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Epidermal filaggrin deficiency mediates increased systemic Th17 immune response

Running head: Systemic Th17 response mediated by filaggrin deficiency

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Bulleted statements

What's already known about this topic? An increased level of Th17 cells is found in skin of flaky tail mice. Th17 cells seem to be involved in the pathogenesis of atopic dermatitis.

What does this study add? We show that loss-of-function mutations in the filaggrin gene could be a driver of increased frequency of peripheral Th17 cells in both human and mice. The increase in Th17 cells is most likely mediated by extrinsic factors.

Summary

Background: Cellular Th17 infiltrates dominate skin inflammation in filaggrin-deficient flaky tail (*ft/ft*) mice, and Th17 cells are found in both skin and blood of patients with acute atopic dermatitis (AD). However, the potential role of loss-of-function mutations in the filaggrin gene (*FLG*) for increased peripheral Th17 cells is unclear.

Objective: To study whether mutations in *FLG* influence the frequency of peripheral Th17 cells.

Methods We studied blood samples from 6 adults with mutations in *FLG* and 5 controls without mutations for frequencies of cytokine producing CD4⁺ T cells. *ft/ft* mice and wild-type (WT)

control mice were evaluated for IL-17-producing CD4⁺ T cells in naïve mice and following DNFB-challenge. In addition, the TCR Vβ-chain repertoire was analyzed by flow cytometry.

Results Human studies showed increased frequency of peripheral Th17 cells in *FLG* mutation carriers when compared to WT individuals. Mouse studies showed increased frequency of peripheral Th17 cells in adult, but not in 2-week old *ft/ft* mice. Moreover, altered TCR Vβ chain repertoire was found in *ft/ft* mice when compared to WT mice. An increased frequency of CD4⁺ Vβ10⁺ T cells producing IL-17 was found in the spleen of adult *ft/ft* mice when compared to WT mice. Finally, DNFB challenge induced an increased number of Th17 in *ft/ft* mice compared to WT mice.

Conclusion Deficiency of filaggrin appeared to be a driver of increased peripheral levels of Th17 cells. This increase must be acquired as peripheral Th17 cells were found in adult, but not in 2-week old *ft/ft* mice indicating involvement of exogenous factors.

(253 word, max 250 words)

Abbreviations

AD Atopic dermatitis

FLG Filaggrin gene

ft/ft Flaky tail

IL Interleukin

OVA Ovalbumin

PBMC Peripheral blood mononuclear cell

PMA Phorbol 12-myristate 13-acetate

SEM Standard error of mean

TCR T cell receptor

WT Wild-type

DNFB 2,4-dinitrofluorobenzene

OOA Olive oil:acetone

Introduction

Atopic dermatitis (AD) is a chronic and relapsing skin condition characterized by pruritus, xerosis and dermatitis in typical anatomical sites ¹. The etiopathogenesis of AD is unclear, but a primary skin barrier abnormality resulting in secondary immune reactivity and further impairment of the epidermal barrier is central in many cases ². Importantly, common filaggrin gene (*FLG*) mutations strongly increase the risk of AD characterized by early-onset, a severe and persistent course as well as atopic co-morbidities and super-infections ^{3;4}.

Flaky tail (*ft/ft*) mice have a frameshift mutation in *FLG* and have been described as a spontaneous model for AD characterized by filaggrin deficiency ⁵. Although *ft/ft* do not develop visible eczematous skin lesions before 28 weeks of age, 8-14 weeks old mice already show increased expression of interleukin (IL)-17 as well as IL-1 β , IL-6 and IL-23 in the skin ^{6;7}. While

several cell types including subsets of innate lymphoid cells, neutrophils, CD8⁺ T cells and $\gamma\delta$ T cells can release IL-17⁸, skin inflammation in *fl/fl* mice after 28 weeks is dominated by Th17 cells^{6;9}. Th17 cells have also been identified in peripheral blood of patients with AD¹⁰⁻¹², and have been associated with the severity of AD as well as with skin infections^{10;11}.

IL-17 mainly induces signaling in cells of non-hematopoietic origin including epithelial cells like keratinocytes. IL-17 induces the production of a variety of cytokines (e.g. IL-1 β and IL-6) and chemokines (e.g. CXCL1, CXCL2 and CXCL5), which largely explain the inflammatory activity of IL-17⁸. Interestingly, the increased colonization with superantigen-producing *Staphylococcus aureus* (*S. aureus*) in patients with AD may stimulate IL-17 production by CD4⁺ T cells^{13;14}.

Thus, increased percentages of Th17 cells have been found in AD patients with skin infections indicating that Th17 reactivity in these patients is partly driven by infection¹⁰. Importantly, filaggrin proteins appear to play an important role in the defense against *S. aureus*, since reduced filaggrin expression results in increased colonization with *S. aureus* and since *S. aureus* α -toxin preferentially targets and destroys filaggrin-deficient keratinocytes¹⁵⁻¹⁷. Moreover, IL-17-mediated skin inflammation in *fl/fl* mice seems to be driven by skin infections as *fl/fl* mice kept under germ-free conditions have less IL-17-mediated skin inflammation than *fl/fl* mice kept under specific pathogen-free conditions¹⁸.

In this study, we show that *FLG* deficiency leads to a systemic increase in the fraction of Th17 cells in humans and mice. Our results indicate that this increase in Th17 cells might be driven by extrinsic factors such as pathogenic microorganisms and chemicals.

Materials and Methods

Dermatitis patients

Participants were recruited from a previous study on AD performed at Gentofte Hospital, Denmark¹⁹. A total of 11 participants, all of Northern European descent, were enrolled (Table 1). Three common loss-of-function mutations in the *FLG* were analyzed (R501X, 2282del4 and R2447X) by multiplex PCR analysis as previously described²⁰. AD was diagnosed using the UK Working Party's Diagnostic Criteria²¹. None of the participants without AD had clinical ichthyosis vulgaris. All participants gave informed and written consent, and the study was conducted in accordance with the Declaration of Helsinki principles for research involving human subjects. The study was approved by the local Ethics Committee (H-1-2011-011) and the Danish Data Protection Agency. Blood samples were taken and peripheral blood mononuclear cells (PBMC) were purified as previously described²².

Mice

ft/ft mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and bred in our animal facility. C57BL6 and BALB/c wild-type (WT) mice were purchased from Taconic (Ry, Denmark). All mice were housed in specific pathogen-free facilities at the Department of Experimental Medicine, Panum Institute, The University of Copenhagen, in accordance with national animal protection guidelines (license number: 2012-15-2934-00663). Mice used in the experiments were either 2 weeks old or 8-20 weeks old.

Reagents and Antibodies

Phorbol 12-myristate 13-acetate (PMA), ionomycin, monensin, and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma Aldrich (St. Louis, MO, US). Fluorochrome-conjugated anti-human IL-17A (eBio64DEC17) and anti-human IL-22 (IL-22JOP) from eBioscience (San Diego, CA, USA) and anti-human CD4, anti-human CD8, anti-human IL-4, anti-human IFN γ , anti-mouse CD4 (RM4-5), anti-mouse CD8a (53-6.7) anti-mouse IL-17A and mouse V β T cell receptor (TCR) screening panel kit were purchased from BD Pharmingen (San Diego, CA, US).

Intracellular cytokine staining

Single cell suspensions from skin draining lymph node and spleen as well as PBMC purified from human blood were stimulated at 4×10^6 /mL with PMA and ionomycin in the presence of monensin for 4 hours followed by intracellular cytokine staining as previously described^{22;23}. Cells were subsequently analyzed on a FACSCalibur (Becton Dickinson) with Cellquest software and FlowJo.

Epicutaneous sensitization and challenge

For sensitization, mice were painted on the dorsal side of both ears for three consecutive days with 25 μ l 0.15% DNFB in a 1:4 olive oil:acetone (OOA) mixture. 21 days after sensitization, mice were challenged once on the dorsal side of both ears with 25 μ l 0.15% DNFB in OOA. Control mice were sensitized and challenged with OOA. Mice were euthanized 24 h after challenge and the cervical lymph nodes were removed for flow cytometric analysis.

Statistical analysis

Statistically significant differences were determined by Student's unpaired, one-tailed t-test. P-values of <0.05 were considered to be statistically significant.

Results

Increased frequency of Th17 cells in FLG mutation carriers

To investigate whether mutations in *FLG* affected the frequencies of Th1 (CD4⁺IFN γ ⁺), Th2 (CD4⁺IL-4⁺), Th17 (CD4⁺IL-17⁺) and Th22 (CD4⁺IL-22⁺) cells, we performed intracellular cytokine staining and flow cytometric analyses on PBMC after stimulation with PMA/ionomycin. We found comparable frequencies of Th1, Th2 and Th22 cells in individuals with and without mutations in *FLG* (Figure 1a). Notably, an approximately two-fold increase in the amount of Th17 cells was found in *FLG* mutation carriers when compared to wild type carriers (Figure 1a and b) (p=0.03).

Increased frequency of Th17 cells in spleen of *ft/ft* mice

Since *ft/ft* mice have an IL-17-dominated skin inflammation, we investigated whether they also have systemic Th17 inflammation⁶. Hence, flow cytometric analysis of the frequency of Th17 cells in the spleen was performed. Pertinently, adult *ft/ft* mice exhibited increased frequency of Th17 cells in the spleen when compared to similarly aged C57BL6 (p=0.0007) and BALB/c mice (p=0.02) (Figure 2a and b).

The increased frequency of Th17 cells in the spleen of *ft/ft* mice is acquired

Different mice strains have different Th profiles with BALB/c mice being more prone to Th2 and Th17 skewed inflammatory responses, and with C57BL6 mice being prone to Th1 skewed immune responses⁶. The increased frequency of Th17 cells in the spleen of *ft/ft* mice could therefore be either an inherited or an acquired phenomenon. We examined the frequency of Th17 cells in the spleens of 2-week old *ft/ft* mice and compared it to 12-20 week old *ft/ft* mice. An approximately 2-fold increase in the frequency of Th17 cells was found in the spleen of 12-20 weeks old *ft/ft* mice when compared to 2-week old *ft/ft* mice ($p=0.0001$) (Figure 3). This observation indicates that the increased frequency of Th17 cells in adult *ft/ft* mice is acquired.

Altered TCR β -chain repertoire and increased frequency of $V\beta 10^+$ Th17 cells in *ft/ft* mice

It was recently suggested that IL-17-mediated skin inflammation in *ft/ft* mice is driven by infections¹⁸. *S. aureus*, a common microorganism found on the skin of patients with AD, produces superantigens that bind to specific TCR β -chains^{13;24}. Flow cytometric analysis showed an altered TCR β -chain repertoire in cells from skin draining lymph nodes in *ft/ft* mice when compared to WT controls by flow cytometric analysis (Figure 4a). As several differences in TCR β -chain repertoire also were found between C57BL6 mice and BALB/c mice, the mixed background of *ft/ft* mice is likely to explain these observations. We found an increased frequency of CD4⁺ T cells in the lymph nodes expressing TCR $V\beta 10$, a receptor that binds *S. aureus* superantigen enterotoxin C2 (SEC2), in adult *ft/ft* mice when compared to adult C57BL6 and BALB/c mice, but also when compared to 2-week old *ft/ft* (Figure 4a and b)²⁴. This indicates an induced expansion of CD4⁺ T cells expressing TCR $V\beta 10$ in adult *ft/ft* mice. In accordance with our findings on lymph node CD4⁺ T cells, we found an up to 2-fold increase in the frequency of

CD4⁺ T cells in the spleen expressing TCR V β 10 in *ft/ft* mice when compared to WT controls (Figure 4c). Next, we investigated whether CD4⁺ T cells expressing TCR V β 10 produced IL-17 following stimulation with PMA/ionomycin. We found that a proportion of CD4⁺ T cells expressing TCR V β 10 produced IL-17 in all three mouse strains, but also that *ft/ft* mice had an increased frequency of IL-17-producing CD4⁺ T cells expressing TCR V β 10 when compared to C57BL/6 (p=0.008) and BALB/c mice (p=0.01) (Figure 4d).

DNFB challenge induced an increased Th17 response in *ft/ft* mice

To further evaluate the Th17 response in *ft/ft* mice as compared to WT mice, we exposed *ft/ft* mice and WT controls epicutaneously to DNFB. In correlation with our findings in the spleen of untreated mice (Figure 2), vehicle-treated *ft/ft* mice had significantly increased amounts of Th17 cells in the draining lymph nodes as compared to WT controls (Figure 5a). Challenge with DNFB induced a significantly stronger Th17 response in the draining lymph nodes of *ft/ft* mice as compared to WT controls; with an almost four-fold increase in the number of Th17 cells in *ft/ft* mice compared to an only two-fold increase in WT mice (Figure 5). Thus, suggesting that epicutaneous antigen exposure induces a stronger Th17 response in *ft/ft* mice compared to WT controls.

Discussion

We found that *FLG* mutations were associated with increased frequency of Th17 cells in the periphery of both humans and mice. We further showed that *ft/ft* mice had an age-dependent increased frequency of CD4⁺ T cells that express TCR V β 10 as well as IL-17 in the spleen when compared to WT controls. Finally, we show that challenge with DNFB induces an increased Th17 number in *ft/ft* mice compared to WT controls. Collectively, these findings suggest that the increased peripheral Th17 frequencies are induced by exogenous factors e.g. pathogenic microorganisms and chemical exposure^{13;25}.

Skin inflammation in *ft/ft* mice is predominantly mediated by IL-17⁶. As RAG2 deficient *ft/ft* mice, which lack all T and B cells, do not develop skin lesions, it seems likely that the IL-17 is produced by T cells⁹. Epicutaneous sensitization of non-stripped skin with ovalbumin (OVA) induced a systemic Th17 response in *ft/ft* mice, whereas no response was observed in BALB/c and C57BL6 mice⁶. In contrast, intraperitoneal OVA immunization induced a Th17 response in all three mice strains, with the lowest response in C57BL6 followed by *ft/ft* mice and the highest response seen in BALB/c mice⁶. Collectively, these observations suggest that the increased systemic Th17 response in *ft/ft* mice is initiated in the skin, possibly due to exogenous factors. In correlation with this, we showed that epicutaneous exposure to DNFB resulted in a significantly stronger Th17 response in the draining lymph nodes of *ft/ft* mice as compared to WT mice. This further indicates that exposure of filaggrin-deficient skin to exogenous factors, in this case a potent allergen, can drive strong Th17 responses.

Importantly, we recognize that spontaneous dermatitis in *ft/ft* mice could be an important driver for the systemic Th17 response. Thus, in addition to the frameshift mutation in *FLG*, *ft/ft* mice also carry a nonsense mutation in the *Tmem79/Matt (ma)* gene resulting in impaired lamellar granule secretory system and spontaneous inflammation^{26;27}. This genetic variation has also been shown to increase the risk of AD in humans²⁸. Therefore, we cannot rule out that the *ma* mutation could have contributed significantly to the Th17 response in the animal study.

However, as we also found an increased systemic frequency of Th17 cells in a small sample of human *FLG* mutation carriers, we find it unlikely that the *Tmem79/Matt (ma)* gene alone could explain our results.

Patients with AD have an increased colonization with *S. aureus* in lesional skin when compared to healthy individuals¹³. This is partly explained by a reduction of filaggrin degradation products as these normally prevent growth of *S. aureus* due to maintenance of a low pH, but also from direct growth inhibiting effects¹⁶. *S. aureus* can produce various types of superantigens that recognize TCR in a V β -specific manner. We showed that CD4⁺ T cells from *ft/ft* mice displayed a diverse distribution of V β in their TCR when compared to both C57BL6 and BALB/c mice. As a diverse distribution of V β in TCR on CD4⁺ T cells was also found between C57BL6 and BALB/c mice it is likely that part of this V β diversity is mediated by differences in the genetic background. However, we found an increased frequency of CD4⁺ T cells expressing V β 10 in adult *ft/ft* mice, but not in 2-week old *ft/ft* mice, nor in C57BL6 mice nor in Balb/c mice. In addition, we found an increased frequency of CD4⁺ T cells in the spleen expressing TCR V β 10 and producing IL-17 in *ft/ft* mice when compared to C57BL6 and BALB/c mice. Taken together, we find it likely that this increase is induced by exogenous factors.

The observed correlation between filaggrin deficiency and a systemic increase in Th17 cells in mice has, to our knowledge, not yet been shown in humans. Our study population was very small which may increase the risk of random error. Also, we had no objective measure for current disease activity such as SCORAD. We emphasize that AD is a recurrent chronic condition that is also often associated with asthma and rhinitis; factors that may affect the cytokine profile at the time of sampling. Larger prospective human studies are therefore required to clarify whether the increase in Th17 can be replicated, and if so, whether the increase primarily is driven by *FLG* mutations in general, or only in the setting of AD or atopic disease. Importantly, two previous studies found an increased frequency of Th17 cells in the PBMC from AD patients when compared to healthy controls ^{11;12}. While Koga et al. showed that the frequency of Th17 cells correlated with disease severity, Czarnewicki et al., showed that the frequency of Th17 cells rather correlated with skin infection and not with disease severity ^{10;11}. Finally, Th17 cells have been linked to systemic low grade inflammation and increased levels of IL-17 are found in obese individuals ²⁹⁻³¹, and AD was recently associated with overweight, obesity and an increased risk of cardiovascular diseases ^{32;33}. In these studies the roles of filaggrin and skin infections were not evaluated. Whether *FLG* mutations play a role in these relationships is currently unknown, but something that should be evaluated in future investigations.

Table 1 Study population characteristics

Participant number	<i>FLG</i> mutation carrier status	A history of AD	Self-reported Asthma or hayfever	Predisposition to asthma/hayfever/AD	Self-reported xerosis	Investigator assessed xerosis	CD4 ⁺ IL-17 ⁺ cells (%)
1027	WT	-	-	-	2	4	0.75
1053	WT	-	-	-	1	3	0.29
1004	WT	-	-	-	2	4	0.58
1043	WT	+	+	-	2	2	0.25
1029	WT	+	+	+	2	3	0.21
1030	Het2282del4	-	-	-	1	2	0.55
1018	Het2282del4	-	-	-	1	2	0.56
1006	Het501X	-	-	+	1	1	0.90
1037	Hom501X	+	+	+	1	1	1.25
1025	Hom501X	+	+	-	1	1	0.72
1062	CompHet R2447X/2282del4	+	+	-	1	1	0.56

FLG mutation carrier status: WT: Wild type, Het: Heterozygote. Hom: Homozygous, CompHet: Compound heterozygous. The filaggrin mutations are based on analysis of the three most common mutations in the European population, 501X, 2282del4, and R2447X.

AD: atopic dermatitis. AD was defined by the UK working party's Diagnostic Criteria²¹.

Participants without AD, but heterozygous, had irritant contact dermatitis (ICD) (participant 1030=ACD, 1018=ICD and 1006= ICD) according to ICDRG guidelines³⁴.

Participants without AD and without filaggrin mutations (participant 1027, 1053 and 1004, were healthy controls).

Self-reported asthma or hayfever was obtained from a questionnaire, and not diagnosed at the department.

Self-reported and objective evaluation of xerosis/skin dryness: 1 = very dry skin, 2 = dry/normal, 3 = normal, 4 = normal/rich, and 5 = rich skin hydration

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Figure legend

Figure 1

Increased frequency of peripheral Th17 cells in *FLG* mutation carriers.

(a) Frequencies of Th1, Th2, Th17 and Th22 cells in the blood of wild-type (n=5) and *FLG* loss-of-function mutation carriers (n=6). Data are presented as mean $\pm$ SEM. (b) Representative flow cytometry plots of CD4⁺IL-17⁺ cells in the blood of a wild-type and *FLG* mutation carrier. *P < 0.05.

Figure 2

Increased frequency of Th17 cells in the spleen of *ft/ft* mice compared to WT mice.

(a) Frequencies of Th17 cells in the spleen of adult (8-20 week old) mice. Data are presented as mean $\pm$ SEM. (b) Representative FACS plots showing the frequency of IL-17 producing cells within the population of CD4⁺ cells. *P < 0.05; ***P < 0.001.

Figure 3

Adult *ft/ft* mice exhibit higher frequencies of peripheral Th17 cells than 2-week old *ft/ft* mice.

Frequencies of Th17 cells in the spleen of 2-week old and 12-20-week old *ft/ft* mice. Data are presented as mean $\pm$ SEM. *** $P < 0.001$.

Figure 4

Altered TCR V β chain repertoire in *ft/ft* mice as compared to WT mice.

(a) TCR V β chain distribution diagrams in CD4⁺ T cell and (b) Frequency of CD4⁺ T cell expressing TCR V β 10 in skin draining lymph nodes (n=6 for *ft/ft* and C57BL6; n=3 for BALB/c). (c) Frequency of CD4⁺ T cell expressing TCR V β 10 in spleen (n=11 for *ft/ft* and C57BL6; n=3 for BALB/c). (d) Frequency of TCR V β 10⁺ CD4⁺ T cells producing IL-17 in spleen (n=9). Data are presented as mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

Figure 5

Increased Th17 responses in draining lymph nodes of *ft/ft* mice as compared to WT mice after DNFB challenge.

Mice were sensitized with 0.15% DNFB or vehicle (olive oil:acetone, OOA) on both ears for three consecutive days. After 21 days, mice were challenged once on both ears with 0.15% DNFB or vehicle. (a) Total number of Th17 cells in draining lymph nodes, 24 h after challenge, determined by cell counting and flow cytometric analysis. Pooled data from two independent experiments are shown, each with 4-5 mice per group. Data are presented as mean $\pm$ SEM. (b)

Representative FACS plots showing the frequency of IL-17 producing cells within the population of CD4⁺ T cells in the draining lymph nodes of mice sensitized and challenged with DNFB. *P<0.05; ***P < 0.001; ****P < 0.0001.

Figure 1
CM Bonefeld *et al.*

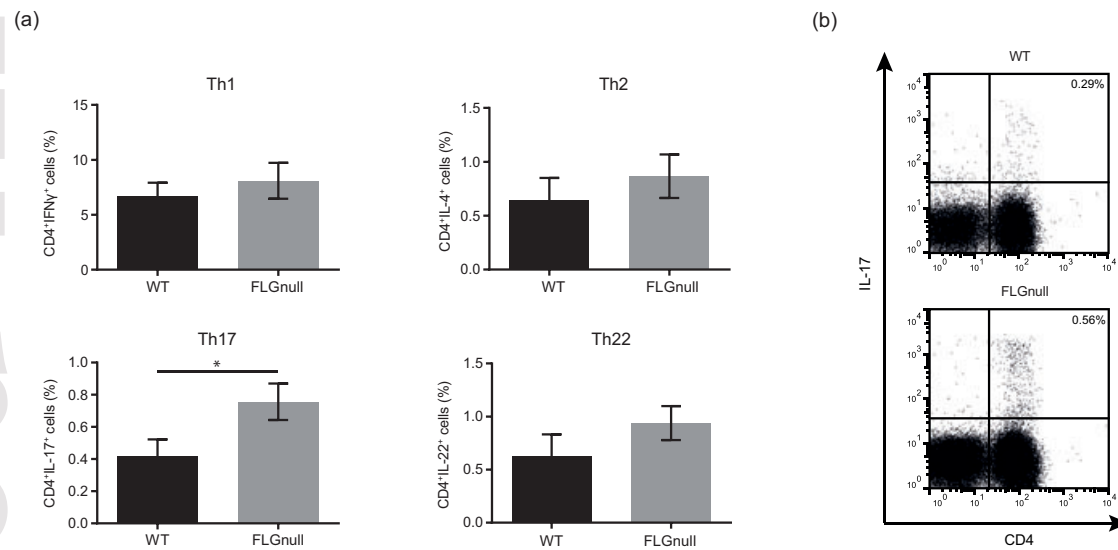


Figure 2
CM Bonefeld *et al.*

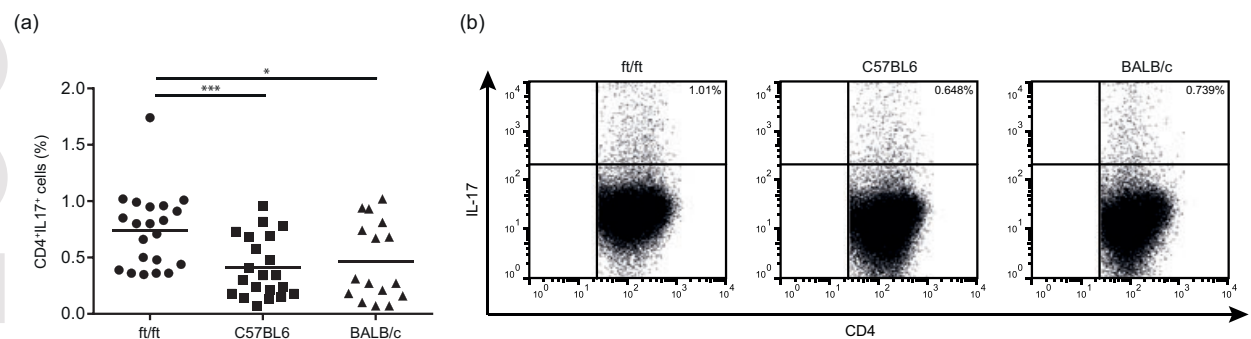


Figure 3
CM Bonefeld *et al.*

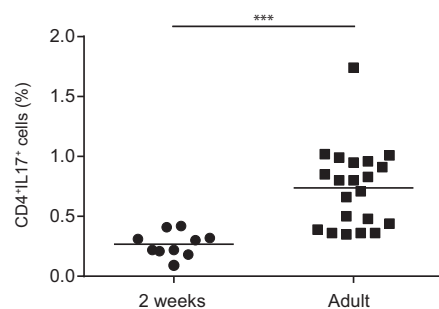


Figure 4
CM Bonefeld *et al.*

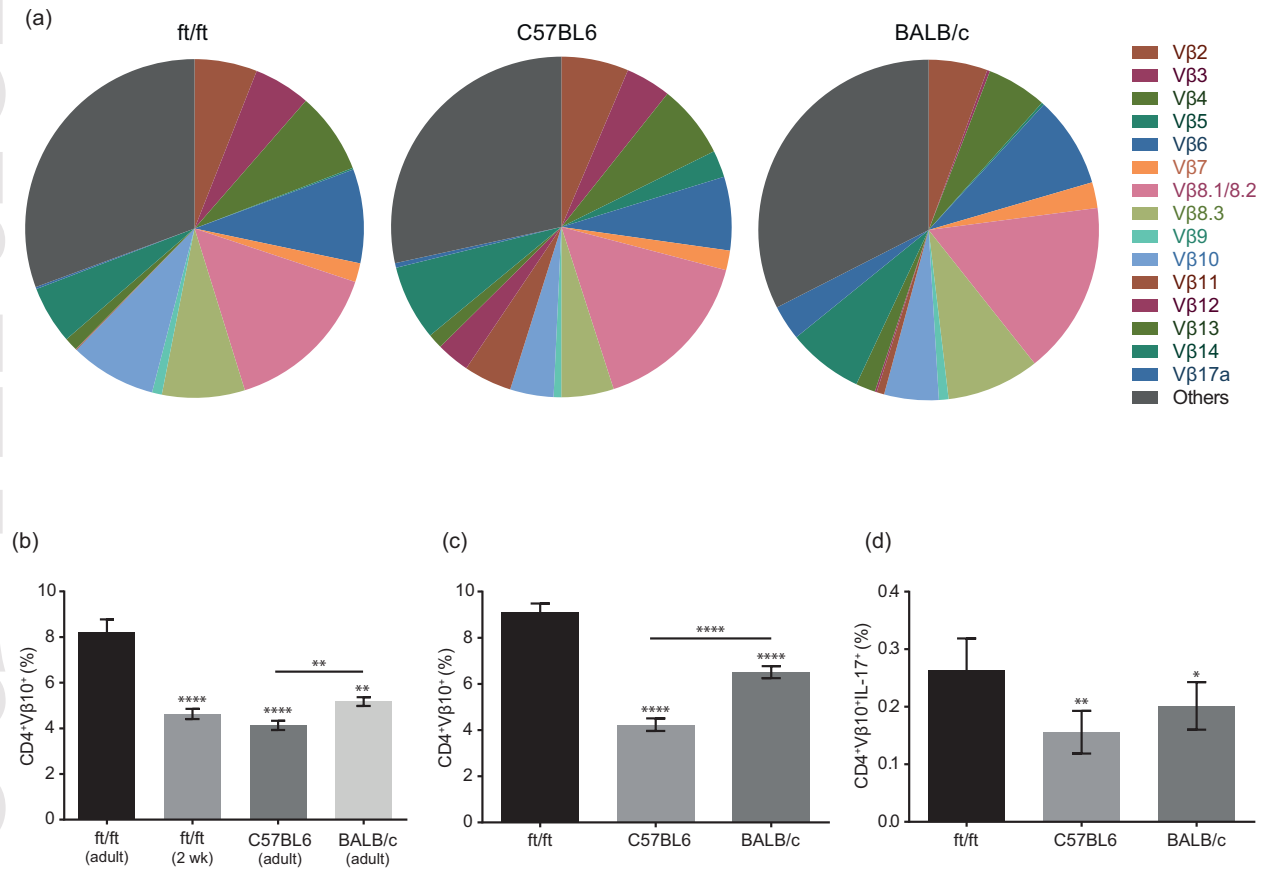


Figure 5
CM Bonefeld *et al.*

