Role of X-Chromosome encoded miRNAs in Autoimmunity: Suppressing the suppressor and Female Predisposition

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Abstract

Role of skewed X inactivation may in part explain high prevalence of autoimmune diseases in women. Human X chromosome encodes approximately 7% of the total microRNAs identified. Recent studies have implicated overexpression of X-linked miRNA in women affected by the autoimmune diseases. Potential biological targets of them include critically important genes such as FOXP3, CTLA-4, PDCD1 and, members of CBL and SOCS family ubiquitin ligases, which play an important role in the immune suppressive mechanisms and maintenance of tolerance. X-linked miRNA mediated post transcriptional suppression of immunosuppressive genes is an open area of study with potential to better understand why women develop many autoimmune disorders more often than men.

Keywords: Autoimmune disease; miRNA; Female-predisposition; X-chromosome; Immune suppression

Abbreviations: AID: Autoimmune Diseases; APC: Antigen Presenting Cells; BCR: B Cell Receptor; CBL: c-Cbl, Cbl proto-oncogene, E3 ubiquitin protein ligase; CBLB: CBL-b, Cbl proto-oncogene, E3 ubiquitin protein ligase B; CTLA-4: Cytotoxic T lymphocyte antigen 4; DC: Dendritic Cells; FOXP3: Forkhead Box P3; GATA-3: GATA binding protein 3; miRNA: microRNA; MS: Multiple Sclerosis; PDCD1: PD-1, programmed cell death 1; PD-L1: Programmed cell Death Ligand 1; PD-L2: Programmed cell Death Ligand 2; SOCS: suppressor of cytokine signaling; TET: T-cell-specific T-box transcription factor; TCR: T Cell Receptor; TGF-β: Transforming Growth Factor, beta; TLR: Toll-Like Receptor; UTR: UnTranslated Region

Introduction

Autoimmunity is generally defined as misdirected immune responses which attacks body’s own healthy cells and tissues. Low level occurrence of autoimmunity is common to all men and women. However, Autoimmune Diseases (AID) occurs when the autoimmunity is progressed from benign to pathogenic levels. Pro-inflammatory environment, defective immune regulatory mechanisms and persistently hyperactive T and B lymphocytes can contribute to auto-reactive functions of immune system. Suppressor mechanisms exist normally to prevent disease development and, immunosuppressive medications are used to reduce inflammation in AID. Some of the prominent AID is Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), type 1 diabetes mellitus, T1DM, Coeliac disease, Crohns Disease and Multiple Sclerosis (MS). Majority of these diseases occur more frequently in women than men. Genetic, hormonal, immune function and behavioral differences in male and female biology can play an important role in female biased disease development. Having two X chromosomes may also predispose women to autoimmunity. Failures in negative feedback mechanisms that inhibit T/B cell autoreactivity and innate immune function are known to contribute to autoimmunity. X chromosome encodes more miRNA genes than most of the autosomes. Some X-linked miRNAs are over expressed in women compared to men, likely due to escape of X chromosome inactivation (XCI). Female-biased downregulation of genes involved in immunosuppressive pathways by X-linked miRNAs can drive chronic inflammatory conditions and autoimmunity in women. This review focuses on how induction of X-linked miRNA expression potentially blocks immune suppressive pathways in female predisposition to autoimmune diseases.

Innate and Adaptive Immune Systems in Autoimmunity

Immune system is evolved to mount strong and efficient immune responses against a pathogen. During this process self-antigens are released by the actions of cytotoxic T lymphocytes or by pathogen-mediated cell destruction. Self-antigens can be taken up and processed by macrophages and dendritic cells resulting in enhanced presentation to T cells and B cells. Historically, the adaptive immune system had been the primary focus as the key player in AID. Abnormal recognition of self antigens by T and B lymphocytes play important roles in pathogenesis. However, it is becoming increasingly clear that synergistic interactions between adaptive and innate immune functions induce autoimmunity [1,2]. Association of the infectious agents with disease manifestation has been described for majority of the AID studied [1-3]. Anti infective mechanisms of the innate immune system; cytolysis processes and generation of harmful agents such as proteases and reactive oxygen species can damage host cells and tissues. Abnormal production of pro-inflammatory mediators produced by an overzealous innate immune response may break immune tolerance [4], and affect T and B cell differentiation. Furthermore, increased production of the chemokines by activated innate immune cells can expand activated T lymphocytes in periphery [5]. Once the pathogen is cleared; immune system is capable of executing multiple inhibitory mechanisms at molecular and cellular levels [6,7] to prevent development of chronic and autoimmune responses. Failures in these inhibitory mechanisms cause a constant risk of anti-self responses from the adaptive immune system [8].

Activation and Negative Regulatory Mechanisms of Immune System

Toll-Like Receptors (TLRs) present on macrophages and Dendritic Cells (DCs) are key receptors that initiate the innate immunity and the subsequent activation of the adaptive immune response. Binding of exogenous or endogenous epitopes to TLRs initiate a signaling cascade

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to induce the release of pro-inflammatory cytokines and chemokines as well as upregulation of co-stimulatory molecules by APCs. Activated APCs could break immune tolerance by enhanced presentation of self-antigens to auto-reactive T and B cells in the adaptive immune system. The role of TLRs in AID has been described extensively by Ann Marshak-Rothstein in 2006 [9]. TLR7 is an X chromosome encoded member of the toll like receptor family of proteins and expressed in macrophages, DCs and B cells. TLR7 present in B cells can bind endogenous RNA and activate autoreactive B cells [10]. TLR7 mediated DC activation reduces the immune suppressive function of regulatory T [Treg] cells [11]. A study using Tlr7 transgenic (tg) mouse model by Walsh et al., has recently demonstrated that the development of Tlr7 induced B cell mediated lupus-like autoimmunity requires B cell interactions with T cells as well as innate immune cells [12]. TLR signaling cascade is negatively regulated at several levels by [1] proteasome-degradation of signal molecules mediated by E3 ubiquitin ligases such as SOCS and CBLB, [2] dephosphorylation of proteins by the cascade in the action of phosphatases, [3] transcription regulation and, [4] miRNA mediated downregulation of signal proteins. mir-155, mir-146a, miR-21 are some of the miRNAs involved in regulation of TLR signaling [13].

Antigen binding to T Cell Receptor (TCR) in the context of the class II Major Histocompatibility Complex (MHC) leads to T cell activation. Optimal T cell activation requires a co-stimulatory signal through CD28 co-receptor activation by interaction with APC counterparts. TCR engagement promotes a protein tyrosine phosphorylation mediated signaling cascade to activate T cell transcriptional machinery which determines T cell fate through cytokine production, cell survival, proliferation, and differentiation. Activated T cells can assist in antibody production by B cells or to carry out cytotoxicity of target cells. CD4+ T helper (Th) cells are involved in B cell maturation and activation of CD8+ cytotoxic T cells and macrophages. Once activated CD4+ T cells can differentiate into Th1, Th2, Th9, Th17, and Th17 cells, or memory T cells. These subsets express specialized cytokines and cell-surface molecules that determine their effector cell capacity. [IFN]-γ-producing Th1 subset of CD4+ T cell has long been considered to be the main effector T cell subset responsible for pathogenesis of autoimmunity. However, recent studies suggest that T helper 17(Th17) cells can mediate sustained autoimmune inflammation in accord with chronic conditions associated with AID [14]. Furthermore, Th17 cells have a greater capacity to provide B cell response compared with Th1 cells [15].

TGF-β signaling and Treg function plays an essential role in maintenance of peripheral T cell tolerance [16,17]. Treg controls autoreactive T cells in the periphery to maintain self-tolerance [18-21]. Defects in Treg function can contribute to autoimmunity [20,21]. Treg development and function are maintained by X chromosome encoded transcription factor FOXP3. CTLA-4 is believed to play an essential role in immunosuppressive functions of Foxp3-CD25+CD4+ natural Treg cells and, its deficiency in Treg cells leads to autoimmunity [18,22]. Surface expression of CTLA-4 on the surface of Treg cells downregulate costimulatory molecules on APCs CD80 and CD86 [23]. The cytokine TGF-β promotes the differentiation of induced Treg (iTreg) cells in the presence of IL2. TGF-β also controls the effector function of Th17 cells by maintaining the Th17 regulatory state rTh17 [24]. Deficiency in TGF-β can result in severe autoimmunity [25]. PD-1(PDCD1), which interacts with PD-L1 and PD-L2, is another co-inhibitory molecule that negatively regulates T cell activation. The PD-1: PD-L signaling pathway inhibits the expression of GATA-3 and TBFET, the transcription factors associated with effector cell function [26,27]. Aged mice deficient in pdc1 has been reported to develop lupus like autoimmunity [28].

B cells are needed for autoantibody production and formation of immune complexes that induce local inflammatory reactions and tissue destruction. In addition, B cells can promote T cell effector function through antigen presentation and various co-signals and cytokines. B cell activation is initiated by binding of antigen to the B Cell Receptor (BCR). BCR activation initiates the formation of kinase mediated ‘signalosome’, which regulates the downstream events resulting in B cell differentiation and proliferation into antibody-producing cells or memory B cells [29]. Recently identified IL10 producing regulatory B cell phenotype (B10) has been shown to downregulate inflammation [30]. They may exert their immunosuppressive function by inhibiting CD4+ T cell proliferation, Th1 differentiation and monocyte activation [31].

Ubiquitin Ligases as Negative Regulators of Immune Response

Ubiquitin mediated proteosomal degradation regulates many cellular functions including immune response [32]. CBL and Suppressor of Cytokine Signaling (SOCS) family proteins are well characterized E3 type ubiquitin ligases that promote negative regulation of innate and adaptive immune cell signaling pathways [33-35]. CBL family proteins act as negative regulators of TCR signaling pathways [http://www.kegg.jp/kegg-bin/show_pathway?map04660]. CBL knockout mice develop severe autoimmune disease phenotype [36]. CBL deficient mice exhibit an increased expression of TCR and its components and enhanced selection of CD4+ thymocytes [37], increased levels of LCK and FYN, and enhanced activity of the ZAP-70 tyrosine kinase [37,38]. TCR engagement of thymocytes and peripheral T cells isolated from CBL deficient mice exhibits increase proliferative response. Furthermore, these studies have indicated that CBL act as a negative regulator of protein tyrosine kinase mediated signal transduction pathways in T cells. Reduced CBL expression in effector memory CD4+T cells lowers the threshold of their functional response [40]. Furthermore, the expression of CBL protein is low in lupus T cells compared with normal controls [41]. CBL activation by LCK has been reported to promote ubiquitlyation, lysosomal targeting and degradation of zeta-chain of TCR/CD3 [42] in Jurkat TAg cells. This study has linked T cell hyper-responsiveness to prolonged survival and sustained signaling from internalized TCR/CD3-complexes, resulting from reduced LCK and CBL activity. CBL mediated ubiquitlyation of zeta-chain through ZAP70 adaptor function has been described in another study [43]. Furthermore, CBL mediated LAT ubiquitlyation and internalization following TCR stimulation has been reported by Balagopalan et al. [44]. Therefore, these facts lend support to the hypothesis that the CBL down regulation in peripheral T cells could have pronounced defects in controlling TCR signaling pathways and contribute to the development of autoimmunity. CBL and CBLB have also been shown to interact with BCR signaling molecules and downregulate BCR signaling pathways [45,46]. In addition, CBL mediated ubiquitlyation controls BCR-mediated antigen processing and presentation [47].

SOCS proteins provide a negative feedback loop to attenuate cytokine action to modulate immune responses by several mechanisms, which involves blocking of JAK-STAT signaling pathway and ubiquitlyation of signaling proteins for proteosomal degradation. They are important regulators of inflammation and adaptive immunity. Their expression is induced by a subset of cytokines including those belonging to the interferon, interleukin and colony-stimulating factor families. The roles of SOCS family proteins are important in preventing uncontrolled T cell activation. In addition, they may play a role in T cell differentiation from naïve cells into Th1, Th2, Th17, and T regulatory
(Treg) functional subtypes by disrupting cytokine signaling pathways. Emerging evidence also suggests that disruption of SOCS expression or activity is associated with several immune and inflammatory diseases [48]. Within the SOCS subfamily, role of SOCS4, SOCS5, and SOCS7 in T cell biology is unclear. SOCS1 is an inhibitor of FNG and STAT1 Signaling pathways [49]. T cells in socs1-/- mice also displayed features of aberrant activation and hyper responsiveness to cytokine induced IFN gamma production [50]. A SOCS1 deficiency in hematopoietic cell induces multi-organ inflammatory disease phenotype [51]. SOCS1 overexpression in activated T cells prevents Th1 differentiation [52]. SOCS2 appear to play a role in regulating Th2 differentiation as the activated SOCS2-/- CD4+ T cells has been shown to produce higher levels of IL-4, IL-5, and IL-13 [53]. In addition, T cell activation in the absence of SOCS2 has resulted in enhanced STAT5 and STAT6 phosphorylation and SOCS1 and SOCS3 expression. SOCS3 has been shown to negatively regulate CD28-mediated IL-2 production [54]. Its inhibition disrupts the negative feedback regulation of STAT3 signaling and enhances the generation of Th17 cells [55]. SOCS6 has been shown to promote proteosomal degradation of active form of T cell-specific protein-tyrosine kinase LCK, to negatively regulate T cell activation [56]. CISH has been shown to physically associate with IL-2 receptor beta chain, inhibit IL2 mediated activation of STAT5, and negatively control IL-2 signaling pathways [57]. It is possible that decrease IL-2 signaling, could disrupt the function of Treg cells [58]. SOCS protein regulation can occur at the transcriptional, translational, and at post-translational levels. Existing knowledge on post transcriptional regulation of SOCS proteins by microRNAs is not complete.

**MicroRNA (miRNA)**

MiRNAs are approximately 22 nucleotide-long, non-coding RNA molecules, which act as post-transcriptional negative regulators of protein synthesis in diverse cellular pathways. They bind to the 3' untranslated regions (3' UTRs) of target gene miRNAs and block the translation, or stimulate rapid degradation of the transcripts. MiRNA genes reside in genome as intragenic (miRNA-coding genes located within protein coding gene introns or exons) or intergenic (miRNA-coding genes located in between protein-coding genes). Approximately 50% of human miRNAs appear to be intragenic [59], and are expected to be co-transcribed with their host genes. Intergenic miRNAs are typically transcribed by RNA polymerase II through the same mechanisms by which protein coding genes are transcribed. Initial transcription of a miRNA or a cluster of miRNAs generates a longer (more than 1kb) primary transcript (pri-miRNA) which includes a precursor miRNA hairpin (pre-miRNA), or sometimes more than one co-transcribed pre-miRNAs. Nuclear cleavage of the pri-miRNA liberates ~60–70 nt long pre-miRNA stem-loop intermediates and exported to the cytoplasm where the final mature miRNA is excised [60]. There is ample evidence for dysregulation of global miRNA expression under various pathological conditions [61,62]. Cellular miRNA expression is regulated at transcriptional as well as post-transcriptional levels in miRNA biogenesis. Epigenetic mechanisms, nuclear receptor [63] and other basic cell signaling pathways [64] could contribute to transcriptional level regulation. Epigenetic events such as DNA demethylation and histone acetylation can reverse repressive chromatin marks to poise miRNA genes for transcription. Nuclear sex hormone receptors bind sex hormones as ligands and act as transcription factors to regulate miRNA gene expression. Estrogen, which functions as a ligand for estrogen receptor, can contribute to female-biased expression of miRNA [65]. Estrogen independent female biased induction of miRNA expression can occur when the miRNA genes located on the X chromosome escape inactivation, and transcribed from both X alleles [65,66]. Post transcriptional steps in miRNA biogenesis pathway such as miRNA processing, RNA editing as well as miRNA-target interactions could further influence the levels of cytoplasmic miRNA [65-67]. It is widely accepted that miRNAs regulate the expression of multiple genes in the cells of origin. In addition, miRNAs can be transferred from cell-to-cell by lipid based carriers and exert their potential role in intercellular communication. Growing evidence suggests that miRNAs excreted from incipient cells and extracellular microRNAs [miRNAs] stably exist in human body fluids [68]. Association of dysregulated miRNA expression with the risk or severity of AID has been summarized recently in several review articles [69-71].

Human X chromosome is highly enriched in miRNAs, second only to the chromosome 1 which encodes 134 miRNAs (Figure 1). Based on miRBase miRNA archive [www.mirbase.org 2012], 113 miRs are encoded by human X chromosome, while Y chromosome encodes only 2. Pinheiro et al. have previously reviewed the importance of X-linked miRNAs in immunity and cancer, and hypothesized that X-linked miRNAs may contribute to sex differences in disease pathogenesis [72]. The purpose of this review is to evaluate X-linked miRNAs for their potential to inhibit genes involved in immunosuppressive mechanisms and potential contribution to autoimmunity. MiRNA mediated downregulation of immunosuppressive pathways may be a contributing factor for AID pathogenesis. Female biased over expression of X-linked miRNAs which target immunosuppressive genes may play an important role in female predisposition.

**Skewed X-Chromosome Inactivation and Female Predisposition to Autoimmune Diseases**

Hormones such as estrogen and prolactin may play a role in female predilection to AID, as does having 2 X chromosomes [73]. In diploid cells, females have two copies of X chromosome and males have only one copy. To neutralize the gene dosage difference of the X chromosome between the genders, one of the two X chromosomes is inactivated in females, silencing the gene expression from inactive X [Xi]. DNA methylation is one of the mechanisms that contribute to repressive chromatin modifications associated with specific silencing of the genes from Xi. Despite this chromosome-wide silencing, about 15% of X-linked genes escape X inactivation in women and are expressed bi-allelically [74]. Previous studies have shown that the X chromosome encoded immune active genes can escape silencing on Xi due to DNA...
demethylation and over expressed in women compared with men [75]. Over expression of immune active genes can cause T cell autoreactivity, a hallmark of lupus disease manifestation. The men with Klinefelter’s Syndrome (47,XXY) develop lupus at the same rate as women also suggest that the presence of 2 X chromosomes contributes to disease development [76].

Suppressing the Suppressor –Mirna Mediated Downregulation of Negative Regulators of Immune System
To date, only a handful of X-linked miRNAs are functionally characterized in autoimmunity. Increased expression of miR-18b has been reported in PBMCs of MS patients experiencing a relapse [77]. MiR-223 is significantly upregulated in peripheral T-cells from RA patients compared with healthy donors [78]. MiR-223 can also potentially target CBLB (Table 1). Mir-221/222 cluster was reported to be upregulated in RA SF of human TNF transgenic mouse model [61]. On the contrary: a recent study has shown that X-linked miR-221 suppress genes involved in proliferation and survival [79] indicating miR-221 as a direct suppressor of immune response. Inhibition of miR-miR-221 increased T cell proliferation and survival indicating that miR-221 indeed serves in a feed back negative regulatory mechanism to control immune response. MiR-221 is also predicted to target several immunosuppressive genes (Table 1), indicating a suppressor of suppressor function. Increased expression of miR-221 in FOXP3+ Treg cells could prevent Treg proliferation and survival leading to an increased immune response. Lu et al. reported over expression of miR-224 in SLE T cells [80]. It is possible that the functional consequence of upregulation of miR-221 is determined by T cell subset and micro environment. Transfection of Jurkat cells with miR-224 suppressed apoptosis inhibitory protein 5 [API5] and induced activation-induced cell death. MiR-510 expression is significantly higher in Treg cells of T1DM patients compared with healthy controls [81]. However, this differential expression between T1DM and healthy controls was not observed in conventional T cells, indicating a T cell subset specific overexpression of miR-510 in T1DM. Mir-510 can potentially target SOCS2 and SOCS3 (Table 1). Suppression of SOCS2 and SOCS3 function by miR-510 may be a contributing factor for chronic inflammation in T1DM. It is generally assumed that there is no gender bias in development of T1DM. However, in a study conducted by Howson et al., a subgroup of type 1 diabetic patients sensitive to a subset of type 1 diabetic patients sensitive to a hallmark of lupus disease manifestation. The men with Klinefelter’s Syndrome (47,XXY) develop lupus at the same rate as women also suggest that the presence of 2 X chromosomes contributes to disease development [76].
mediated regulation of FOXP3 expression. However, TargetScan predictions indicate X-linked miRNA binding sites on 3'UTR region of FOXP3 transcript. Balancing act of FOXP3 overexpression due to DNA demethylation and miRNA-mediated downregulation appears to play an important role in determining Treg function associated with AID. Furthermore, TargetScan is one of many bioinformatics tools that are available for predicting interactions with miRNAs, and none are considered to be more accurate than the others. Therefore, experimental validation of these miRNAs: mRNA pairs are needed to confirm true biological function. However, these predictions are intended to help design strategies in identifying their effects on disease mechanisms. Since miRNAs can be excreted from incipient cells and taken up by other immune cells, they can function as translational inhibitors in recipient cells as well. Finally, the overexpression of miRNAs from X chromosome and their compounding effects in blocking immunosuppressive pathways can be a contributing factor to immune system hyper responsiveness and female predisposition to AIDs.

Conclusion

Human X chromosome is highly enriched in miRNAs. However, we are still in the early stages of understanding how female-biased X-linked miRNA expression contributes to autoimmunity. Overexpression of X-linked miRNA due to skewed X inactivation could lead to unbalanced miRNA levels between sexes. Initial studies indicate that E3 Ubiquitin ligases such as CBL and SOCS family members as well as FOXP3, CTLA-4 and PDCD1 are potential biological targets of many X-linked miRNA. These molecules play an essential role in immunosuppressive mechanisms to maintain immune tolerance. Female-biased expression of X-linked miRNA and their suppression of protein production of the immunosuppressive genes may contribute to over active immune function and consequently, to female predilection in autoimmunity. The identification of X-linked miRNAs targeting immune suppressive genes may provide us a new arena for exploration of mechanisms for female-biased autoimmunity. It will also provide us new insight in the development of miRNA based therapies to autoimmune diseases.

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