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Original Article

Emerging role of C5a/C5aR IL-17A axis in cGVHD

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Abstract: Chronic graft-versus-host disease (cGVHD) manifests with features characteristic of autoimmune disease with organs attacked by pathogenic Th17 cells. However, the mechanism of Th17 cells generation in the setting of cGVHD is still unclear. Here we defined C5a/C5aR-IL-17Aaxis as a novel signaling that required in the pathologies of cGVHD. We firstly found a positive link between complement activation and the Th17 cells in patients with cGVHD. C5a, a critical component of complements, promoted the generation of Th17 cells *in vitro* and inhibition of the receptor for C5a (C5aR) reduced the Th17-bias response. Of note, C5aR blockade by PMX53 could suppress the generation of IL-17A-expressing Th17 cells and retard the onset and progression of cGVHD *in vivo*. Overall, our results provide new mechanistic insights that activation of C5a-C5aR signaling was required for IL-17A-induced immune responses in cGVHD and define novel molecular targets for developing effective therapeutics for cGVHD.

Keywords: Complement C5a, Th17 cells, Interleukin-17A (IL-17A), Chronic graft-versus-host disease (cGVHD)

Introduction

Chronic graft-versus-host disease (cGVHD) is the major long-term complication of allogeneic hematopoietic stem cell transplantation (HSCT), occurring in approximately 50% of patients [1-3]. It presents as a chronic inflammatory and sclerotic autoimmune-like condition that most frequently affects the skin, oral mucosa, liver, eyes and gastrointestinal tract [4]. Currently, the glucocorticoids with or without calcineurin inhibitors is the standard initial treatment with limited efficacy and novel therapies are warranted [5]. Over the past decade, our understanding of cGVHD pathogenesis and basic biology has improved rapidly. Donor T cells were recognized to contribute to immune pathology in cGVHD through innate and adaptive immune mechanisms in a complement pathway-dependent manner [6-8]. Complement plays a critical role at the interaction of antigen-presenting cells (APCs) and T cells, and the modulation of complement activation may provide a potential mechanism to regulate the response of donor T cells and the treatment of cGVHD [9, 10]. However, direct evidence of this modulation has not been demonstrated.

Recently, many reports perceived skewing of cGVHD toward a Th17 process [11-13]. It remains unclear, however, how autoreactive pathogenic Th17 cells are generated from antigen-experienced T cells during cGVHD. Complement system plays an important role in the innate immunity and can be activated by three pathways including the classical pathway, the alternative pathway, and the lectin pathway. These signaling converge on the activation of C3 and C5, leading to various inflammatory diseases [14]. Moreover, it has been shown that C3a and C5a regulate adaptive immunity via interactions with their respective receptors on both innate and adaptive immune cells. C5aR is identified in neutrophils, monocytes and APCs like dendritic cells [15]. The impact of C5aR signaling on the T cells activation, differentia-

Table 1. Clinical characteristics of the study population

	Healthy Controls	Non-GVHD	cGVHD
Male to female ratio	5:5	4:6	11:4
Age	29.60±5.77	31.40±12.47	30.27±8.47
Primary disease before HSCT	NA	ALL:2	ALL:6
		AML:7	AML:7
		CML:1	CML:2
Donor			
Sibling	NA	8	8
Matched unrelated	NA	2	7

Abbreviations: ALL = acute Lymphoblastic Leukemia; AML = acute myeloid leukemia; CML = chronic myelogenous leukemia; NA = not applicable.

tion and function was required for the effective antigen presentation from APCs to regulates MHC class II (MHC-II) and costimulatory molecule expression. During cGVHD, APCs are activated in response to total body irradiation and present MHC-mismatched peptides, consequently initiate donor T cell activation, which has the capacity to attack the recipient [12, 16]. Given the crucial role of C5aR signaling in the induction and regulation of adaptive T cell responses, we proposed that C5aR may have the potential to induce and intensify cGVHD.

The bidirectional regulation of IL-1β, TGF-β, IL-6 and IL-23 is affected by complement activation and could determine the differentiation and commitment of $CD4^+$ T cells [17, 18]. IL-6 is a pleiotropic cytokine with critical role in regulating Th17/Treg balance by inducing the generation of Th17 cells from naïve T cells together with TGF-β while inhibiting TGF-β-induced Treg differentiation [19, 20]. IL-23 is incapable of differentiating Th17 cells from the naïve T cells but has the potential to promote the expansion of the Th17 population [21]. A new concept is emerging in which IL-6 and IL-23 induced the Th17 cells differentiation through regulating C5a production and C5aR signaling activation, forming a positive feedback loop. As the gene encoding C5 has been identified as a susceptibility gene for cGVHD [22], we explored the possibility that C5a signaling is the key player in initiating and regulating excessive Th17 cell responses in cGVHD.

Here, we found higher level of C5a and IL-17A and a positive correlation between C5a and IL-17A expression in patients with cGVHD. Moreover, the IL-17-expressing Th17 cells were significantly elevated in the cGVHD group, either compared with HSCT patients without cGVHD or normal controls. The deficiency retrained the development of Th17 cells, whereas C5a activation promoted the differentiation of Th17 *in intro*. Furthermore, we found the C5aR blockade by mAb PMX53 attenuated the pathology of cGVHD and improved the survival of the cGVHD mice, accompanied with increased IL-6 and IL-23 expression and decreased Th17 cells generation. Our data of the link between complement activation and the Th17 cells may provide insights on the underlying pathogenesis of cGVHD.

Methods

Patients

Fifteen patients with cGVHD, ten patients without GVHD after HSCT, and ten healthy controls in Guangdong General Hospital were enrolled in this study (**Table 1**). The diagnosis of cGVHD was determined according to the NIH consensus criteria for cGVHD. The data of interest were: gender, age, primary diseases before HSCT, and donor source. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and our protocol was approved by the Ethics Committee of Guangdong General Hospital.

cGVHD mouse model and histology

B10.D2 (H-2d, Jackson Laboratories, Bar Harbor, USA) and BALB/cJ (H-2d, Beijing Vital River Laboratory Animal Technology Co., Ltd, China) mice were used as donors and recipients to establish the cGVHD model. All experimental procedures and protocols used in this study were approved by the Institutional Animal Care and Use Ethics Committee. Recipient BALB/cJ mice received 700-850 cGy from a cesium irradiator and were reconstituted by tail vein injection with 8×10^6 bone marrow cells with 8×10^6 spleen cells from B10.D2 mice. PMX53 (Tocris Bioscience, Minneapolis, USA) solubilized in PBS was injected intraperitoneally every three days at the dose of 1 mg/kg when the established cGVHD model showed the clinical

scores above 0.6 at Day 29 after BMT. Control mice receive equal amounts of PBS injection. When mice either died or were euthanized for humane reasons, their disease severity scores at time of death remained included in subsequent mean scores. For histological assessment of cGVHD, the representative samples from skin and liver were isolated and fixed in 4% formaldehyde and embedded in paraffin. Tissue sections of 6 μm were stained using H&E to study the tissue damage of cGVHD with or without PMX53 treatment.

Splenocytes cultures and stimulation

The splenocytes were isolated and cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.005% 2-mercaptoethanol (Gibco), 0.01% penicillin G and gentamycin (Sigma) at 37°C, 5% CO_2. Cell concentration was standardized to 3×10^6 cells/ml and stimulated with IL-2 (40 ng/ml), immobilized anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) for 3 days. Supernatants were collected for Th17-related cytokines production detection and Th17 cells were determined by flow cytometry.

Flow cytometry

Th17 cells were determined by multicolor flow cytometric analysis. The used antibodies include Anti-human CD4 FITC, Anti-human IL-17 APC-Cy7, Anti-mouse CD4 FITC, and Anti-mouse IL-17 PE (eBioscience, USA). The flow cytometry data were analyzed by *FlowJo* software.

ELISA and luminex technology

C5a level in plasma or production of cultured splenocytes were assessed using enzyme-linked immunosorbent assay (Elisa kit, Abcam, USA) according to the manufacturer's instructions. The Th17-related cytokines were analyzed using Luminex MAGPIX system (Luminex Corp., Austin, TX) according to the manufacturers' specifications. Samples were detected in triplicate relative to standards supplied by the manufacturer and analyzed for significant differences between different groups.

Statistical analysis

Statistical analysis was performed with SPSS software version 13.0 (Inc., Chicago, IL, USA). Group comparisons of flow cytometry data, ELISA, Luminex data were analyzed by Student's *t* test or One-way analysis of variance. Bivariate correlation analysis was performed to find the relationship of the expression of C5a and frequency of $CD4^+IL\text{-}17^+$ T cells in cGVHD. Survival curves were plotted using a Kaplan-Meier curve and analyzed by log-rank test.

Results

Th17 cells are elevated significantly in the patients with cGVHD

To explore the potential role of Th17 cells in cGVHD, we firstly detected the percentage of Th17 cells and their relation to pathogenesis of cGVHD in patients. Patients diagnosed as cGVHD were enrolled in the study and non-GVHD patients after HSCT and healthy individuals were used as controls. The clinical characteristics of candidates are listed in **Table 1**. As expected, the $IL\text{-}17^+CD4^+$ Th17 cells were significantly elevated in the cGVHD group, either compared with HSCT patients without cGVHD or normal controls (**Figure 1A**, **1B**). The serum was collected and IL-17A level was analyzed by ELISA. Consistently, patients with cGVHD presented with increased protein level of IL-17A in comparison to both controls (**Figure 1C**).

Expression of C5a positively correlates with numbers of Th17 Cells

Next, we detected the level of C5a in the plasma or serum and found that C5a expression was markedly elevated in cGVHD patients (**Figure 1F**). Bivariate correlation analysis was used to evaluate the correlation between the expression of IL17A and C5a in the cGVHD patients. Obviously, there was a significant positive correlation between C5a and IL-17A expression (**Figure 1G**). In addition, IL-6, a pleiotropic cytokine with critical role in skewing Th17 response, was elevated (**Figure 1D**), indicating the possibility of Th17 cells dominant. IL-23, capable to promote the expansion of the Th17 population, was also up-regulated in cGVHD patients (**Figure 1E**). Together, a link between complement activation and the Th17 cells was identified in cGVHD.

C5a signaling contributed to Th17 cells development in vitro

To assess the contribution of the C5a-C5aR signaling on initiating and orchestrating $CD4^+$

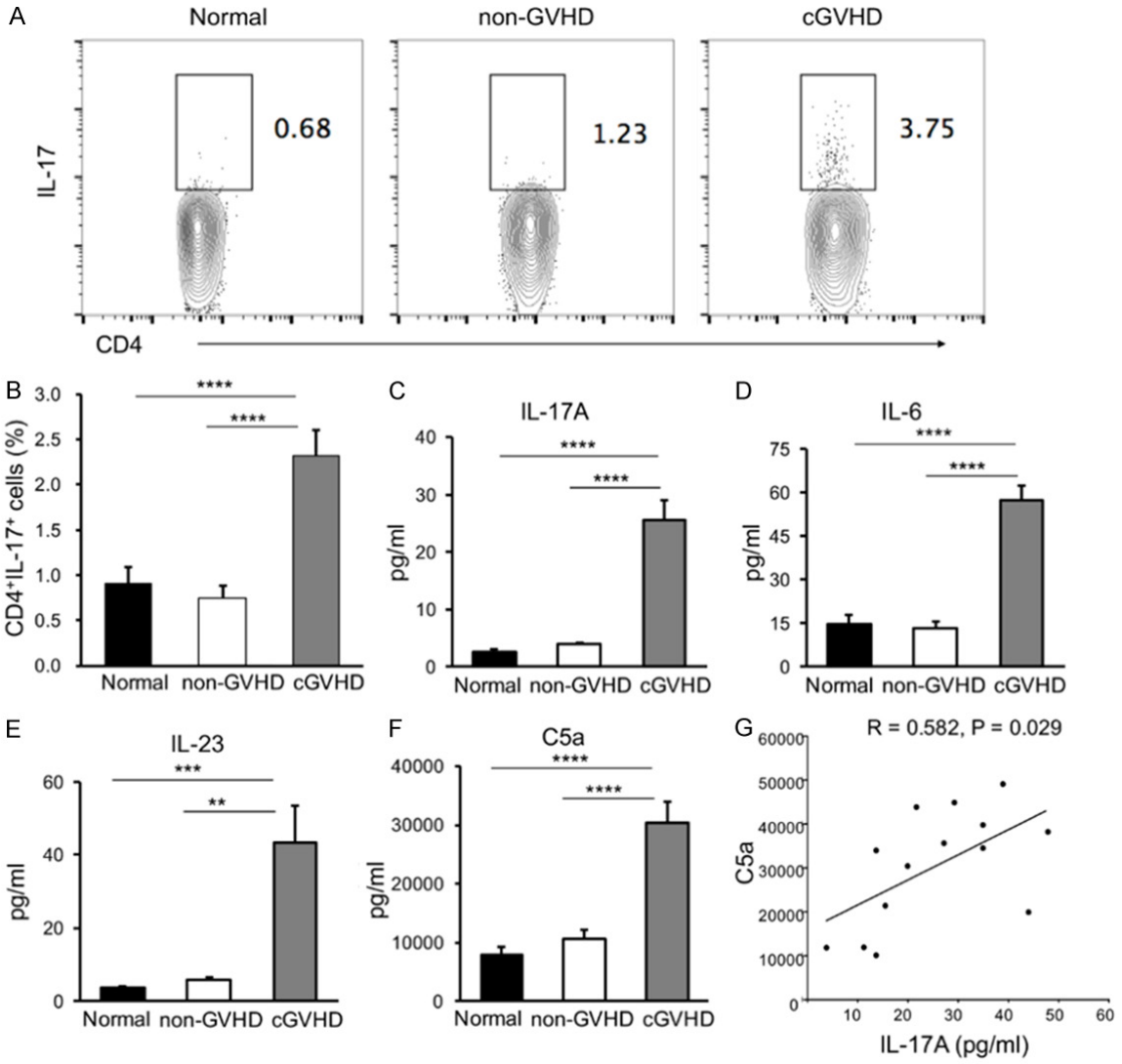

Figure 1. Th17 cells are elevated significantly in the patients with cGVHD. A, B. The flow cytometry results revealed that the $CD4^+IL\text{-}17^+$ cells were significantly elevated in the patients with cGVHD, either compared with HSCT patients without cGVHD or normal controls. C. The IL-17A protein expression in serum was increased marked in patients with cGVHD compared with the other two groups. D. The IL-6 expression was also elevated. E. The up-regulated IL-23 was observed in cGVHD group. F. The C5a expression was found to be elevated significantly in patients with cGVHD. G. The correlation analysis data showed the positive correlation of IL-17 expression and C5a level in cGVHD patients. $^{**}P<0.01$; $^{***}P<0.005$; $^{****}P<0.0005$.

T cells immune responses, the splenocytes were stimulated with IL-2, anti-CD3 and anti-CD28 antibodies and gain- and loss-function assay were performed. In response to extrinsic C5a, the splenocytes differentiated into IL-17-expressing Th17 cells significantly (**Figure 2A**). Th17-related cytokines including IL-17A, IL-6, and IL-23 were also increased markedly (**Figure 2B-D**). In the contrast, upon combined treatment with PMX53, a potent, highly selective C5aR antagonist, we found substantial reduction of Th17 cells and downregulation of IL-17A, IL-6, and IL-23 production (**Figure 2B-D**). Similarly, C5a-C5aR signaling blockade using the splenocytes from C5aR-/- mice resulted in marked reduction of Th17 cell generation as well as IL-17A, IL-6, and IL-23 production. Taken together, these data suggested that C5a-C5aR was required for the induction of a Th17-promoting microenvironment.

PMX53 administration diminishes clinical and histopathological damage and improved the survival of cGVHD mice

To evaluate the potential role of C5a-C5aR-IL-17A axis in the pathogenesis of cGVHD, the

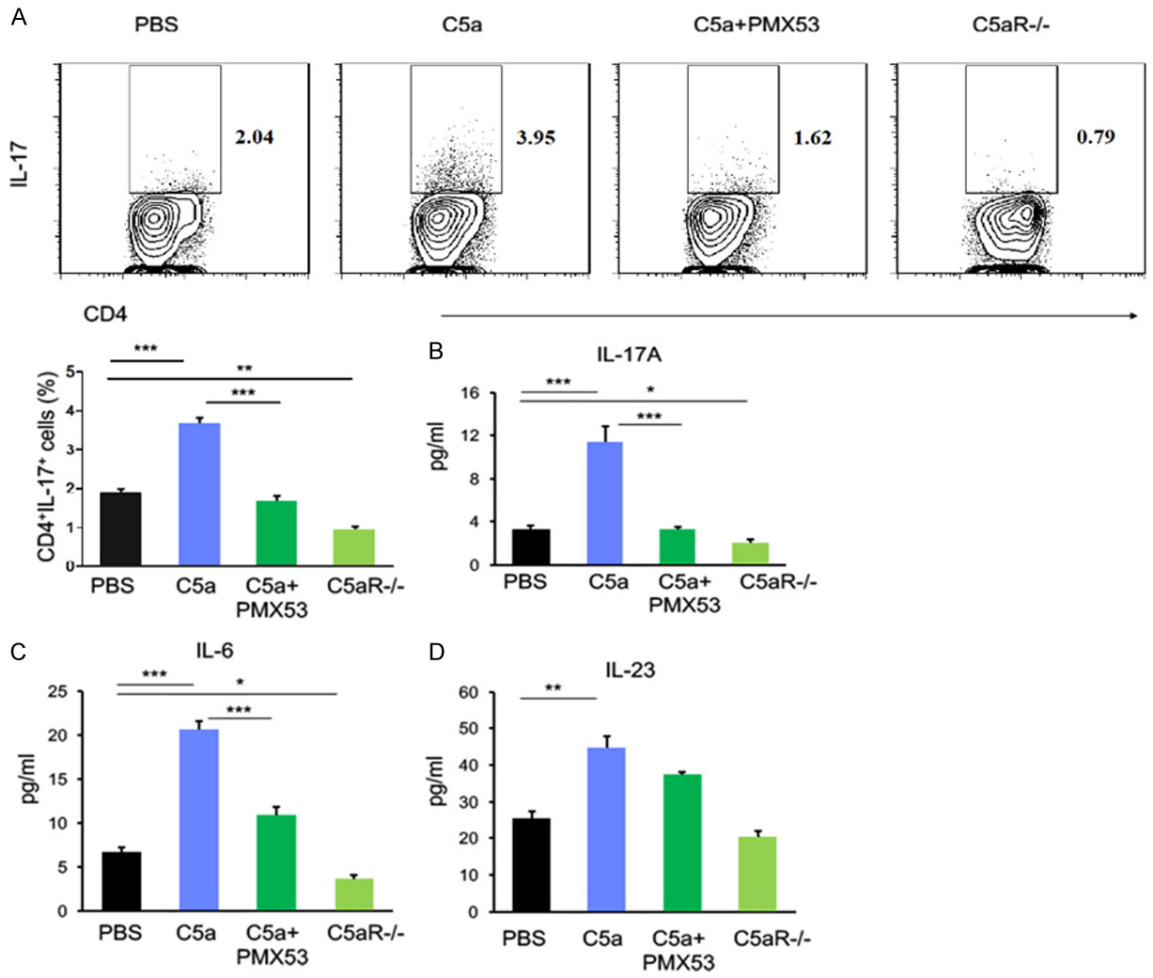

Figure 2. C5a signaling contributed to Th17 cells development *in vitro*. (A) The flow cytometry results demonstrated that lack of $CD4^+IL\text{-}17^+$ cells in the splenocytes in response to anti-CD3 and anti-CD28 antibodies while the C5a stimuli upregulated the percentage of Th17 cells. However, PMX53, a selective C5aR antagonist, could abolish the elevation of Th17 cells at presence of C5a. In addition, absence of Th17 cells was observed in the splenocytes from the C5aR-/- mice. (B-D) The Th17 cells related cytokines of IL-17A (B), IL-6 (C), and IL-23 (D) were increased in response to C5a induction. In the contrast, downregulation of IL-17A, IL-6, and IL-23 production was seen upon combined treatment with PMX53. *P<0.05; **P<0.01; ***P<0.005.

mouse model of cGVHD was established and PMX53 was used to block C5aR signaling. Strikingly, PMX53-treated mice developed less disease, as evidenced by reduced clinical scores (**Figure 3A**). Similarly, PMX53-treated mice experienced improved survival than controls (**Figure 3B**). The representative images showed the severe fur loss, scabbing, flaking, hunched posture, and thickened skin in cGV-|HD model while PMX53 treatment clearly attenuated the injuries (**Figure 3C**). The histological results revealed that the cGVHD control mice presented with epidermal hyperplasia, dermal fibrosis, serocellular crusting, lymphohistiocytic infiltration and less hair follicle, which were not observed in skin samples from the PMX53-treated group (**Figure 3D**). In addition, a marked reduction in portal inflammatory response and bile duct destruction was seen in sections taken from PMX53-treated mice compared with controls, in which marked periportal inflammation is observed (**Figure 3D**).

C5aR blockade alleviated production of IL-17A and other Th17-related cytokines

We then analyzed the number of Th17 cells ($CD4^+IL\text{-}17^+$ T cells) by flow cytometry. As expected, a decrease in Th17 numbers was found

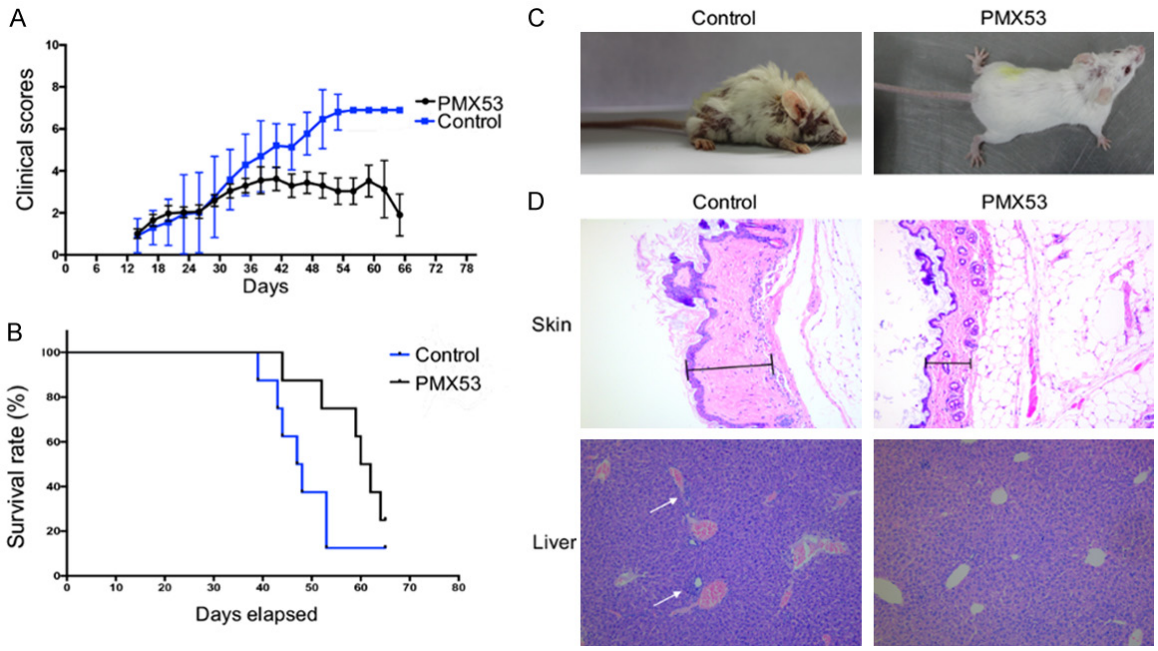

Figure 3. PMX53 administration diminishes clinical and histopathological damage and improved the survival of cGVHD mice. A. Average clinical disease score for mice affected with cGVHD was analyzed and reduced clinical scores were observed in the PMX53 treatment group. B. Kaplan-Meier plot of cGVHD survival curve revealed improved survival in the PMX53-treated mice compared with controls. C. A representative image showed cGVHD mice suffered from severe fur loss, scabbing, flaking, hunched posture, and thickened skin while PMX53 treatment clearly attenuated these injuries. D. The histological results revealed that the cGVHD mice presented with thickened epidermal layers, less fat tissues, serocellular crusting, lymphohistiocytic infiltration and less hair follicle, which were not observed in skin samples from the PMX53-treated group. In liver sections, less infiltration of inflammatory cells around the portal and relative completed structure of bile duct was seen in PMX53-treated mice compared with controls.

in the PMX53-treated group compared with the control group (**Figure 4A**). Th17 related cytokines were also analyzed, and PMX53-treated animals had significantly reduced IL-17A, IL-6, and IL-23 production in circulation compared with animals in the control group (**Figure 4B**). Importantly, we found the significant reduction of IL-17 expression in spleen tissues as well as the targeted skin and liver organ from the PMX53 treatment group (**Figure 4C**).

Discussion

The complement is a critical orchestrator at the interface of innate and adaptive immunity that it plays an instrumental role in the pathogenesis of cGVHD [10]. Here, we reported a pivotal role for C5a in the regulation of Th17 cell-mediated immune responses in cGVHD. The aberrant Th17 response and excessive IL-17A production in patients with cGVHD is associated with C5a upregulation. The *in vitro* data provided the direct evidence of C5a/C5aR-driven Th17 generation and differentiation. Finally, the *in vivo* data demonstrated that the severe cGVHD phenotype is driven by C5a activation via inducing Th17 responses. These data suggested that pharmacologic targeting of C5aR would suppress cGVHD disease by reducing the numbers of Th17 cells.

A growing number of evidence demonstrates the central role of the complement system in shaping T cell responses [7, 23]. Complement components and activators could be produced not only by APCs, including monocyte, macrophages, and dendritic cells, but also by T cells, indicating that complement is actively involved in the regulation of T cell effector immune responses [24]. There are two possible mechanisms, on the one hand, the complement system exert direct effects on T cells themselves; on the other hand, it affects T cell biology by regulating the function of APCs, which induce T cells [25]. In this report, we found that the elevated common complement product C5a was

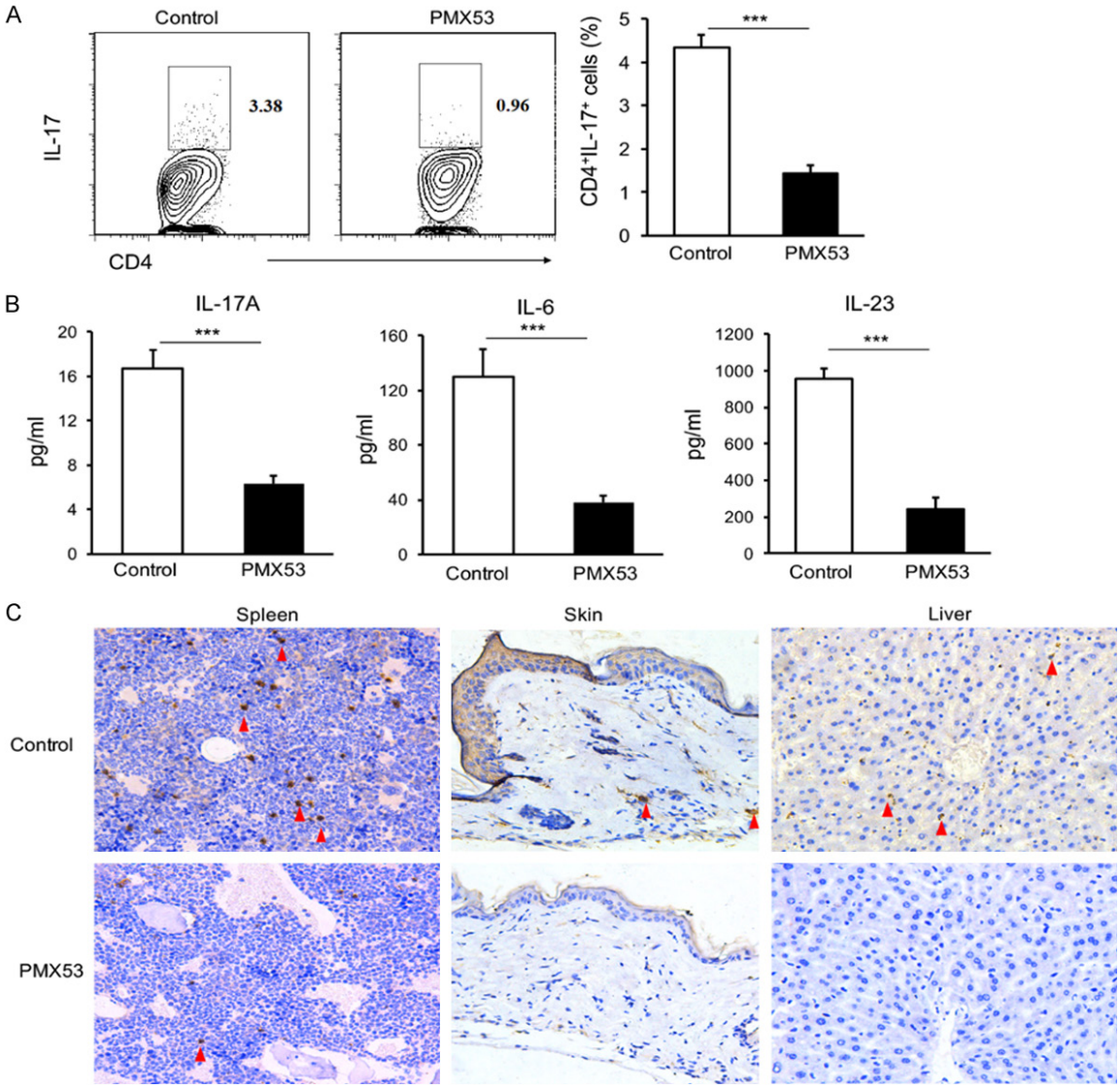

Figure 4. C5aR blocked alleviated IL-17A production and increased the accompanied expression of Th17 related cytokines. A. The flow cytometry revealed the $CD4^+IL\text{-}17^+$ T cells was decreased in the PMX53-treated group compared with the control group. B. Th17 related cytokines, including IL-17A, IL-6, and IL-23 were also reduced after PMX53 treatment. C. The immunohistochemical results showed the IL-17 expression in spleen, skin and liver organ was significantly reduced in the PMX53 treatment group. ***P<0.005.

correlated with IL-17 expression, suggesting that the complement participated in the regulation of Th17 differentiation in cGVHD may be by complement C5a activation.

The complement system has been identified as a crucial orchestrator in modulating T lymphocyte responses. C5a-C5aR signaling activation in APC-$CD4^+$ T cell interaction provides a pivotal innate immune signal that regulates differentiation of naïve T cells into Th17 and Treg [11, 26-28]. Moreover, the C5aR activation plays an integral role in suppressing dominant immunologic tolerance, which inhibits both Th1 and Treg responses. Some studies have reported that C5aR signaling may provide proinflammatory signals to immature DCs in absence of pathogens that it is essential for biasing T cell differentiation into a Th17 response [29]. IL-17, a cytokine produced primarily by Th17, has been reported to contribute to disease severity in several models of autoimmune and chronic inflammatory diseases [18]. Some data suggested that the C5a-C5aR signaling regulates Th17 differentiation through DC-derived IL-6 and IL-23 [19, 20]. Indeed, our work showed the

effects of C5aR on Th17 cells generation and cytokine production. We found that the expressions of IL-6 and IL-23 were elevated in the patients with cGVHD. Furthermore, C5a accelerant augmented the production of IL-6 and IL-23 whereas C5aR blockade reduced. Taken together, these studies reveal that C5a modulates Th17 cell differentiation and its related cytokines production.

C5aR antagonist has been tested in classical inflammatory diseases including rheumatoid arthritis and Gaucher disease [30, 31], and a widening of the indications for C5aR antagonist to traditionally, non-inflammatory diseases such as cancer [15, 23]. PMX53, a highly selective C5aR antagonist, has been tested significantly reduce C5a-mediated inflammation in these diseases [32]. We evaluated the efficacy of PMX53 administrated by intraperitoneal injection every three days in cGVHD mice. PMX53-treated mice developed less disease, as evidenced by their reduced clinical scores. Similarly, PMX53-treated mice experienced improved survival than controls. In addition, a marked reduction in portal inflammatory response and bile duct destruction was seen in sections taken from PMX53-treated mice compared with controls, in which marked periportal inflammation is observed. Further, C5aR blockade could alleviate aberrant IL17A production, suggesting a potent suppressive role of PMX53 in attacking T effector cells in cGVHD through reduced Th17 cells generation and function.

In this study, we cultured the splenocytes from C5aR deficient mice and WT mice and performed the gain- and loss-function assay. In response to extrinsic C5a, we found significant upregulation of Th17 cells and its related cytokines of IL-17A, IL-6, and IL-23 as compared with unstimulated controls. In the contrast, upon combined treatment with PMX53, we found substantial reduction of Th17 cells and downregulation of IL-17A, IL-6, and IL-23 production. Similarly, C5a-C5aR signaling blockade using the splenocytes from C5aR-/- mice resulted in marked reduction of Th17 cell generation as well as IL-17A, IL-6, and IL-23 production, confirming the requirement of C5a-C5aR for the induction of a Th17-promoting microenvironment. The inductive effect of a lack of C5aR may be exploited as a potent method to lessen Th17 cells and suppress cGVHD diseases.

In summary, we have reported that the aberrant Th17 response and excessive IL-17A production in patients with cGVHD were associated with C5a upregulation. As severe cGVHD has proven difficult to treat with existing therapies, the encouraging efficacy of PMX53 in this study suggested that modulation of C5a/C5aR signaling may hold promise for the treatment of cGVHD. We also envision that application of PMX53 has implications beyond cGVHD, such as other Th17-mediated diseases.

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Disclosure of conflict of interest

None.

Abbreviations

cGVHD, Chronic graft-versus-host disease; HSCT, Hematopoietic stem cell transplant; IL-17A, Interleukin-17A.

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