell Antibody Class Switch in Autoimmune Disease. *J Immunol* 2018; 201(1):31-40.
- [71] Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* 2012; 209(2):243-50.
- [72] Liu Y, Wan S, Sun X, Zhang W, HE J, Liu C. Effect of Follicular Helper T Cells on the Pathogenesis of Autoimmune Hemolytic Anemia. *Labeled Immunoassays and Clinical Medicine* 2016; 23(2):126-8.
- [73] Ding T, Su R, Wu R, Xue H, Wang Y, Su R et al. Frontiers of Autoantibodies in Autoimmune Disorders: Crosstalk Between Tfh/Tfr and Regulatory B Cells. *Front Immunol* 2021; 12:641013.
- [74] Zhu Q, Rui K, Wang S, Tian J. Advances of Regulatory B Cells in Autoimmune Diseases. *Front Immunol* 2021; 12:592914.