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Table of contents

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Enriching $CD4^+$ $C25^+$ Foxp3 Regulatory T Cells: Hypothetical Scenarios for Strengthen the Immunity Against Pathogens

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Regulatory T cells-mediated suppression play a vital role in the maintenance of immune cell homeostasis. These regulatory T cells are influenced by the overexpression of phospholipase D enzymes. These enzymes are implicated in acute viral and chronic infections and cancer. This article sheds light on these topics, and discusses some hypothetical scenarios for strengthen the immunity against pathogens. These scenarios include the utility of small-molecule ligands such as halopemide compounds and butylated hydroxyltoluene as well as the coordination with the active sites of phospholipase D enzymes (HKD) and their fragmentation to diminish their activities.

Keywords: Regulatory T cells, Immune homeostasis, Foxp3, T helper cell, Phospholipase D, Immunotherapy.

1. Introduction

The immune system has developed to initiate an effective defense against pathogens and to diminish metabolic inflammatory disorders. To avoid immune-mediated pathology and unrestricted clonal expansion of responder T cells, the immune system has subsets of T cells, regulatory T cells (Tregs), that are dedicated to regulate or suppress other cells in the immune system. Tregs are vital to the maintenance of immune cell homeostasis which suppress the immune response launched by cytotoxic T cells helping to prevent or retard over-reactive responses that can lead to autoimmune diseases and auto-inflammatory disorders. Tregs are highly enriched in suppressor activity. In general, $CD4^+$ T cells are classified into two distinct linages: Treg and conventional T helper (T_H) cells. Conventional T_H cells control the adaptive immunity by activating, in an antigen-specific fashion, other effector cells such as $CD8^+$ cytotoxic T cells, B cells and macrophages. Tregs are divided into natural or adaptive Tregs; natural Tregs, produced by a normal thymus, are $CD4^+$ $CD25^+$ T cells which develop and emigrate from the thymus to achieve their vital role in immune homeostasis. Whereas, adaptive Tregs, formed by differentiation of naïve T cells outside the thymus, *i.e.* the periphery, are non-regulatory $CD4^+$ T cells which acquire the interleukin (IL)-2 receptor α-chain (CD25) expression outside of the thymus, and are induced by autoimmunity diseases and inflammation.[1-6]

ISSN 2590-0811
March/April 2020 | volume 3 | number 3

Treg cells constitutively express high levels of the cell surface molecule CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor–related protein (GITR). They also express a unique intracellular protein, the nuclear transcription factor Forkhead box P3 (Foxp3), which acts as a master regulator gene for inducing a Treg phenotype and lineage.[7] Expression of the Foxp3 protein is essential for Treg cell differentiation in the thymus and the level of Foxp3 protein expression in Treg cells is critical for suppressor function and defines the Treg cell lineage.[8,9]

2. The role of Treg cells in immune homeostasis

Many T cell types have immune regulatory function. The most important Treg cell subsets express the transcription factor Foxp3 and develop in the thymus or can be induced in peripheral sites including the mucosa-associate hymphoid tissue (MALT). Another important type of Treg cell secretes the immunosuppressive cytokine interleukin-10 (IL-10) and may develop from conventional $CD4^+$ T cells by activation in the presence of IL-10 or may develop from T helper 1 (T_H1) or T_H2 cell subsets. Other T cell subpopulations including natural killer T (NKT) cells and $CD8^+$ T cells can also exert suppressor functions in certain settings.

The family of $CD4^+$ T cells, including Treg cells, possesses a somatically rearranged T-cell receptor (TCR), which is functionally suppressive and in antigen-primed state already in the thymus. The hallmark feature of $Foxp3^+$ Treg cells is their constitutive high expression of CD25 which forms the high-affinity interleukin-2 (IL-2) receptor, and the co-inhibitory molecule CTLA-4. $Foxp3^+$ Treg cells are not only positively selected by the thymic self-peptide-major histocompatibility complex (MHC) molecule but also driven to differentiate into functionally competent antigen-specific suppressive cells within the thymus.[4] These properties allow Treg cells to quickly sense IL-2 produced by self-reactive T_H cells in the early stages of an immune response and co-localize to prevent their further activation, thereby dominantly suppressing potentially harmful activities of T_H cells and preventing autoimmunity.[10]

There is increasing evidence that Tregs manifest their function through a myriad of mechanisms that include cell–cell contact-mediated suppression, which inhibits stimulatory properties of dendritic cells (DC) and occurs via the engagement of Treg cell inhibitory receptors such as CTLA-4 and LAG-3 with CD80/86 and MHC molecules on the DC, respectively. The metabolic disruption of effector T (Teff) cells is another suppressive mechanism, which is mediated by Treg cell delivery of cAMP to effector Tcells via gap junctions. Besides, Treg cells secrete inhibitory cytokines such as IL-10, IL-35, and TGF-b1, which inhibit both T cells and DCs.[11] Figure 1 explains how Treg cells control the responder Tconv cells and induce of long-term tolerance.

Treg cells need functional stability and also adaptability to effectively control inflammation. Such functional adaptability plays an important role in Treg biology and in particular their ability to home to the sites of inflammation. However, in some cases, functional adaptability may proceed to lineage instability, causing the Treg cells to lose Foxp3 expression and become potentially pathogenic.[10]

ISSN 2590-0811
March/April 2020 | volume 3 | number 3

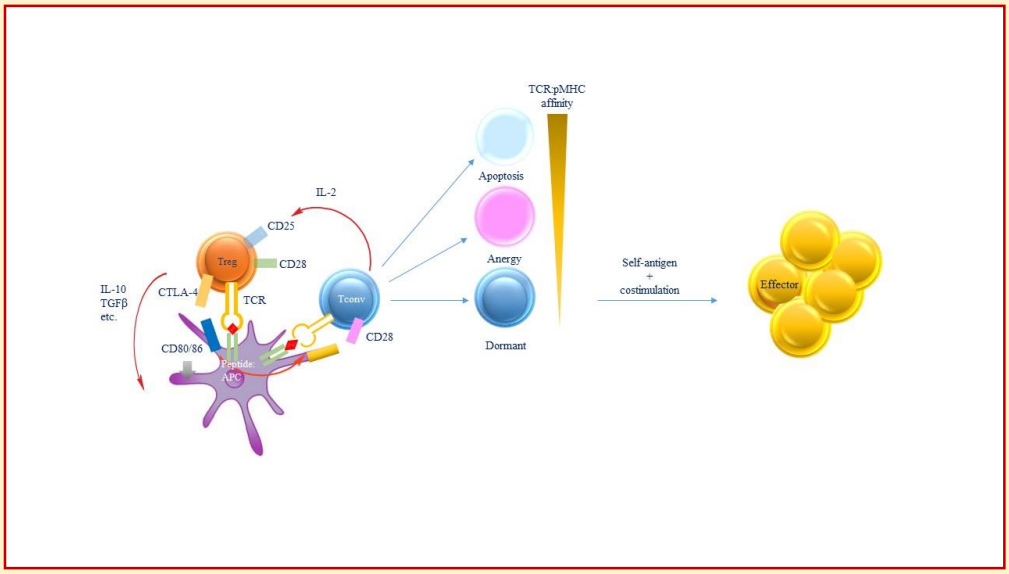


Figure 1. Regulatory T cell-mediated suppression: Treg cells activated by TCR stimulation downregulate the expression of CD80/86 on antigen presenting cells (APCs) via CTLA-4, reducing the availability of co-stimulation molecules for self-reactive Tconv cells. Treg cells express high levels of CTLA-4 and CD25, and control the cell fate and survival of self-reactive T cells establishing long-term tolerance. Tconv cells are differentially affected by Treg suppression. Tconv cells with high-affinity TCRs for the presented antigen die by apoptosis when stimulated without co-stimulation, those with intermediate affinities are driven to anergy, while those with low affinity survive and stay dormant as naïve Tconv cells.

3. The function of Foxp3

Foxp3 is the most important subset of Treg cells which develops in thymus and can be present in lymphoid tissues.[12] Mice and humans with genetic deficiencies of Foxp3 develop severe abnormalities of immune homeostasis. The expression of Foxp3 is mostly restricted to $CD4^+$ T cells, but some $CD8^+$ T cells do express Foxp3 as well.[13] The exact function of Foxp3 as a transcription factor is unknown. However, it has been proposed that Foxp3 may serve as a repressor of transcription with function of regulating the amplitude of the response of $CD4^+$ T cells to activation.[14] Furthermore, all human $CD4^+$ and $CD8^+$ T cells may upregulate Foxp3 and acquire suppressive properties upon activation.[15] Evidences have been shown that Foxp3 may act as both a transcriptional activator and repressor.[16,17]

In the lymph nodes and spleen, some of the transferred Treg cells had differentiated into interferon-γ (IFN-γ)-producing conventional effector T_H1; IL-4-producing T_H2 cells and IL-17-producing T_H17 cells that induced lung inflammatory disease in recipient mice.[18] Under certain condition, $Foxp3^+$ Treg cells can downregulate their expression of Foxp3, lose suppressor functions and manifest some of the functions of conventional effector T_H1, T_H2, T_H17 and T follicular helper (T_{FH}) subsets, which have been shown to suppress each other.[19,20] The key causes of this loss of Foxp3 expression include inflammatory environments

with high levels of cytokines that are normally involved in the induction of effector T cells, such as IL-6 and interferon-γ (IFNγ). Furthermore, Treg cell-specific deletion of certain transcription factors that are shared between Treg cells and effector cell subsets (e.g., the T_H2 cell-specific factor IRF4) results in impaired suppression of T_H2 cell responses by the Treg cells. T_H1, T_H2 and T_H17 cells may all produce IL-10, which suppresses proliferation and cytokine production by various T-cell subsets.[21-23] Reported studies suggested that Foxp3 may be expressed by T_H cells that produce pro-inflammatory cytokines such as IFN-*γ* and IL-14. Moreover, $Foxp3^+$ T cells may represent T_H that are not fully differentiated.[24,25]

$Foxp3^+$ T cells have been shown to influence the outcome of immune responses in a variety of tissues. Increasing Foxp3 promoter methylation levels may cause Treg cells to lose their immunosuppression function leading to abnormal immune tolerance through the downregulation expression of the Foxp3 protein.[26] During infection Treg cells execute a delicate balance act; preventing immunity would lead to the inability to clear the pathogen, whereas unrestrained immunity would lead to unwanted immune-mediated tissue destruction.

It is important to highlight the critical role of IL-10 during immune responses to microbial pathogens. Many bacterial and viral infections stimulate host IL-10 production, which is ultimately beneficial or harmful, depending upon the type of infection. Evidences showed that IL-10 impairs the initial T_H cell function that is required for effective virus-specific antibody production, and hence contributes to susceptibility to primary influenza virus infection.[23]

On the other side, dendritic cells (DCs) are extremely prone to extrinsic signals that modify the functions of antigen-presenting cells (APCs). Maturation of DCs induced by diverse pro-inflammatory conditions promotes immune responses. However, some signals induce tolerogenic functions in DCs, that help to moderate immune responses. While others, under certain conditions exhibit inherent tolerogenic properties, that help to promote tolerance to peripheral innocuous antigens (Ags). By extending tolerance initially established in the thymus, these functions of both “induced tolerogenic DCs” and “natural tolerogenic DCs” help to regulate autoimmune and other immune responses.[27]

4. The correlation between regulatory T cells and phospholipase D

Antigen stimulation of lymphocytes induces upregulation of the activity of phospholipase D (PLD) enzyme, which most of $CD4^+$ T cells require the activity of PLD. However, it was shown that the proliferation of $CD4^+CD25^+$ Treg cells do not require PLD activity, in contrary to the proliferation of mouse $CD8^+$ T cells and $CD4^+CD25^-$ T cells which PLD signaling is essential.[28] Inhibition of PLD signaling blocked effector T-cell proliferation after T cell–antigen receptor (TCR) engagement.

Based on this finding, one would thus expect that inhibiting PLD activity may be an effective strategy to preferentially strengthen the proliferation of $CD4^+$ $C25^+$ $Foxp3^+$ regulatory T cells function, which may lead to improve the immunity against pathogens. So, I further discuss hypothetically how it may be possible to inhibit PLD activity.

ISSN 2590-0811
March/April 2020 | volume 3 | number 3

5. Possible scenarios to enrich $CD4^+$ $C25^+$ $Foxp3^+$ regulatory T cells in a population of $CD4^+$ T cells

5.1. Phospholipase D

The phospholipase D superfamily (PLD) is phosphodiesterase enzyme that catalyzes the hydrolysis of a head group of amine-containing glycerophospholipid leaving phosphatidic acid as an intracellular signaling species, e.g., the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline. Mammalian cells encode two isoforms of PLD: PLD1 and PLD2. The sequence homology between plant and mammalian enzymes have a consistent molecular structure, with four small regions of sequence similarity of highly conserved amino acid sequences (I-IV). Motifs II and IV of these sequences contain duplicate catalytic sequences, termed the HKD domain, characterized by the sequence $HxKx_4Dx_6G(G/S)$, where x denotes amino acids between the histidine (H), lysine (K), and aspartic acid (D) residues (Figure 2).[29-31] These two HKD motifs confer hydrolytic activity to PLD, and are critical for its enzymatic activity both in vitro and in vivo. Hydrolysis of the phosphodiester bond occurs when these HKD sequences are in the correct proximity.

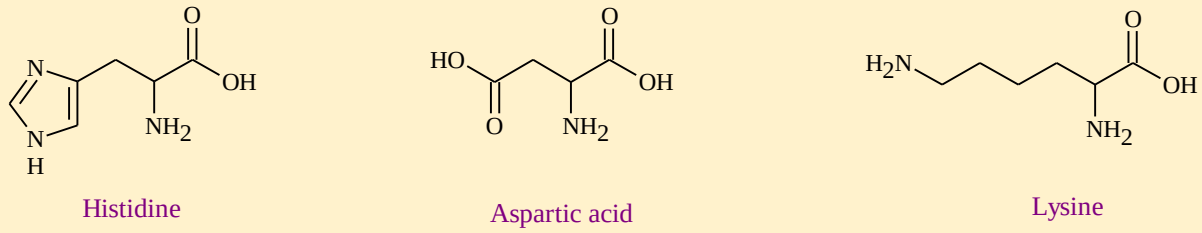


Figure 2. The structures of histidine, lysine and aspartic acid.

The PLD enzymes are ubiquitously expressed and are found in almost all mammalian tissues. PLD activities have been enriched from almost every region of the cell, including the plasma membrane, cytoplasm, nucleus and 'endomembranes', which consist of the Golgi, endoplasmic reticulum (ER) and endosomes.[29] The activity of PLD is regulated by lipids, neurotransmitters, G protein-coupled receptors, and other small molecules that bend to their corresponding domains on the enzyme. Most PLDs require phosphatidylinositol 4,5-bisphosphate (PIP_2), as a cofactor for activity.[31]

Evidences have been shown that overexpression of PLD isoforms is associated with enhanced tumorigenesis.[31,32] Many cancer types require PLD and its product, PtdOH, for sustained survival under stress conditions.[33]

In order to discuss the possible scenarios that lead to the inhibition of PLD signaling, it is important to point out the mechanism of PLD. A two-step "ping pong" reaction mechanism has been proposed for PLD-catalyzed hydrolysis, in which the histidine residue of one HKD motif acts as a nucleophile and attacks the

phosphate group of phosphatidyl-choline to form a covalent phosphatidyl histidine intermediate, while the choline-leaving group extracts a proton from the second HKD motif. In the subsequent step, a water molecule donates a proton to the second histidine, while the hydroxide ion hydrolyzes the phosphatidyl histidine intermediate to yield PtdOH. The lysines and aspartic acids may participate in the stabilization of phosphatidyl histidine intermediate.[31]

5.2 Discussion of possible scenarios

The available employed techniques for inhibition of the activity of PLD include the use of primary alcohols to produce the inactive phosphatidyl alcohols such as *n*-butanol to form phosphatidylbutanol ; RNA interference (RNAi) to silence the expression of PLD mRNA; and expression of catalytically inactive PLD mutant proteins that function in a dominant-negative manner.[31] In this context, I introduce some possible scenarios which may inhibit PLD activity, and may result, hence, in enriching the proliferation of $CD4^+$ $C25^+$ $Foxp3^+$ regulatory T cells function, that may strengthen the immunity against pathogens.

5.2.1 Halopemide compounds

As small-molecule ligands that have the potential to modulate PLD function and to prevent the lipid PtdOH formation, halopemide compounds and their analogs have proven to serve as potently dual PLD1/PLD2 inhibitors. Among the developed ligands was the Molecular Libraries Probe Production Centers Network probe (MLPCN) ML395 (VU0468809) (Figure 3). This compound is a potent because it provided a > 80-fold PLD2 selective allosteric inhibitor (cellular PLD1, IC50 >30,000 nM, cellular PLD2, IC50 = 360 nM), with improved physiochemical properties, no cytotoxicity, high CNS penetration and a favorable DMPK profile.[34] Furthermore, it has been found to possess interesting activity as an antiviral agent in cellular assays against a range of influenza strains (H1, H3, H5 and H7).

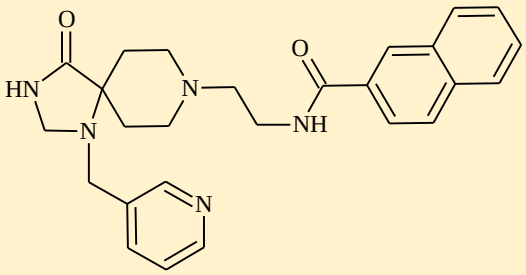
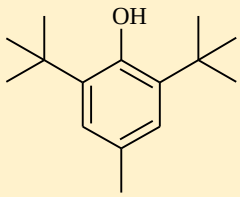


Figure 3. Structure of ML395.

ISSN 2590-0811
March/April 2020 | volume 3 | number 3

5.2.2 Butylated hydroxyltoluene

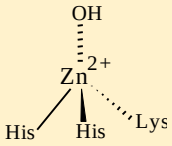
Butylated hydroxyltoluene (BHT) is an antioxidant compound. The steric bulkiness, hydrophobic property, and unique reactivity of the two *tert*-butyl (*t*-Bu) groups highlight the importance of BHT in terms of bioactivity. I speculate that the tree-dimensional orientations of the bulky *t*-Bu groups may play an important role in allosteric activation of PLD through hydrophobic interactions and may restrict its conformational flexibility. Thus, BHT may have the ability to inhibit the overexpression of PLD.



Butylated hydroxyltoluene

5.2.3 Coordination with HKD

The active sites of HKD are critical for PLD enzymatic activity. These amino acids have a highly affinity toward zinc (Zn^{2+}) ions. Zn^{2+} ions have the ability to coordinate electrostatically with the functional groups of His, Lys and Asp moieties. Such coordination by Zn^{2+} may restrict the activity of PLD and may diminish its function in signaling the proliferation of $CD4^{+}$ T cells that depend on its activity leading to selectively enrichment of $CD4^{+}$ $C25^{+}$ $Foxp3^{+}$ regulatory T cells.



Furthermore, Zn^{2+} ions could participate in complexation with phosphatidic acids through pyridine carboxaldoxime-Zn(II) complex, which may lead to their fragmentation (Figure 4). The proposed mechanism for such interaction can be explained as follow. In the first step, the oxygen of phosphate group would interact electrostatically with Zn(II) ion, which would allow the hydroxyl group of the oxime to be an extremely efficient nucleophile and may also polarize the scissile carbonyl group of one of the chain of PC. In the second step, deacylation of this chain would take place due to the attack of the hydroxyl group of the oxime

on the scissile carbonyl group of PC. Herein, the dual role of Zn(II) ion would come into play, i.e., the first would be an orientation or template effect, while the second is a concentration (of the nuceophile) effect at the site of reaction. Such reaction is pH dependent. Expectedly, at pH values above 6, Zn(II) ion would catalyze the hydrolysis of the acyl chain with large rate enhancements. The resulting Zn(II)-substrate complex would need to be decomposed by a reducing agent.

On the other hand, the peptides and proteins esters may be susceptible to catalytic action of Zn^{2+} ions in the presence of hydroxyl ions, due to the electron-withdrawing effect of positively charged zinc ions.

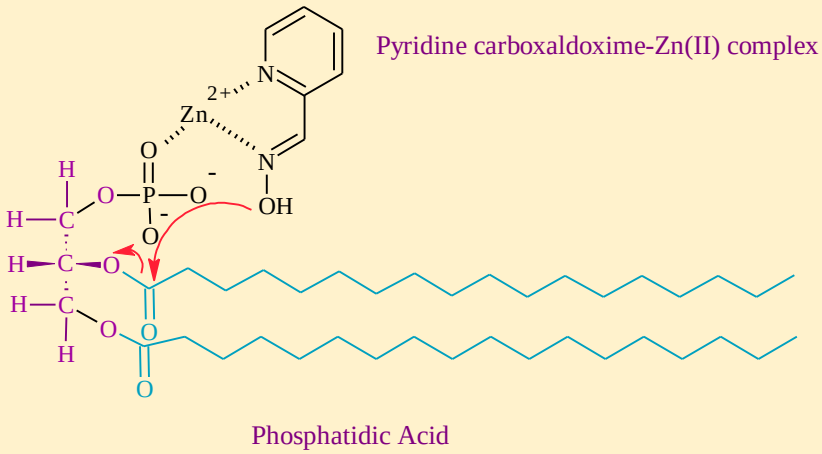


Figure 4. The complexation of phosphatidic acids by pyridine carboxaldoxime-Zn(II) complex.

In a realistic scenario, one would expect the role of Zn (II) ions: i) at physiological pH 7.4, they could partially inhibit the catalytic activity of PLD, and may enhance the preferentially expand $CD4^+$ $C25^+$ $Foxp3^+$ regulatory T cells at the expense of other $CD4^+$ T cells which in turn prevent autoimmune diseases in an effective way; ii) they could hinder phosphatidic acid activities; and iii) they could promote the hydrolysis of the peptides amides and esters.

5.2.4 Fragmentation of PLD

Using *cis*-β-hydroxoaquatriethylenetetramine cobalt(III), abbreviated $[Co(trien\ H_2O)OH]^{2+}$ may be another option to realize the target goal. It is well known that cobalt ions can exert catalytic properties on amide hydrolysis due to their efficiently of polarizing the carbonyl group of an amide bond. The expected scenario is that the rate-determining initial step may involve the replacement of a coordinated water molecule of cobalt(III) complex by the NH_2 group of histidine (Figure 5). The remaining OH-ligand may act as a

nucleophile to promote the hydrolysis to form the coordinated cobalt(III)-histidine and the fragmented part containing the active sites, lysine and aspartic acid. In this process, water and OH group are the two ligands replaced by the substrate. The resulting Co(III)-substrate complex would need to decompose by a reducing agent. It is expected that the promoter action of Co(III) complexes in such hydrolytic reaction would result in high rate enhancements relative to alkaline hydrolysis of the amide function. As a hypothetical scenario, Co(III) complexes may efficiently operative to inhibit the activity of PLD at physiological conditions.

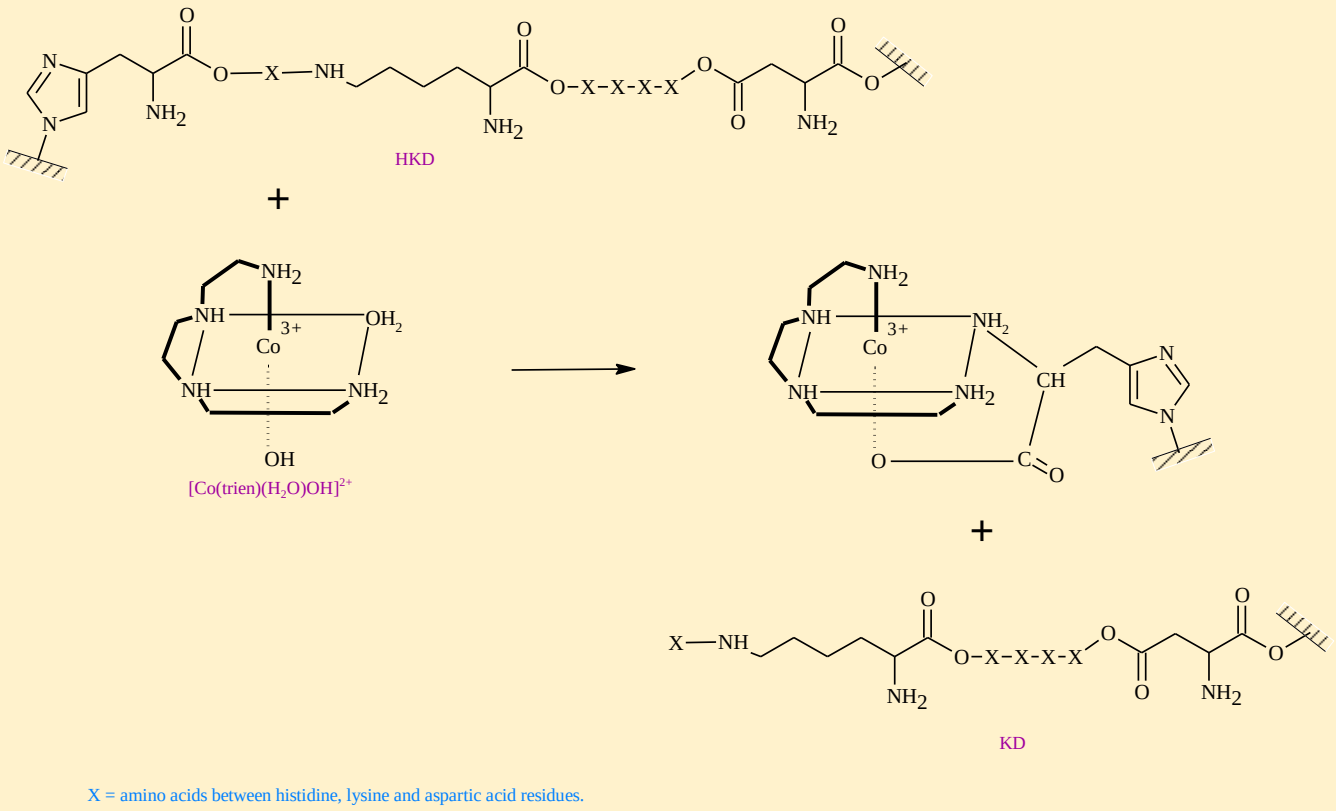


Figure 5. Fragmentation of PLD by *cis*-β-hydroxoaquatriethylenetetramine cobalt(III).

6. Conclusion

The potential roles of Treg cells in the maintenance of immunological self-tolerance and homeostasis paved the way for analysis of heterogeneity within Treg cells and their adaptation to and stability in various viral infections. The adverse implication of the overexpression of PLD enzymes on regulatory T cells opens

ISSN 2590-0811
March/April 2020 | volume 3 | number 3

an interesting research avenue in the development of therapies against pathogens and certain cancers. As hypothetical scenarios to inhibit PLD functions, I discussed the utility of ML 395 which has shown potent activity as an antiviral agent in cellular assays against a range of influenza strains (H1, H3, H5 and H7). Butylated hydroxyltoluene may play a role in allosteric activation of PLD through hydrophobic interactions and may restrict its conformational flexibility. In addition, the coordination with the active sites of PLD by Zn^{2+} ions may diminish its activity in signaling the proliferation of $CD4^+$ T cells that depend on its activity leading to selectively enrichment of $CD4^+$ $C25^+$ $Foxp3^+$ regulatory T cells. Using pyridine carboxaldoxime-Zn(II) complex as a coordination agent with PLD's product, phosphatidic acids, may be an option. Fragmentation of PLD may be an effective strategy that relies on employing *cis*-β-hydroxoaquatriethylene-tetramine cobalt(III) as a chelate compound. I expect this work will yield novel insights into the development of new therapeutic antiviral agents.

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ISSN 2590-0811
March/April 2020 | volume 3 | number 3

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