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Review Article
Interaction between Th9 cells, Interleukin-9 and Oxidative Stress in Chronic Lymphocytic Leukemia

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Abstract

Chronic Lymphocytic Leukemia (CLL) is a cancer of B lymphocytes, originating within the bone marrow and poignant most body tissues. The T helper 9 (Th9) cell is the chronic T helper cells related to Th2 immunity and secretes IL-9 which make these cells contribute to each protecting immunity and immunopathological malady. This review focused on the role of Th9 cells and oxidative stress in cancer immunity especially in CLL. Also, it summarizes the relation between oxidative stress and T-cell function and the interaction between Th9 cells, interleukin-9 (IL-9) and oxidative stress in CLL. The T helper 9 cells have pleiotropic effects in anti-tumor immunity. They activate innate and adaptive anti-cancer immunity and induce tumor cell cycle arrest and apoptosis. However in CLL, IL-9 seems to participate in the pathogenesis of the disease by both enhancing proliferation and inhibiting apoptosis of cancer cells. Oxidative stress has been linked to the etiology and development of carcinogenesis. The redox state at the interface between Antigen Presenting Cell (APC) and T-cells within the immunological synapse also impacts on T-cell activation, proliferation and differentiation. Higher level of oxidative stress was observed in CLL patients that correlated with bad prognosis and the disease outcome. Oxidative stress can promote the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) which up regulates the expression of proinflammatory cytokines, including IL-9, which may be involved in the pathogenesis of CLL.

Key words: Chronic lymphocytic leukemia, T helper 9 cell, interleukin-9, oxidative stress, prognosis, immunity

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INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a slow growing blood cancer that affects developing certain white blood cells (called lymphocytes). The term "chronic" in CLL comes from the fact that it typically progresses more slowly than other types of leukemia. The term "lymphocytic" in CLL means that it starts from cells that will become B-lymphocytes in the bone marrow. The CLL most commonly affects older adults[1].

Immune cells could play a dual role in cancer development and survival. They can make physiological conditions within cancer tissue to either prevent tumor growth or promote tumor progression[2]. Effector immune cells are short-lived activated cells that defend the body against foreign substances and eliminate immunogenic cancer cells[2]. They include effector B cells that secrete antibodies and called plasma cells and activated T-cells which carry out cell-mediated responses including cytotoxic T-cells and helper T-cells[3]. Indeed, regulatory and effector immune cells have opposite effects on most human tumors[4]. Regulatory immune cells usually participate in maintenance of immune tolerance through suppression of immune responses in majority of cancers and also contribute to tumor progression and worse prognosis[5,6].

T helper 1 cells reveal anti-cancer effects through secretion of IFN-γ, which prevents tumor angiogenesis, enhances immunogenicity of cancer cells by up regulating the MHC I and II classes expression, recruits CD8 T and Natural Killer (NK) cells which were both responsible for tumor elimination and stimulates macrophages anti-cancer activity[7]. Conversely, tumor-infiltrating T helper 2 (Th2) cells promote tumor growth due to their secretion of IL-4 and IL-13 which promote the differentiation of tumor-infiltrating monocytes and macrophages into pro-angiogenic (M2-like) tumor-associated macrophages (TAMs)[8]. Compared with the other subsets of Th cells, Th9 cells which are defined by their secretion of interleukin-9 (IL-9) are a new addition[9] and recently that scientists have begun to understand the factors that control their development and function. Th9 cells develop from CD4 precursors in response to transforming growth[10] factor-β (TGFβ) and IL-4. Interleukin-9 enhances immunosuppressive potency of Treg cells by suppression of immunologic memory development[11]. The IL-9 has been linked with the promotion of certain cancers, particularly lymphomas and other hematological malignancies[12,13]. *In vitro* studies indicated that IL-9 promotes tumor growth by both enhancing proliferation and inhibiting apoptosis of tumor cells[14]. On the other hand, anti-tumor effect of Th9 cells was documented in a mouse model of melanoma which was superior to all other $CD4^+$ T-cell subsets tested, including Th1 and Th17 cells[15].

A balance between free radicals and antioxidants is critical for correct physiological perform[16]. If free radicals overwhelm the body's ability to control them, a condition referred to as oxidative stress ensues[17-20]. Oxidative stress is reported to be involved in induction of chronic inflammation, metastatic cancer and angiogenesis[21]. Oxidative stress can promote the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) which up regulates the expression of pro-inflammatory cytokines, including IL-9, which may be involved in the pathogenesis of cancer[22,23].

According to the previous findings, the exact role of IL-9 in tumor is not clear. Interleukin-9 was identified as the signature cytokine of Th9 cells[9]. Therefore in this review, recent findings about the interaction between Th9 cells, IL-9 and oxidative stress particularly in CLL were discussed.

CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

Chronic Lymphocytic Leukemia (CLL) is a sort of blood cancer that affects B cell lymphocytes. B cell lymphocytes originate within the bone marrow, develop within the lymph nodes and ordinarily fight infection by manufacturing antibodies[24]. It is called leukemia because the cancerous lymphocytes (or CLL cells) are found in the blood and in the bone marrow. It is described as 'chronic' because it lasts for a long time and the affected cells can mature partly, but not completely. These cells survive longer than normal cells and do not fight against infections, ranging from simple colds to pneumonia and other serious infections, as normal white blood cells do[1].

Incidence of CLL: The estimates for leukemia in the United States showed that, about 60,300 new cases were diagnosed with leukemia and about 24,370 deaths occur from all kinds of leukemia[25] in 2018. About 20,940 new cases were diagnosed with CLL and result in 4,510 deaths. The CLL accounts for about 25% of adult leukemia's in the western world. The common person's lifespan risk of obtaining CLL is regarding 1 in 175 (0.57%) and the 5-year survival rate is 81.7%. Over 75% of fresh diagnosed CLL patients were over the age of 50 (Fig. 1) and the majority was men. However teenagers and youngsters may also be affected in rare cases[25].

Prognostic factors for CLL: Nodular or interstitial pattern of spread of CLL cells, where the cells are in small groups, in the bone marrow usually indicated a better outlook for CLL than a diffuse pattern, where the cells are scattered throughout the

marrow[26]. Lymphocyte Doubling Time (LDT) is the time for blood lymphocytes to duplicate its count and LDT less than one year indicated a higher risk[26]. Serum levels of β2 microglobulin and lactate dehydrogenase (LDH) may estimate tumor burden, since high levels indicate a poorer prognosis[27].

Patients with the 17p or 11q deletions have the worst prognosis, with a median survival of 3 and 7 years, respectively, whereas patients with a normal cytogenetic status or trisomy 12 have a median survival of 9 years and those who carry the 13q deletion have a median survival of 11 years[28]. The prognostic significance in the 17p deletion is related to the loss of the cell cycle regulating protein p53. The del (17p) can occur with or without TP53 mutations on the second allele and TP53 mutations can also be present without deletions[29]. Other adverse prognostic factors, such as; high expressions of CD38, unmutated IGHV and zeta-associated protein-70 (ZAP-70) expression are observed in higher frequency in patients with del (17p)[30]. Expression level of CD38 on T-cells negatively correlated with treatment-free survival in male CLL patients, but not in female patients[31,32].

A significant prognostic factor for CLL is the mutation status of IGHV region. The CLL can evolve either from pre-germinal or post-germinal B cells. The B cells passed through the germinal center have a mutated phenotype, where somatic mutation are made for encoding antibodies after antigen exposure[33]. A CLL cell is considered as mutated if IGHV rearrangement citing sequences are more than 2% non-homologous to the corresponding germ-line sequence. Unmutated IGHV status (45% of patients) is the more aggressive form of the disease as well as unmutated CLL cells are more adhesive and less migratory, that causes adenopathy and tumor burden[34].

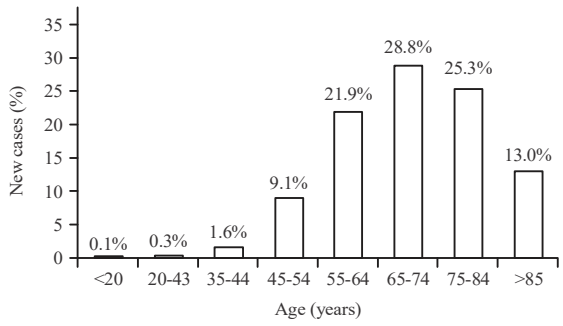

Fig. 1: Majority of CLL patients are over the age of 50 years, More than 75% of newly diagnosed CLL patients are over the age of 50
Source: Siegel *et al.*[25]

CD38, a transmembrane glycoprotein expressed on normal B cells, plays an important role in apoptosis[35]. However CD38 promoted survival and proliferation of B cells on their way to and after neoplastic transformation. More than 30% proportion of CD38-expressing CLL cells (i.e., 30% of patients) indicated worse prognosis[36].

More than 20% positive CLL cells for ZAP-70 (60% of patients), a cytoplasmic tyrosine Kinase expressed by T-cells and not expressed by B cells, is associated with a lower survival rates and worse prognosis[37]. Prognostic factors are summarized in Table 1.

Treatment of CLL: The CLL is a disease that can be monitored for a number of years without treatment with a "watch and wait" strategy especially in patients who are in earlier stages (Rai 0-II or Binet A)[39]. Treatment can improve the disease outcome for patients with Rai III and IV or Binet B and C stages. Early treatment of asymptomatic CLL patients doesn't result in any survival benefit and treatment is necessary only after the presence of symptoms associated with CLL like "B" symptoms or progressive complications from hepatomegaly, splenomegaly, lymphadenopathy, worsening anemia and/or thrombocytopenia, autoimmune cytopenias and LDT less than one year[40].

The patient's risk factors determined the best type of first-line treatment. Patients with adverse prognostic factors may benefit from combination chemotherapy for CLL or

Table 1: Prognostic factors in CLL. The prognostic information can be categorized into 3 categories according to their risk (good, intermediate and bad)

Prognostic factor	Good	Intermediate	Bad
Stage	Binet A (Rai 0)	Binet B (Rai I-II)	Binet C (Rai III-IV)
Sex	Female	-	Male
LDT	Slow (<1 year)	-	Rapid (≥1 year)
Bone marrow biopsy pattern	Nodular or interstitial	-	Diffuse
Chromosomes	Del (13q)	Trisomy 12	Del (17P)
		Normal cytogenetics	Del (11q)
			Complex karyotypes
IGHV genes	Hypermutated	-	Unmutated
ZAP expression	Low (<20%)	-	High (≥20%)
CD38 expression	Low (<30%)	-	High (≥30%)
β2 microglobulin and LDH levels	Normal	-	Raised

Source: Baliakas *et al.*[38]. LDT: Lymphocyte doubling time, IGHV: Immunoglobulin heavy variable, ZAP: Zeta-associated protein, LDH: Lactate dehydrogenase

allogeneic transplantation early in their disease course, as this is the best curative therapy[39]. Currently, no prospective data exist to support the early treatment of patients with adverse prognostic features, although it seems likely that attainment of complete molecular remission may lead to longer survival. Treatment for older patients and those with no adverse prognostic features should focus on minimizing the disease symptoms[41].

Treatment of CLL is diverse and individualized. The routine treatment is a combination of an anti-CD20 antibody (rituximab, GA101 (obinutuzumab) or ofatumumab) and chemotherapy (fludarabine/cyclophosphamide (FC), bendamustine and chlorambucil). The therapeutic choice is mainly determined by physical condition (fitness and comorbidity) of the patient, prognostic factors and Rai stage of CLL[39].

CD4+ T HELPER (TH) CELLS

The adaptive immune system comprises of two arms, effector T-cell and effector B cell arms[42]. The T-cells originated in bone marrow and developed into progenitor cells, then migrated to the thymus to mature into naïve T-cells (i.e., mature T-cells that have never been exposed previously to antigen). They circulate to secondary lymphoid sites to be activated by recognizing antigens presented on the Major Histocompatibility Complex (MHC) molecule by Antigen-presenting Cells (APC). Dendritic Cells (DC) are the main APC, but macrophages and B cells can also do this role. The APCs engulf antigens and express epitopes at their surface on MHC class molecules[43]. The APCs create an immunological synapse (Fig. 2) with T-cells: First, the T-Cell Receptor (TCR) interacts with antigen-bearing MHC molecules; second, the co-stimulatory molecules, especially CD80 and CD86, bind co-receptor CD28 on surface of T-cells and third, naïve T-cells differentiate into effector T-cells under the effect of local cytokines[44].

$CD4^+$Th cells do not neutralize infections, but coordinate the adaptive immune response in infection, inflammation and autoimmunity. These include subtypes, the most important are; Th1: which mediate immune responses against intracellular pathogens and has a role in some autoimmune diseases, Th2: Which mediate host defense against extracellular parasites (helminths), Th9: Which is important in chronic allergic condition such as; asthma, Th17: which mediate immune responses against extracellular bacteria and fungi and participate in autoimmunity; regulatory T (Treg) cell, which maintain self-tolerance; and follicular helper T (Tfh) cell, which is the key helper cell for the B cell germinal centers. Each has its specific network of transcriptional regulators and unique cytokine profiles[10,45-47].

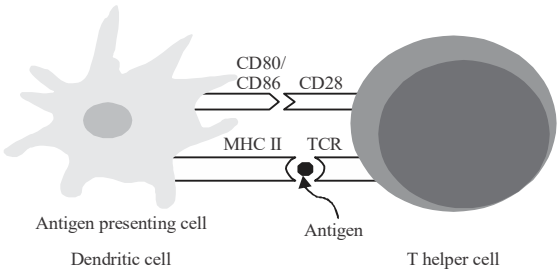

Fig. 2: Immunological synapse of T helper cells. Antigen-Presenting Cells (APCs) engulf antigens and express epitopes at their surface on Major Histocompatibility Complex (MHC) molecules to create an immunological synapse with T cells: First, T-cell receptor (TCR) interacts with antigen-bearing MHC molecules; second, the co-stimulatory molecules, especially CD80 and CD86, bind co-receptor CD28 on surface of T-cells and third, naïve T cells differentiate into effector T cells under the effect of local cytokines
Source: Gutcher and Becher[47]

T helper 1 cells reveals anti-cancer effects through secretion of IFN-γ, which prevents tumor angiogenesis, enhances immunogenicity of cancer cells by upregulating MHC I and II classes expression, recruits CD8 T and Natural Killer (NK) cells which were both responsible for tumor elimination and stimulates macrophages anti-cancer activity[7]. Conversely, tumor-infiltrating T helper 2 (Th2) cells promotes tumor growth due to their secretion of IL-4 and IL-13 which promoted the differentiation of tumor-infiltrating monocytes and macrophages into pro-angiogenic (M2-like) tumor-associated macrophages (TAMs)[8]. Compared with the other subsets of Th cells, Th9 cells, which are defined by their secretion of interleukin-9 (IL-9) are a recent addition[9] and recently the scientists have begun to understand the factors that control their development and function. Th9 cells develops from CD4 precursors in response to transforming growth[10] factor-β (TGFβ) and IL-4. Interleukin-9 enhanced immunosuppressive potency of Treg cells by suppression of immunologic memory development[11]. IL-9 has been linked with the promotion of certain cancers, particularly lymphomas and other hematological malignancies. *In vitro* studies indicated that IL-9 promoted tumor growth by both enhancing proliferation and inhibiting apoptosis of tumor cells[14]. The anti-tumor effect of Th9 cells was documented in a mouse model of melanoma which was superior to all other $CD4^+$ T cell subsets tested, including Th1 and Th17 cells[15].

Th9 cells

Function of Th9 cells: Th9 cell is closely related to Th2 cells due to plasticity between the two cell types. Thus, Th9 cell is

the chronic T helper cells related to Th2 immunity[48]. As IL-9 could be a pleiotropic protein, Th9 cells would possibly contribute to each protecting immunity and immunopathological malady[49]. However, Th9 cells are n't the sole source of IL-9 during an immune response and accordingly, the relative importance of Th9 cells *in vivo* has been difficult to define[10]. Th9 cells promote allergic disease[50], autoimmune disease[51,52] and transplant rejection[53]. Among the beneficial role of Th9 cell is its ability to initiate immunity against parasitic infection[54] and effective anti-tumor immunity[55].

Role of T helper cells in tumor immunity

Role of Th1, Th2 and Th17 cells in tumor immunity: Th1 cells, through its secretion of interferon-γ (IFNγ), enhance $CD8^+$ cytotoxic T lymphocytes (CTLs) activity to be the most efficient $CD4^+$ T-cell subset in generating anti-tumor immunity[56]. Anti-tumor effect of Th2 cells is mediated by recruitment of tumoricidal eosinophils and macrophages into the tumor microenvironment[57]. Th2 cells are also known to inhibit cell-mediated immunity by secreting IL-4 and IL-10 to promote tumor progression[58]. Tumor-promoting and tumor-suppressing activities have been reported for Th17 cells[59,60]. Th17 cells have also been shown to better mediate destruction of B16 melanoma than Th1 cells. This Th17-mediated anti-tumor result is critically addicted to conversion of Th17 to Th1 cells and/or achievement of alternative arms of the system and IL-17A solely marginally or partly contributes to this effect[61]. Increased level of circulating Th17 cells and serum levels of IL-17 and IL-21 were reported in acute leukemia patients, which returned to normal levels in patients who achieved complete remission after induction therapy[62].

Role of IL-9 and Th9 cells in tumor immunity: The role of Th9 cells and IL-9 in cancer immunity might be of interest in the therapy of malignancy. IL-9 can drive tumor progression in a number of solid tumors, including lung adenocarcinoma and melanoma (Fig. 3)[63]. Innate anti-cancer immunity of Th9 cells

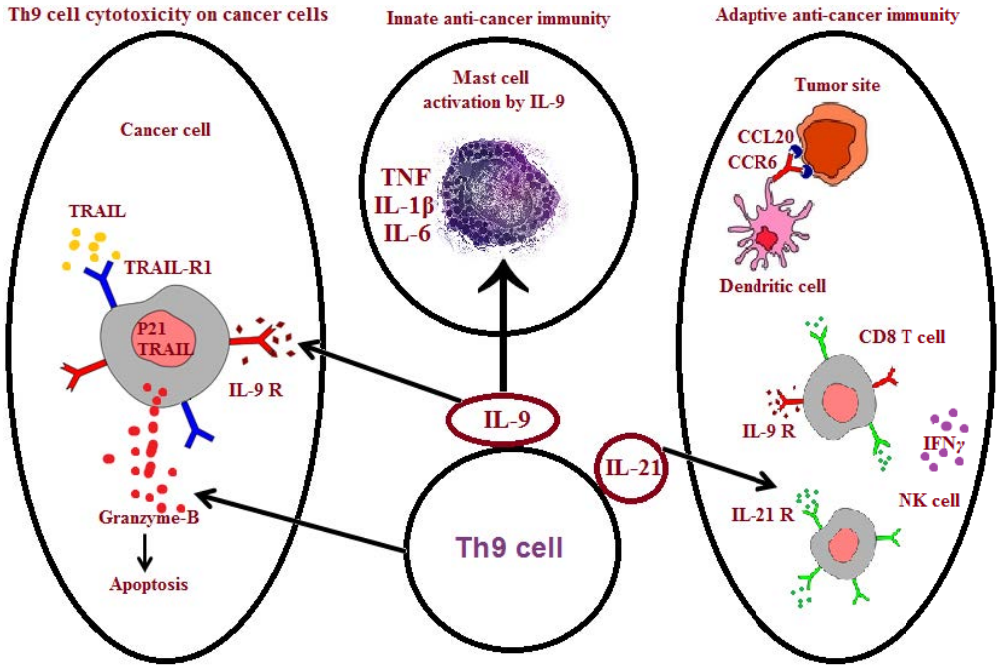

Fig. 3: Anti-tumor effect of Th9 cells. Innate anti-cancer immunity of Th9 cells is found through activation of mast cells by their secretion of IL-9. Adaptive anti-cancer immunity of Th9 cells is found through induction of the expression of CC-chemokine ligand 20 (CCL20) within the tumor site and CC-chemokine receptor 6 (CCR6) on leukocytes by IL-9 to facilitate tumor infiltration and rejection. Also IL-9 induces elevated frequencies of tumor-specific CD8 T-cells in the tumor tissues. IL-21,secreted by Th9 cells, stimulates IFNγ secretion from both natural killer (NK) and CD8 T-cells, which were both responsible for tumor elimination. Through the production of Granzyme-B, Th9 cells trigger cancer cell death. Th9-derived IL-9 leads to upregulation of p21 and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), thus inducing tumor cell cycle arrest and apoptosis

Source: Vargas *et al.*[63]

was found to be through activation of mast cells by their secretion of IL-9. Adaptive anti-cancer immunity of Th9 cells was found to be through induction of the expression of C-C chemokine ligand 20 (CCL20) within the tumor site and C-C-chemokine receptor 6 (CCR6) on leukocytes by IL-9 to facilitate tumor infiltration and rejection. Also, IL-9 induced elevated frequencies of tumor-specific CD8 T-cells in the tumor tissues[63]. IL-21, secreted by Th9 cells, stimulates IFNγ secretion from both natural killer (NK) and CD8 T cells, which were both responsible for tumor elimination[64]. Through the production of granzyme-B, Th9 cells trigger cancer cell death[15]. Th9-derived IL-9 leads to upregulation of p21 and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), thus inducing tumor cell cycle arrest and apoptosis[65].

Th9 cell-driven activation of innate anti-cancer immunity: The anti-tumor effect of Th9 cells was investigated in a mouse model of melanoma[15] (Table 2). They documented that anti-cancer efficacy of Th9 cells was superior to all other $CD4^+$ T-cell subsets tested, including Th1 and Th17 cells. The authors found, in contrast to published studies in hematological cancers, that IL-9 blockade using neutralizing antibodies or absence of IL-9 receptor signaling prevented the beneficial effect of adoptive Th9 cell transfer. Also authors found additional IL-9 limited Lewis lung carcinoma tumors *in vivo* but not *in vitro*. They injected Th9 cells into tumor-bearing mice, which lack T and B cells and found that the anti-tumor potential of Th9 cells was conserved in the absence of adaptive immunity, suggested that other immune effectors are involved in the anti-cancer effects observed. Also, Th9 cells were illustrated to prevent tumor growth through activation of mast cells by their secretion[66] of IL-9.

Th9 cell-driven activation of adaptive anti-cancer immunity: Enhanced infiltration with CD4 and CD8 cells as well as Dendritic Cells (DCs) was found after Th9 cell transfer to prevent the development of lung tumor in mice, suggested the induction of an adaptive immune response[67]. This hypothesis was further reinforced by the observation that CD44 expression on T-cells was upregulated in mice receiving Th9 cells. They further found that IL-9 can induce the expression of CC-chemokine ligand 20 (CCL20) within the tumor site and CC-chemokine receptor 6 (CCR6) on leukocytes to facilitate tumor infiltration and rejection. They noted elevated frequencies of tumor-specific CD8 T cells in the tumor tissues, in contrast to control mice or Th1-treated mice[67]. Depleting CD8 T cells using anti-CD8 antibodies prevent the anti-cancer effects of the transferred Th9 cells. The ability of Th9 cell-derived IL-9 to drive the activation of anti-cancer CD8 T cells were further confirmed recently[68].

It was found that IL-1β enhanced IL-9 and IL-21 secretion levels from differentiating Th9 cells[69]. These observations relied on the signal transducers and activators of transcription-1 (STAT1)-dependent activation of the transcription factor interferon-regulatory factor 1 (IRF1), which bound to both IL-9 and IL-21 promoters in differentiating Th9 cells. IL-21 stimulates IFNγ secretion from both NK and CD8 T cells, which were both responsible for tumor elimination. Th9 cells also secrete IL-3 that could favor DC survival[64].

Th9 cell cytotoxicity on cancer cells: Through the production of granzyme-B, Th9 cells trigger cancer cell death[15]. Th9-derived IL-9 leads to upregulation of p21 and TNF-related apoptosis-inducing ligand (TRAIL), thus inducing tumor cell cycle arrest and apoptosis upon activation[65] of IL-9R.

Pro-tumor activity of Th9 cells: The anti-cancer effects of Th9 cells might be restricted to solid tumors, such as; melanoma and lung adenocarcinoma. Numbers of Th9 cells in the blood and the skin are significantly reduced in those patients when compared with healthy individuals. The IL-9 has been linked with the promotion of certain cancers, particularly lymphomas and other hematological malignancies. *In vitro* studies indicated that IL-9 promoted tumor growth by both enhancing proliferation and inhibiting apoptosis of tumor cells[14]. It is unclear whether Th9 cells are a relevant source of IL-9 during lymphoma development. The basis for IL-9 and potentially Th9 cells, to have discriminatory effects on different cancers is unexplored but could be linked to the differential expression of IL-9R on the cancer cell type[10].

Table 2: Role of Th9 cell and IL-9 in tumor immunity

Type of cancer	Findings	References
Melanoma	Anti-cancer efficacy of Th9 cells was superior to Th1 and Th17 cells	Purwar *et al.*[15]
Lewis lung carcinoma	IL-9 limited Lewis lung carcinoma tumors *in vivo* but not *in vitro*	Purwar *et al.*[15]
Lung tumor	Th9 cell induces an adaptive immune response	Lu *et al.*[67]
Lymphoma	IL-9 promotes tumor growth by both enhancing proliferation and inhibiting apoptosis of tumor cells	Chen *et al.*[14]
CLL	Th9 cell and IL-9 correlated with bad prognostic markers of disease and advanced stages	Sabry *et al.*[70]

CLL: Chronic lymphocytic leukemia

Prognostic role of T helper cells in CLL: Higher levels of Th1, Th2, Th17, regulatory T (Treg) cell have been detected in CLL patients when compared with healthy controls with no significant difference between progressive and non-progressive patients[71]. Increased frequency of T follicular helper (Tfh) cells was identified in CLL patients with higher levels found in advanced stages of the disease suggested its involvement in the pathogenesis and progression of CLL[72]. This imbalance of T-cell subsets in CLL indicates immune deregulation and increased risk for infections.

Prognostic role of Th9 cells in CLL: Malignant CLL cells secrete IL-4 that induces STAT-6 phosphorylation and activation to promote IL-9 upregulation and secretion[14]. Higher level of IL-9 gene expression and its protein level were detected in CLL patients[14]. The upregulated IL-9 was correlated to bad prognostic markers of CLL such as advanced stages, β2 microglobulin expression, ZAP-70 expression and immunoglobulin heavy chain variable region (IGHV) status of the patients[14]. The IL-9 participates in the pathogenesis of CLL by both enhancing proliferation and inhibiting apoptosis of cancer cells[10]. Most recently, increased level of IL-9 along with no significant change in Th9 cell frequency in CLL was detected that speculated other sources for IL-9 other than Th9 cells during CLL development[70]. Moreover, over expression of IL -9 at both its messenger ribonucleic acid (mRNA) and protein levels was described in CLL patients that correlated with worse prognosis of disease[73].

OXIDATIVE STRESS

The term 'oxidative stress' is defined as a state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them leading to potential cellular damage or as a shift to electron deficient (i.e., oxidized) equilibrium[74]. Most of the cells can tolerate a mild degree of oxidative stress, because they have sufficient antioxidant defense capacity and repair systems, which recognize and remove molecules damaged by oxidation[75]. Exercise of sufficient intensity and duration such as; moderate running can increase the formation of reactive oxygen and nitrogen species due to increase of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and Nitric Oxide (NO)[76]. Reactive Oxygen Species (ROS) are molecules that include oxygen free radical, which are more highly reactive than the original oxygen molecule, such as; superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$) or are stable non-radical oxidants, such as hydrogen peroxide (H_2O_2)[77].

The most important Reactive Nitrogen Species (RNS) are nitric oxide radical ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$)[78]. The $NO^{\bullet}$ is biosynthesized from L-arginine by the enzyme NO synthase (NOS) through two step oxidation of L-arginine to L-citrulline, with concomitant production of $NO^{\bullet}$ (Fig. 4). The formation of $ONOO^-$ from $NO^{\bullet}$ and $O_2^{\bullet -}$ is controlled by superoxide dismutase (SOD), which can lower the concentration of superoxide ions as shown in Eq. 1[79]:

$$NO^{\bullet}+O_2^{\bullet -}\rightarrow ONOO^- \quad (1)$$

Consequences of oxidative stress: The extent of damage to biological targets depends on a range of factors including the concentration of particular targets, the rate constant for reaction of oxidant with target, the location of the target relative to that of the oxidant, the occurrence of secondary damaging events like chain reactions, intra and inter-molecular transfer reactions and the possibility and extent of repair and oxidant scavenging reactions[80]. As mentioned previously, lipids, proteins, carbohydrates and DNA are all capable of reacting with ROS and antioxidants[81] (Fig. 5). Lipids have important structural and functional role in cell membranes. Oxidation of lipids is detrimental to the structure and function of cell membranes and proceeds by a free radical chain reaction[82]. Oxidized proteins are more sensitive to proteolysis and an increase in oxidized proteins may be responsible for the loss of their biochemical and physiological functions[83,84]. The DNA fragmentation is frequently seen in cells subjected to oxidative stress[85]. The ROS causes a non-specific oxidative degradation of

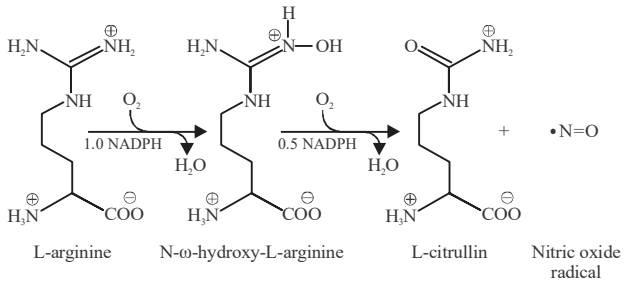

Fig. 4: The NOS-catalyzed reaction. The reaction consumes 1.5 mol of NADPH and 2 mol of oxygen per mol of L-citrulline formed. The proposed mechanisms involve an initial hydroxylation of L-arginine, leading to the formation of N-ω-hydroxy-L-arginine, which can also act as a substrate for NOS. This is followed by oxidation of the intermediate (N-ω-hydroxy-L-arginine) by using a single electron from NADPH to form L-citrulline and $NO^{\bullet}$

Source: Andrew and Mayer[79]

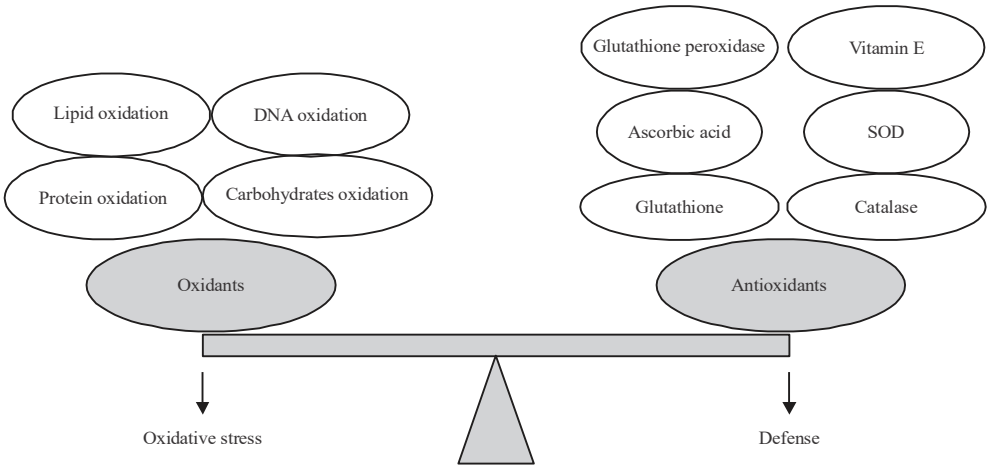

Fig. 5: Oxidative stress and antioxidant defense. Oxidative stress is a state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them leading to potential cellular damage as a result of oxidation of cellular components including lipids, proteins, carbohydrates and DNA. Endogenous antioxidant defenses include a network of enzymatic and non-enzymatic molecules that are usually distributed within the cytoplasm and various cell organelles

Sources: Sies *et al.*[74] and Schmidt *et al.*[87]. SOD: Superoxide dismutase

carbohydrates, accompanied by glycosidic hydrolysis leading to polysaccharide depolymerisation[86,87].

OXIDATIVE STRESS AND T-CELL FUNCTION

Phagocytic cells (e.g., neutrophils and macrophages) which are part of the innate immunity generate ROS and release them intracellularly and extracellularly in the form of "oxidative burst" to destroy pathogens and clear debris. The redox states at the interface between antigen presenting cell (APC) and T-cells within the immunological synapse also impacts on T-cell activation, proliferation and differentiation[88].

Prolonged exposure to high ROS or RNS concentrations can inhibit T-cell proliferation and lead to apoptosis[89,90]. Oxidative stress-induced modification to selective molecules involved in T-cell receptor (TCR) signaling reduces T-cells responses to activating stimuli. However, small amounts of ROS are important for T-cell function to induce the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and gene expression of cytokines and receptors essential for T-cell proliferation, e.g., IL-2 and IL-2 receptor[91].

Upon T-cells activation, they exhibit increased cell surface thiol levels suggested that a reduced extracellular environment is associated with T-cell activation. Depletion of reduced glutathione (GSH) in the absence of other small molecular weight thiols in human and murine T-cells attenuates proliferative response of T-cells to antigenic stimulation and reintroduction of GSH restores it suggested that GSH is the rate limiting step for T-cell proliferation[92].

In order for T-cells to synthesize intracellular glutathione (iGSH), they require cysteine. However, circulating levels of cysteine are low and T cells lack cysteine, the oxidized form of cysteine, transporter and so are unable to import cysteine. T-cells rely completely on APCs to provide them with cysteine. APCs, in contrast to T-cells have cystine transporter and reduces it to cysteine once it enters the cell which is then secreted into the extracellular space. Cysteine is thus accessible both intra- and extracellularly for T-cells and allows reducing environment necessary for their proliferation[93].

Treg cells account for 5-10% of $CD4^+$ T-cells[44]. One way in which Tregs exert their suppressive function is by altering the redox potential at the interface between DC and naïve T-cells at the immune synapse, leading to reduced cysteine availability for naïve T-cells[94]. In a more recent study, Treg cells were demonstrated to reduce extracellular cysteine concentration through cell to cell interaction between cytotoxic T-lymphocyte antigen-4 (CTLA-4) on Tregs and CD80/CD86 on dendritic cells, this inhibits DC iGSH synthesis and thus reduces extracellular cysteine generation[93]. In addition to this, Tregs compete with effector T-cells at the immune synapse for extracellular cysteine, which they preferentially catabolize to sulfate. This limits the amount of cysteine available for effector T-cells and necessary for their function and immune response[93].

Tregs can withstand higher levels of oxidative stress than other subsets of $CD4^+$ T-cells, Tregs secrete greater

levels of thioredoxin (Trx), a 12 kDa oxidoreductase enzyme substrate which contains a dithiol-disulfide active site, providing it with potential to scavenge ROS and metabolize H_2O_2[95].

The role of chronic oxidative stress in the polarization of Th2 responses was investigated[96]. The author found that exposure of primary human $CD4^+$ T-cells to low levels of $O_2^{\bullet-}$ leads to up-regulation of the entire family of Th2-specific cytokines, as well as modulation of chemokine receptors associated with T-cell polarization. Moreover, in the absence of APC, reactive protein carbonyl (PC) and MDA, which are oxidative products of proteins and lipids, promote differentiation towards a Th2 phenotype[95].

The NO can suppress the Th17 cells proliferation and function in human and mice via the down regulation of the expression of aryl hydrocarbon receptor which participates in the induction[97] of Th17. Unlike the effect of NO on Th17, NO markedly enhances the polarization and function of Th9 cells by elevating the expression of p53, which in turn increases the IL-2 production and activates the downstream events including phosphorylation of STAT5 and the expression of IRF4 and activation of IL-9 gene promoter[97].

Oxidative stress and cancer: Oxidative stress is reported to be involved in induction of allergies, autoimmune disorders, neurodegenerative diseases, chronic inflammation, metastatic cancer and angiogenesis[21,81,98]. Injury to the cellular elements like lipids, proteins, genes and blood vasculature is behind such alterations[99]. The early stages in cancer development and ageing are the results of damage to the DNA in a cell caused by oxidative stress. The involvement of free radicals with injury to tumor suppressor genes and proto-oncogenes counsel their role in development of various human cancers[99]. Major damage to these genes is the break in one or both strands of the DNA, due to the alterations in the purine or pyrimidine ring leading to genetic mutations as well as chromosomal alterations and thus contribute to cancer development along with alteration in apoptotic pathway. This adduct formation interferes with normal cell growth by causing genetic mutations and altering normal gene transcription[100].

Infection by *Helicobacter pylori* leads to increased production of ROS and RNS in human stomach which is thought to be causative factor of gastric cancer[101]. Antioxidants vitamins C, E and A from supplements have been shown to reduce the risk of breast cancer by suppressing the state of oxidative stress[102].

Oxidative stress and leukemia: Increased free radicals generation, especially $O_2^{\bullet-}$ in leukemia patients as well as increased antioxidant defense enzymes are indicative of mild oxidative stress. In previous studies in all types of leukemia, higher levels of superoxide dismutase (SOD) enzyme in leucocytes was reported, which decreased to normal when patients achieved remission. No relationship was reported between SOD level and the type of leukemias[103].

Likewise, there is GSH depletion in lymphocytes isolated from the blood of patients with CLL[104]. These findings recommend that there are alterations in the enzymatic antioxidant system, which can interfere in the direct removal of free radicals (pro-oxidants) and in the protection for biological sites[105].

Although oxidative stress has been linked to the etiology and development of carcinogenesis[106], many chemotherapeutic drugs have been shown to exert their biologic activity through induction of oxidative stress in affected cells.

Chemotherapy may increase oxidative stress beyond the level produced by the malignant cell. However, only a thin line separates the beneficial level and deleterious level of oxidative stress in treatment of cancer. For instance when the oxidative stress in malignant cells is doubled by pro-oxidant treatment, malignant cells conversely trigger lipid peroxidation, proteins oxidation and DNA oxidation leading to increased tumor burden[107]. Extra-mitochondrial antioxidants such as; vitamin C can act both as pro-oxidant and as antioxidant[108].

PROGNOSTIC ROLE OF OXIDATIVE STRESS IN CLL

Higher oxidative stress level was observed in CLL patients[109]. Scientists used d-ROMs, stands for derivatives of the reactive oxygen metabolites, test and Biological Antioxidant Potential (BAP) test to evaluate oxidative stress in CLL patients[109] and found an increased oxidative damage (d-ROMs test) and a reduced antioxidant level (BAP test) in CLL patients than healthy controls. Higher values of d-ROMs were correlated with increased TLC, Absolute Lymphocytic Count (ALC), β2-microglobulin, un-mutated IGHV, unfavorable cytogenetics (trisomy 12, del (11q) and del (17p)) and more advanced clinical stage (Table 3). Also they demonstrated higher oxidative stress levels in patients with shorter time to start treatment (TST) and reduced overall survival. The MDA is the end products of lipid peroxidation and its measuring is a significant indicator of oxidative stress. Also MDA represents as co-carcinogenic agent due to its high inhibitory effect on

Table 3: Role of oxidative stress parameters in prognosis of CLL

Parameters	Level	Findings	Reference
d-ROMs test (oxidative damage)	Increased	d-ROMs test correlated with increased TLC, absolute lymphocytic count (ALC), β2-microglobulin, un-mutated IGHV, unfavourable cytogenetics (trisomy 12, del(11q) and del(17p)) and more advanced clinical stage	D'Arena *et al.*[109]
BAP test (antioxidant level)	Decreased	BAP test decreased in patients with shorter time to start treatment (TST) and reduced overall survival	D'Arena *et al.*[109]
MDA	Increased	Malondialdehyde correlated with lymphocytic doubling and more advanced stage	Zelen *et al.*[111], Pujari and Jadkar[112] and El-Aziz *et al.*[113]
Catalase	Increased	Catalase associated with progression of the disease	Zelen *et al.*[111]
GPx, SOD activities and glutathione (GSH)	Decreased	No statistically significant difference among CLL patients with different Rai stages in these parameters	Bakan *et al.*[114]

protective antioxidant enzymes in the cell[110]. Increased plasma level of MDA was reported in CLL patients[111-113]. Higher plasma level of antioxidant enzyme catalase was detected in CLL patients when compared with controls; also, catalase activities were associated with progression of the disease[111]. In contrast, they didn't find differences in plasma activities of SOD and glutathione peroxidase (GPx) between CLL and control groups. Nevertheless, in a previous study, lower serum levels of GPx, SOD activities and glutathione (GSH) concentration as well as higher concentration of MDA are observed in CLL patients when compared with healthy subjects[114]. However, they found no statistically significant difference among CLL patients with different Rai stages in these parameters.

INTERACTION BETWEEN TH9 CELLS, IL-9 AND OXIDATIVE STRESS IN CLL

It was shown previously that IL-9 inhibited the production of ROS by human blood monocytes[115]. More recently, it was reported that oxidative stress can promote NFκB activation to further activate pathways necessary for gene expression and synthesis of antioxidant enzymes[22]. The NFκB activation also up regulates the expression of pro-inflammatory cytokines, including IL-9[23], which may be involved in the pathogenesis of CLL. Scientists hypothesized that increased levels of MDA can induce significant increases in inflammatory cytokines, that could increased the activation of lymphocytes and elevating[116] the ALC.

CONCLUSION

Th9 cells helps in protecting immunity and immune pathological malady in cancer immunity. Th9 cells may also contribute to the pathogenesis of CLL through its secretion of IL-9 by both enhancing proliferation and inhibiting apoptosis of cancer cells. Oxidative stress in CLL can promote NFκB activation to enhance IL-9 expression and secretion by Th9 cells and other sources.

SIGNIFICANCE STATEMENT

This study presented the interaction between Th9 cells, interleukin-9 and oxidative stress in chronic lymphocytic leukemia that can be beneficial as a potential therapeutic target. This study will help the researchers to uncover the critical areas of novel mechanisms for the Th9 cells and IL-9 in CLL that many researchers were not able to explore. Thus a new theory on Th9 cells and IL-9 effect in CLL may be arrived at.

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