Oral administration of Coenzyme Q10 has the capacity to stimulate innate lymphoid cells class two during Experimental cerebral malaria

Abstract

Cerebral malaria is a complex neurological syndrome, whose pathology is mediated by inflammatory processes triggered by the immune system of the host following infection with *P. falciparum.* Coenzyme Q10 (CoQ10) is an obligatory cofactor in the electron transport chain and a potent antioxidant which has been identified as a modulator of gene expression, inflammation and apoptosis. However, the modulatory effects of CoQ10 during PbA infection process and risk occurrence of ECM have not been determined. In the present study we sought to determine the role of CoQ10 in regulation of innate lymphoid cells during pathogenic immune responses of ECM. We observed significant increase in the percentage of ILC2 in the spleens of Co-Q_{10} supplemented PbA-infected mice; whereas the frequency of ILC1 and ILC3 were comparable in the spleens upon PbA infection. The results also show Splenic ILC2 from CoQ_{10} mice are avid co-producer of IL-13 (T_h2 phenotype cytokine) during ECM. Our data collectively demonstrates that Coenzyme Q10 administration was very effective in stimulating Innate lymphoid class two (ILC2), which are known to play a protective role during ECM.

Key words: Coenzyme Q10; Experimental cerebral malaria and *Plasmodium berghei ANKA.*

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1. Introduction

Malaria is a global health disaster, which claims many lives, according to the WHO reports (2018) during the year 2017; 219 billion people were at risk of infection and 435,000 died from malaria [1]. Malaria is a vector-transmitted disease and is caused by four species of *Plasmodium* parasites that are known to infect humans, namely: *P. falciparum, P. vivax, P. malariae and P. ovale.* Among these, *P. falciparum* is the most dreaded due to high numbers of neurological complications and deaths attributed to it. Cerebral malaria (CM) is one of the most life threatening complications of *P. falciparum* infection often affecting infants and travellers from non-endemic areas. CM is characterized by delirium, body ache, fever, coma and ultimately

impaired consciousness in patients - thus a complex neurological syndrome whose pathology is mediated by inflammatory processes following infection with the parasite [2]. It is well-accepted that sequestration of the parasite in microvasculature due to the adherence of pRBCs in the endothelial lining of the brain and also other organs is the main reason of pathogenesis and changes in tissue pathophysiological [3]. Additionally, pro-inflammatory and inflammatory cytokines also play a major role in the pathogenesis of CM. Importantly, in well-described animal models of CM; it has been shown that pathogenesis of experimental CM (ECM) that is induced in C57BL/6 mice upon infection with *P. berghei ANKA* is aggravated by cytotoxic CD8+ T lymphocytes [4].

Innate lymphoid cells (ILCs) are unique lineage of lymphocytes capable of expressing surface receptors and effector molecules of T cell subsets that are under the regulation of transcription factors, accentuating their resemblance to adaptive lymphoid cells [5]. Importantly, it is well recognized that ILCs are effector cells that play a significant role in the regulation of autoimmune inflammation, tissue remodeling and metabolic homeostasis. Moreover, ILCs play more intricate functions throughout the period of immune responses, orchestrating the shift from innate to adaptive immunity and contributing to chronic inflammation. Note that, if the role of ILCs is not properly regulated; then this will lead to chronic pathologies that are associated with tissue inflammation, autoimmunity and allergies [6].

Therefore, the importance of ILCs in the maintenance of metabolic homeostasis has started to emerge, underlining their importance in fundamental physiological processes beyond infection and immunity. However, the role of ILCs in regulation of innate and adaptive immunity in ECM remains elusive. This is in particular interesting as the induction of inflammatory responses during *Plasmodium* infection is a double-edged sword with the absolute requirement of T cells and inflammatory mediators for proper parasite burden elimination on the one hand *versus* detrimental consequences resulting from overwhelming production on the other hand. Recently, it has also been demonstrated that IL-33 mediated protection against ECM is associated with the induction of ILC2 and alternatively activated macrophages of the M2 phenotype [7]. This observation in particular is very important for our experiments because it will help delineate the role and mechanisms of ILCs in the pathogenesis of ECM.

The question now arises, whether ILCs might have a protective role in PbA infection or whether they rather contribute to pathology via the recruitment of inflammatory monocytes.

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Despite the importance of ILCs in the development of immunity against intracellular infections and being implicated in the pathogenesis of inflammatory conditions, their role in regulation of immune-pathogenesis, generation of effector molecules and antigen specific T-cell responses in ECM is poorly understood. Therefore, improved understanding the role of ILCs in immune regulatory mechanism will provide insight to the role of this novel type of innate lymphocytes, which might be helpful for the understanding of the molecular basis of malaria and help to develop novel therapies.

Coenzyme Q10 (CoQ10) is an anti-oxidant and free-radical scavenger which is like vitamins C, vitamins E and glutathione acts against oxidative [8-9]. It has been applied for a potential supplementary treatment of hypertension, neurodegerative disorders [10] and recently we demonstrated the potential role of CoQ10 on Human African Trypanosomiasis disease [11]. However, the role of CoQ10 in the pathogenesis of inflammatory conditions, their role in regulation of immune-pathogenesis and generation of effector molecules in ECM currently has not been determined. Therefore, this study sought to determine the role of CoQ10 in regulation of innate lymphoid cells during pathogenic immune responses of ECM.

2. Material and Methods

2.1 Mice and ethical clearance

Three-four weeks-old female C57BL/6J mice were purchased from International Livestock Research Institute (ILRI) mice were bred under specific pathogen free conditions in the house of experimental therapy (HET) of the Technical University of Kenya. All experimental procedures and protocols involving mice were also reviewed and approved by Institutional review Committee (IRC) of Institute of Primate Research Karen, Kenya. The mice were maintained on mice pellets and water *ad libitum* at room temperature. Wood-chippings were provided as bedding material.

2.2 Experimental design

The mice were allocated randomly into cages with n=5-6 mice per group while survival experiment were conducted with 8 mice. Experimental mice were sub-divided into three groups: Group one was wild type (WT) naive mice which served as control, group two: was WT + *Plasmodium berghei ANKA* (PbA) infected mice and group three: WT + Coenzyme-Q_{10}+PbA-infected mice.

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2.3 Treatment of mice with 200mg/kg CoQ10

Oral administration of 200mg/kg of CoQ10 was done daily for one month prior to infection with PbA to the experimental group three. Administration was done of CoQ10 was done using a gavage needle and continued thereafter until six days post infection when the mice were sacrificed. CoQ10 solution (Zambom Group S.p.A., Italy) was prepared by directly dissolving it in olive oil. The solutions were prepared immediately before use and were protected from the light before administration to the animals.

2.4 *Plasmodium berghei* ANKA (PbA) infection

All experiments in this study made use of wildtype and of transgenic *Plasmodium berghei* ANKA (PbA). All experimental were infected by intravenous (i.v) infection with 5x10^4 pRBC that was obtained from a syngenic donor mouse previously infected with stock solution of PbA, which was stored in liquid nitrogen as pRBC in solution containing 10% glycerol. Parasitaemia in infected mice was monitored daily with 5% Giemsa stained blood smears.

2.5 Preparation of spleen

To obtain single spleen cells suspension, mice were sacrificed on day 6 of ECM, the spleen was perfused with a syringe containing collagenase A, followed by slicing the organ into small pieces; then the spleen was digested in collagenase for 30 min at 37 °C. Ten ml of magnetic activated cell sorting (MACS) buffer was added to the spleen and spleen tissue was crushed using syringe piston through a sieve in a petri dish with sterile PBS (Life Technologies Corporation, Grand Island, NY, USA). Cell preparations were depleted of erythrocytes with ammonium-chloride-Tris-buffer (ACT buffer) and resuspended in appropriate Roswell Park Memorial Institute (RPMI) medium and counted. After counting the cells of each mouse with CASYton the suspensions were adjusted to 1 x 10^7 splenocytes per ml with RPMI medium.

2.6 Flow cytometric analysis of ILCs and cytokines

Splenocyte cells ($1x10^5$) were cultured and either stimulated with or without PMA-ionomycin (50ng/ml PMA and 1μM ionomycin) for about 5hrs after which protein secretion was blocked by addition of 100ng/ml of monesin (Golgi stop) solution (BD Biosciences) according to the manufacturer's protocol. Cells were then stained for surface proteins such as ILC1 and ILC2 and permeabilized using the e-Bioscience permeabilization reagent for 30 min. After a washing step, cells were stained using the corresponding antibody towards IFN-γ, TNF-α, IL-13 and IL-4 or their isotype controls after 30 min. Cells were then washed twice and acquired using the FACS

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LSR Fortessa of BD Biosciences flow cytometer. ILCs live cells were analysed through a forward and side scatter and approximately 100,000 events were acquired through FACS for cells analysis for each experiment. The data was analyzed with FACS Diva® software (Becton Dickinson). A master mix for the cytokine antigens, the single stained controls and the FMOs were prepared and applied to the cells for the purpose of gating identification of the correct cell population.

2.7 Statistical analysis

One way ANOVA to compare treatment groups with controls Bonferronis post hoc tests was done for internal comparison. Results were given as mean± with significance set as $p<0.05$. The statistical analyses was done using Graphpad prism version 5 software package

3. Results

3.1 Coenzyme-Q_{10} supplementation leads to dramatic alteration of splenic innate lymphoid cells during ECM

Innate lymphoid cells (ILCs) are a unique lineage of lymphocyte effector cells that have an important role in the regulation of autoimmune inflammation, tissue remodeling, cancer and metabolic homeostasis [12]. Despite being implicated in shaping adaptive immune responses, relatively little is known about their function in regulation of innate and adaptive immunity in experimental cerebral malaria (ECM) in a complete T-cell compartment. Therefore, the dynamic patterns of splenic ILCs in Co-Q_{10} administered mice during PbA infection and WT PbA-infected mice were examined. Specifically, the localization and characterization of ILCs in WT PbA-infected with or without Co-Q_{10} supplementation and the immune responses in the spleen via flow cytometry (FACS) was determined. Splenic ILCs were identified as Lineage negative and positive for the innate lymphoid cell transcription markers T-bet (ILC1), GATA-3 (ILC2) or RORgt (ILC3) (Fig. 1A-C). Significant increase in the percentage of ILC2 was noted (GATA-3) (Fig. 1D) in the spleens of Co-Q_{10} supplemented PbA-infected mice; whereas the frequency of ILC1 and ILC3 were comparable in the spleens upon PbA infection (Fig. 1E-F). This study demonstrates that Co-Q_{10} supplementation might influence the recruitment of ILC2 into the spleen from mucosal barriers, which contributes to improved protection in these mice.

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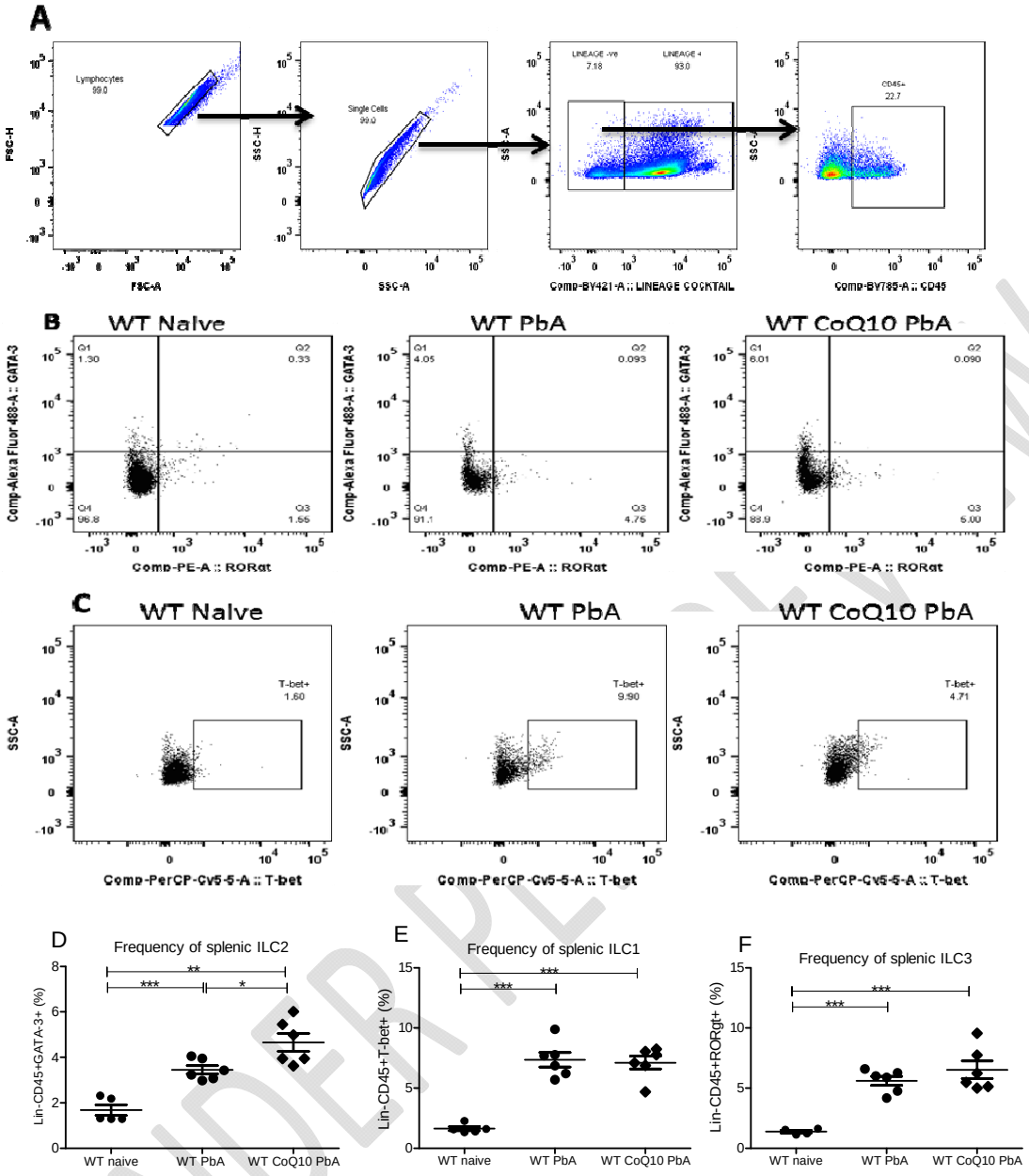

Figure 1: Comparison of splenic levels of innate lymphoid cells among mice either supplemented with or without Co-Q_{10} during PbA infection

C57BL/6J mice were either supplemented with or without Co-Q_{10} or then infected with $5x10^4$ PbA iRBC. At day 6 of ECM, splenic cell from the individual mice were prepared and analysed by FACS. (**A**) Flow cytometric gating strategy for identification of positive ILCs (**B-C**) The displayed dot plots shows the levels of GATA-3+ and RORgt+ and T-bet+ cell population. (**D**) Calculated percentage of splenic Lin-CD45+GATA-3+ (ILC2). (**E**) Calculated percentage of splenic Lin-CD45+T-bet+ (ILC1). (**F**) Calculated percentage of splenic Lin-CD45+RORgt+ (ILC3). Bars show the mean of each group ± SEM and are representative of at least two independent experiments. Percentage of ILCs was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: *P ≤ 0.05; **P ≤0.01;***P ≤0.001). n = 5-

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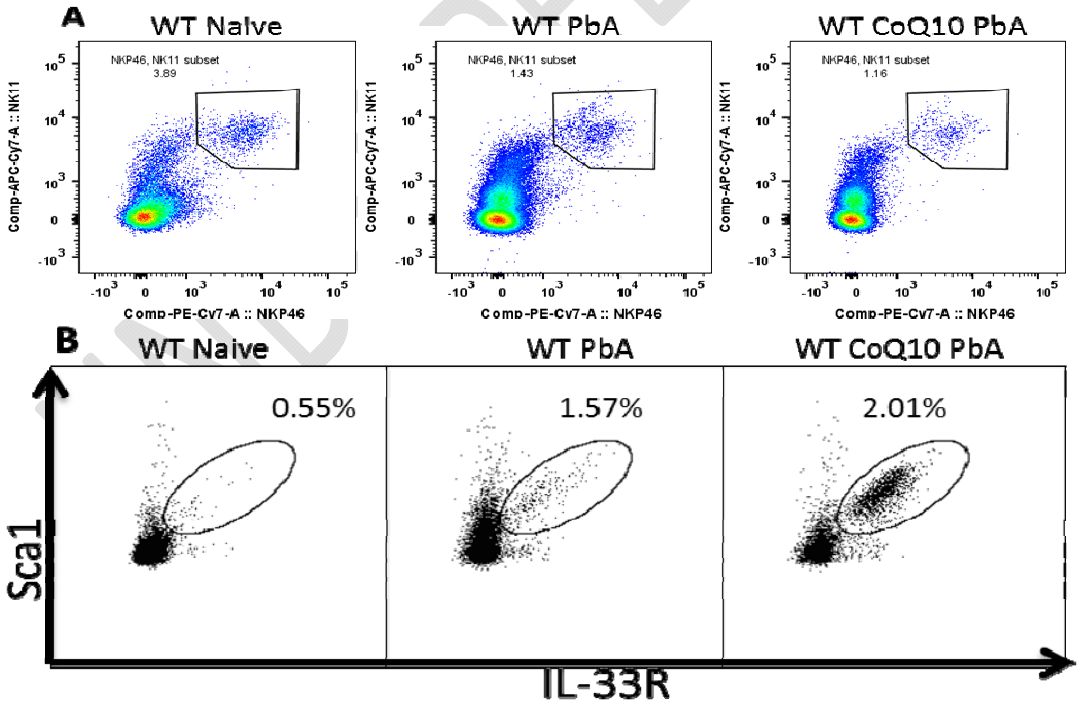
6mice per group.

3.2 Varying frequency of ILC1&2 in the spleen during PbA infection

ILC subsets can be categorized based on the transcription factors, cell-surface markers and functional properties of cytokine production. Innate lymphoid cells have been shown to express extracellular markers which can aid there identification. Therefore, it is possible to identify them based on the cell surface markers and also on the type of cytokine they produce.

Hence, this study sought to determine how these lymphocytes are modulated in an ECM model, where some mice were supplemented with CoQ10. Extracellular markers were used to characterize ILC1 and ILC2 in the spleen as $Lin^{-}CD45^{+}NK1.1^{+}NKP46^{+}$ and $Lin^{-}CD45^{+}Sca1^{+}IL\text{-}33R^{+}$ respectively (Fig. 2A-B). A small population of ILC1 in the spleen that was marked as lineage negative (Lin^{-}) cells that lacked surface markers for myeloid cells, T and B cells but were positive for expression of NK1.1 and NKP46 receptor (Fig. 2C), was identified. Intriguingly, I also observed a slight increase in the frequency of ILC2 in Co-Q_{10} supplemented mice, though not significant (Fig. 2D). The elevated levels of ILC2 in the present study may be linked to protection against ECM in Co-Q_{10} supplemented mice consistence with early studies in which ILC2 was shown to protect mice against ECM [7].



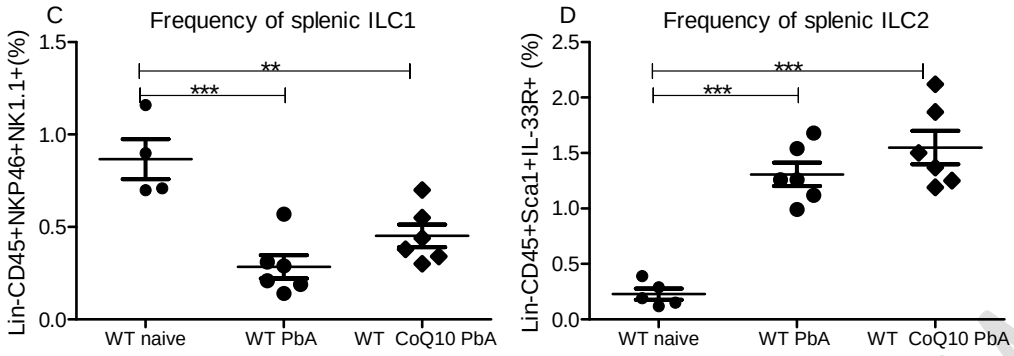


Figure 2: Comparison of splenic levels of innate lymphoid cells based on surface markers among mice either supplemented with or without Co-Q_{10} during PbA infection

C57BL/6J mice were either supplemented with or without Co-Q_{10} then infected with $5x10^4$ PbA iRBC. At day 6 of ECM, splenic cell from the individual mice were prepared and analysed by FACS. (**A-B**) The displayed dot plots shows the levels of ILCI (NK1.1+NKP46+) and ILC2 (IL-33R+SCA1+) cell population. (**C)** Calculated percentage of splenic Lin-CD45+NK1.1+NKP46+ (ILC1). (**D**) Calculated percentage of splenic Lin-CD45+IL-33R+SCa1+ (ILC2). Bars shows mean of each group ± SEM and are representative of at least two independent experiments. Percentage of ILCs was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: **P ≤0.01;***P ≤0.001). n = 5- 6mice per group.

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3.3 Splenic ILC2 from Co-Q_{10} mice are avid co-producer of IL-13 (T_h2 phenotype cytokine) during ECM

Protection of intracellular infections by ILC1 as avid co-producers of IFN-γ and TNF is well established [12]. Other previous studies have also shown that ILC2 is responsible for protection against ECM [7]. Already this study has noted varying frequencies of ILC1 and ILC2 during PbA infection. On this basis, it was quite compelling to speculate that the protective activity mediated by ILCs was associated with production of key signature cytokines. Therefore, the functional profile of ILC1 and ILC2 for cytokine production was assessed. In order to verify this, splenic like ILC1 and ILC2 were sorted out by FACS on the basis of extracellular surface markers and then cultured with PMA/Ionomycin/recombinant IL-2/IL-33. Thereafter, cytokine release was measured by flow cytometry. Surprisingly, as shown in Fig. 3, splenic ILC2 from Co-Q_{10} supplemented mice showed a higher propensity to produce IL-13 (Fig. 3B) and IL-4 production (Fig. 3C). The production of IFN-γ and TNF-α cytokine from splenic ILC1 with or without Co-Q_{10} supplementation during PbA infection was comparable among the groups (Fig. 3D-E). These data suggest a differential role of ILC1 and ILC2 during ECM.

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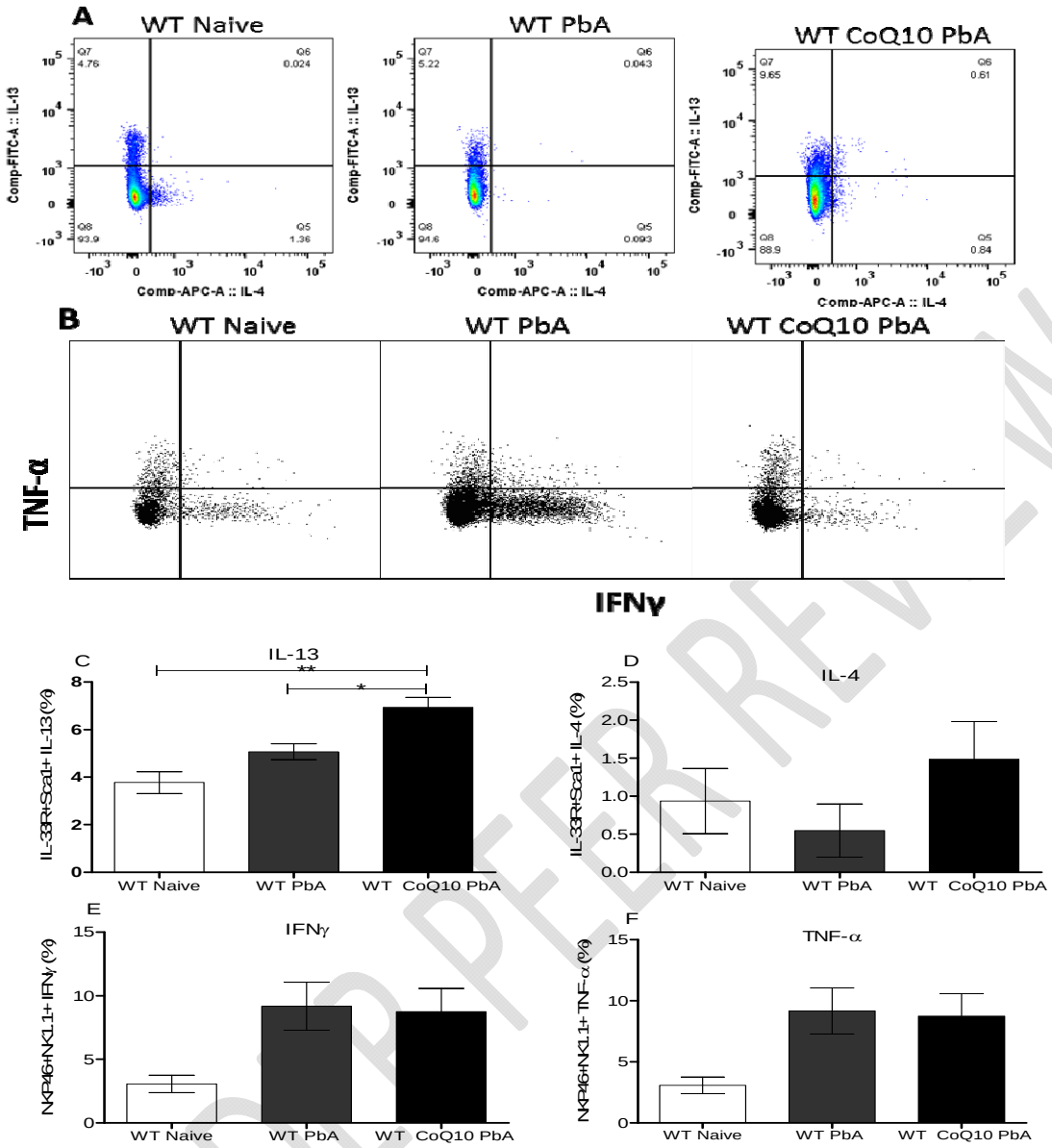

Figure 3: Comparison of splenic cytokine levels from innate lymphoid cells among mice either supplemented with or without Co-Q_{10} during PbA infection

C57BL/6J mice were either supplemented with or without Co-Q_{10} or then infected with $5x10^4$ PbA iRBC. At day 6 of ECM, splenic cell from the individual mice were prepared and analysed by FACS. (**A-B**) The displayed dot plots shows the levels of ILC2 cytokine (IL-4 Vs IL-13) and ILC1cytokine (TNF-α Vs TNFγ) cell population. (**C**) Calculated percentage of splenic IL-33R+SCa1+IL-13+. (**D**) Calculated percentage of splenic IL-33R+SCa1+IL-4. (**E**) Calculated percentage of splenic NK1.1+NKP46+IFNγ. (**F**) Calculated percentage of splenic NK1.1+NKP46+TNF-α. Bars shows mean of each group ± SEM and are representative of at least two independent experiments. Percentage of ILCs cytokine was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: *P ≤0.05;**P ≤0.01). n = 5-6 mice per group.

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4. Discussion

He we demonstrate for the first time that Coenzyme Q10 administration was very effective in stimulating Innate lymphoid class two (ILC2), which are known to play a protective role during ECM. Innate lymphoid cells (ILCs) are a unique lineage of lymphocytes whose discovery was made recently. These immune cells have been identified to be present in the bone marrow, liver, and spleen. More importantly, the mucosal surfaces are enriched with high population of ILCs [13]. It is well recognized that ILCs play a crucial role in organ homeostasis, regulation of glucose and lipid metabolism and generation and repair of the epithelial [14].

ILCs have also been associated with inflammation and inflammatory-driven diseases; a recent study demonstrated ILC1-mediated protection against *T. gondii* infection through IFN-γ production [12]. However, it is currently not clear the exact role played by type 1 innate lymphoid cells in the generation of immune-pathology during ECM. On the other hand, type 2 innate lymphoid cells (ILC2), are part of the dynamic immune cells that are essential for protecting mice against ECM [7]. Specifically, administration of exogenous IL-33 drives expansion of ILC2 increase with concomitant production of IL-4, IL-5, IL-9 and IL-13. Additionally, ILC2 represent the main initial producer of IL-5 and IL-13 during allergic inflammation and worm infection [15-16]. Expansion of ILC2 is indispensable for worm expulsion [17]. In contrast, type 3 innate lymphoid cells have been previously shown that they do not play any functional role in ECM development [18]. In this study, a significant increase in the percentage of ILC2 in the spleens of Co-Q_{10} supplemented PbA-infected mice was noted. Moreover, sorted out ILC2 were avid producers of IL-13 cytokine which is crucial in driving M2 Macrophages (Besnard *et al*., 2015). This observation is further supported by our finding showing that Co-Q_{10} administered mice had high levels of M2 phenotype.

Taken together, improved protection from ECM in Co-Q_{10} administered mice is clearly linked to enhanced expression of splenic ILC2 that produces high amount of IL-13, ultimately polarizing macrophages into M2 phenotype that exhibit anti-inflammatory properties. Nevertheless, it remains unclear how Co-Q_{10} modulates gene expression that are associated with innate lymphoid cells. Therefore, a more detailed gene analysis of how ILCs turn up during ECM is warranted.

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