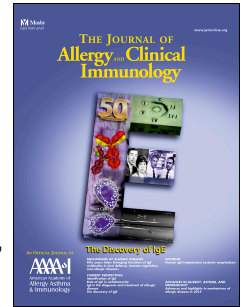


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Title: α_{s1} -casein Elucidate Major T Cell Responses in Cow's Milk Allergy

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10 **Short summary:** Cow's milk allergy (CMA) is increasing in prevalence, affecting
11 approximately 4% of children. Cow's milk (CM) is a common cause of fatal/ near fatal
12 anaphylactic reactions. Understanding of CM-specific CD4⁺ T-cells responses to milk allergens
13 should help elucidate the pathological mechanisms of persistent CMA. Milk allergen epitopes
14 specific T-cells were examined in CMA subjects. Frequencies and phenotypes of these T-cells
15 were found to be different between older and younger subjects.

To the Editor:

Cow's Milk Allergy (CMA) is the most common food allergy in children⁽¹⁾, approximately 42% of children can outgrow their CMA by 8 years of age⁽¹⁾. Whey (β -lactoglobulin the most abundant) and casein proteins are the major milk allergens⁽²⁾. The composite allergen casein consists of several isoforms: α _s1-casein, α _s2-casein, β -casein and κ -casein⁽³⁾. IgE sensitization is particularly frequent against α _s1-casein, inducing strong immediate or delayed allergic reactions⁽⁴⁾. Previous studies have shown that patients with persistent CMA showed IgE reactivity to epitopes from the casein group, specifically to α _s1-casein, as compared to patients who developed clinical tolerance⁽⁵⁾. Thus, we hypothesized that patients with persistent, IgE mediated CMA may have abnormal T-cell responses to casein. Additionally, little is known about specific T-cell responses toward these allergens in adults and children with CMA and non-allergic subjects.

A total of 26 allergic subjects, including 18 CM allergic subjects >8 years of age and 8 CM allergic subjects $\leq$ 8 years of age with persistent CMA (including subjects that are tolerant to baked milk products (n=8)) were recruited for this study (see Methods section in this article's Online Repository at www.jacionline.org). Characteristics of the recruited milk-allergic subjects are shown in **Table E1**. 18 HLA-matched subjects including 13 non-atopic control subjects, and 5 patients with peanut or walnut allergy without milk allergy were recruited as control subjects (**Table E1**). Tetramer-guided epitope mapping approach was utilized to identify Bos d-specific-CD4⁺ T-cell epitopes. A total of 23 epitopes were identified: 3 β -lactoglobulin-, 5 α _s1-casein-(including 4 previously identified⁽⁶⁾), 3 α _s2-casein-, 8 β -casein- and 4 κ -casein-T-cell epitopes (see **Fig E1** and **Table E2** for HLA-restriction information and epitope specificity between cohorts in this article's Online Repository at www.jacionline.org). Seven of the current identified epitopes have been previously reported in IEDB.

The anti-phycoerythrin (PE) magnetic bead tetramer enrichment approach was utilized to determine the frequencies of Bos d-specific T cells *ex vivo*. Bos d epitope-specific memory (CD45RA⁻) CD4⁺T-cells

were detected in high frequencies in allergic subjects, while lower frequencies were observed in non-allergic subjects (**Fig 1A and 1B**). A significant difference in frequencies of Bos d-specific cells between CM allergic subjects >8 years of age and children ≤8 years of age with CMA was observed (**Fig 1A and B**). Regarding each allergen individually, β-lactoglobulin, α_s1-casein, α_s2-casein, β-casein and κ-casein-epitope specific memory T cells responses were detected in 6/23(26%), 13/23(57%), 7/23(30%), 11/23(48%) and 4/23(17%) of CMA subjects, while responses for subjects ≤8 years of age were 0/7 (0%), 4/7(57%), 1/7(14%), 3/7(42%) and 0/7(0%) and for subjects >8 years were 6/16(38%), 9/16(56%), 6/16(38%), 8/16(50%) and 4/16(25%) respectively. Responses for subjects with baked milk tolerance were 3/7(43%), 4/7(57%), 3/7(43%), 2/7(29%) and 1/7(14%) and for subjects with baked milk intolerance were 3/16(19%), 9/16(56%), 4/16(25%), 9/16(56%) and 3/16(19%) respectively. These results show that α_s1-casein and β-casein responses were the most prevalent Bos d-specific T cell responses in CMA subjects and suggest a possibility of epitope spreading in older subjects as well as differential allergen recognition between the baked milk sensitized and the baked milk tolerance subjects. CD154 upregulation assay was also used to examine the overall T-cell responses towards these allergens in 8 of 26 milk allergic subjects. Strong α_s1-casein-specific responses were also observed, confirming our MHC class II tetramer staining (see **Fig E2** in this article's Online Repository at www.jacionline.org). In total, these results suggested that majority of milk-specific CD4⁺ T cell responses in CMA subjects were directed against α_s1-casein and β-casein. The current data is also in agreement with a previous study that showed frequency of casein-specific T cells was higher in CMA compared to non-CMA children⁽⁷⁾.

Chemokine receptor and differentiation marker expression of Bos d-specific T-cells was analyzed by *ex vivo* tetramer staining of PBMC (**Fig 1C, 1D and 1E**). Detectable Bos d-specific CD4⁺T-cells in allergic subjects were CCR4⁺, suggesting a T_H2 phenotype. In addition, CRTH2⁺ and CD27⁻ T cells were observed (**Fig 1C and 1D**), which indicates a terminally differentiated T_H2 phenotype⁽⁸⁾. CCR4⁺ T cells that co-expressed CCR6 and Integrin β7 were also observed in subjects with CMA and were more common in children ≤8 years of age than older subjects with persistent CMA, suggesting that a sub-

population of these cells account for a T_H17 -like response (**Fig E3A & B**). $CCR6^+CRTH2^+$ T-cells were detected but to a lesser degree (**Fig E3C and D**). However, we did not observe differences between allergic adults and children for these populations. On the other hand, higher CCR7 and CXCR3 expression (**Fig 1C and 1E**) were observed in subjects >8 years of age. The presence of more CXCR3⁺ and CCR7⁺ milk specific cells imply T_H1 and T_{CM} (central memory T cells as defined by expression of CD27 and CCR7) milk specific cells are more common in CM allergic subjects >8 years of age than in children ≤8 years of age with CMA. No difference in frequencies and phenotypes amongst subjects with baked milk tolerance or intolerance was observed (Data not shown).

The CD154 upregulation assay was used to analyze the cytokine profiles of Bos d specific T-cells (**Fig 2A**). In subjects with CMA, a dominant T_H2 response was observed, which accounted for 58% of the overall response (**Fig 2B**). Amongst the T_H2 cytokine producers, cells can be classified into IL-4 producers (3%), IL-4/IL-13 (44%) producers, and IL-4/IL-5/IL-13 (11%) producers. We did not detect cells that produced IL-5 or IL-13 alone. Interestingly, a subdominant T_H2/T_H17 response was also observed, which accounted for 27% of the overall T-cell response (**Fig 2B**). Amongst IL-17A producers, cells can be classified into IL-17A producers (4%), IL-4/IL-17A producers (2%) and IL-4/IL-13/IL-17A producers (21%). Interestingly, higher proportions of IL-4/IL-5/IL-13 producers (30%) and IL-4 producers (33%) were detected in CM allergic subjects >8 years of age (**Fig 2C**), in contrast higher proportions of IL-4/IL-13/IL-17A producers (61%) were detected in children ≤8 years of age (**Fig 2C**). There was no difference in the cytokines profiles of baked milk tolerant and intolerant subjects (Data not shown). Conversely, T-cells from non-allergic subjects produced IFN- γ , IL-10, or both, with low IL-17A (**Fig 2A and 2B**). These results confirmed the observed T_H2 and T_H2/T_H17 phenotypes in our *ex vivo* experiments and our previous studies that these phenotypes could be a potential trademark in food allergy⁽⁸⁾.

The current study implicates an important role of Bos d-specific T-cell responses in the persistence of CMA. In older children and adults with CMA, a committed T_H2 response was observed (**Fig. 2B**,

IL4/IL5/IL13 triple cytokine producers). On the other hand, in younger children with CMA, T_H2/T_H17 responses were more prevalent, suggesting that these T-cell populations are not fully committed into the T_H2 phenotype and could explain loss of CMA in younger children. Moreover, T_{CM} are less susceptible to deletion by allergen specific immunotherapy in a murine model⁽⁹⁾. Accumulation of $CCR7^+CD27^+$ Bos d-epitope-specific T cells (T_{CM}) in adults might be indicative of CMA persistence and also complicate possible oral immunotherapy for CMA. Knowledge of CM-epitope-specific T-cell responses will be useful in devising novel strategies to halt and reverse the progression of CMA.

Word Count: 1156 words.

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Key Words: Key words: Food allergy, Cow's milk allergy, Bos d, α S1-casein, T-cells, MHC class II tetramers, epitopes.

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Figure 1. Frequency and phenotype of Bos d-epitope-specific CD4⁺ T-cells. **A**, Frequencies of Bos d-epitope-specific CD45RA⁺CD4⁺T-cells in subjects with and without CMA. **B**, Comparison of Bos d-specific CD4⁺T-cell frequency for each epitope described (data point) between milk allergic (n=23; filled squares) and non-allergic subjects (n=13; opened circles); and adults, teenagers and children >8 years old (n=16; triangles) and children ≤8 years old (n=7; downside triangles) with CMA. **C**, PBMC from a non-allergic and allergic subjects were stained with PE-labeled DRB1*10:01/Bos d 9₁₄₅₋₁₆₄ tetramers and a panel of antibodies. The percentages of memory Bos d-specific T cells that expressed the surface marker of interest are as indicated. **D**, Phenotype of Bos d-specific T-cells in allergic and non-allergic subjects and in **E**. CMA >8 years old and CMA children ≤8 years old. Symbols in **D** and **E** are identical to those used in **B**. A Student *t* test was used in the statistical analysis. **P*<0.05, ***P*<0.01, ****P*<0.001, NS. Not significant.

Figure 2. Cytokine profiles of Bos d-reactive T-cells. **A**, *First row*, Cytokine profile in a DRB1*01:01 non-allergic subject. *Second row*, Cytokine profile in a DRB1*01:01 allergic adult. *Third row*, Cytokine profile in a DRB1*01:01 allergic child. The percentages of memory CD154⁺ Bos d-reactive cytokine producing T-cells are as indicated. **B**, and **C**, Cytokine profiles of Bos d-reactive T-cells in non-allergic (n=13) and allergic subjects (n=21), and between adults, teenagers and children >8 (n=14) and children ≤8 years of age (n=7) with CMA. Data are presented as the mean frequency of cytokine producing T-cells from each group in pie charts.

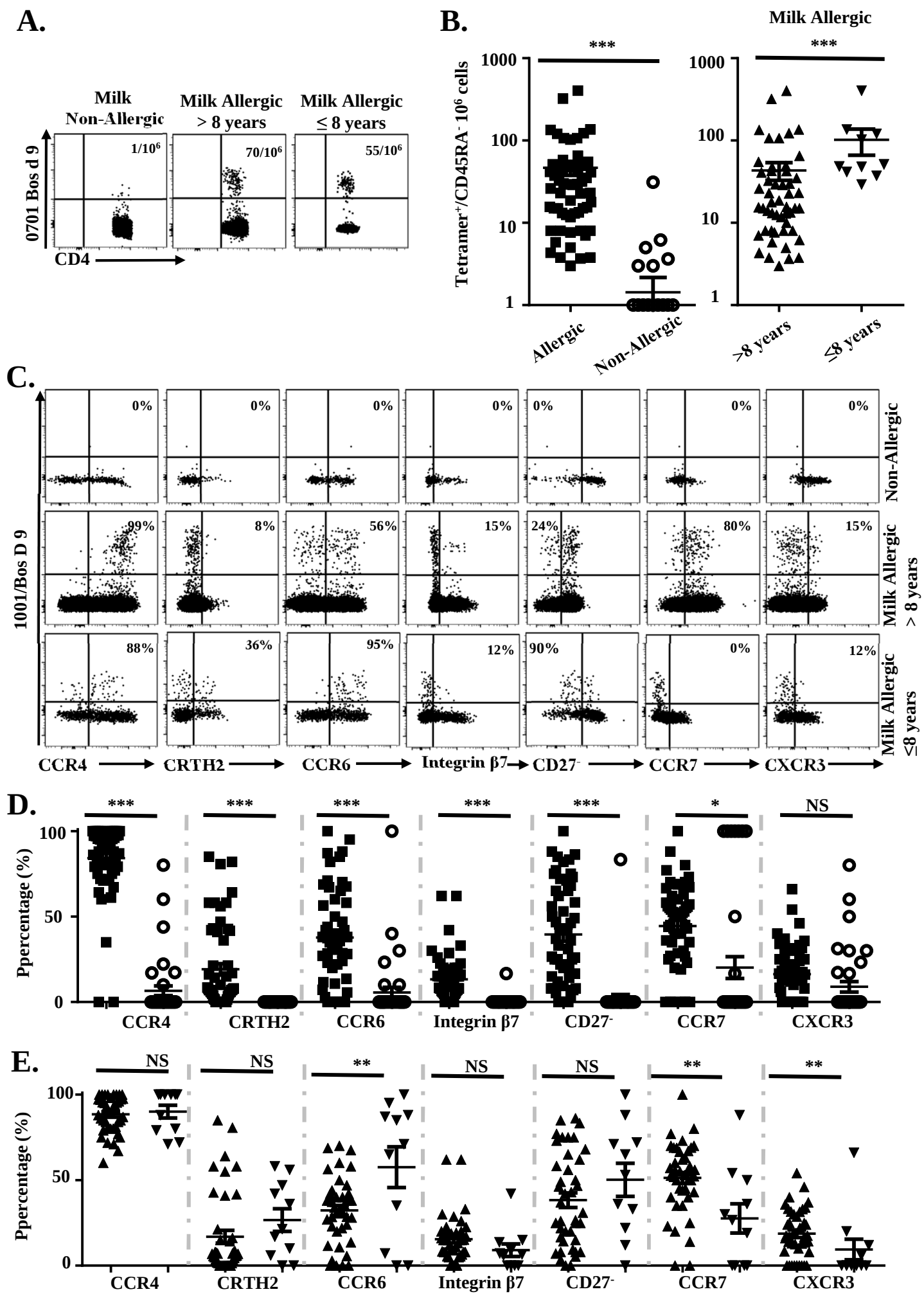


Fig. 1

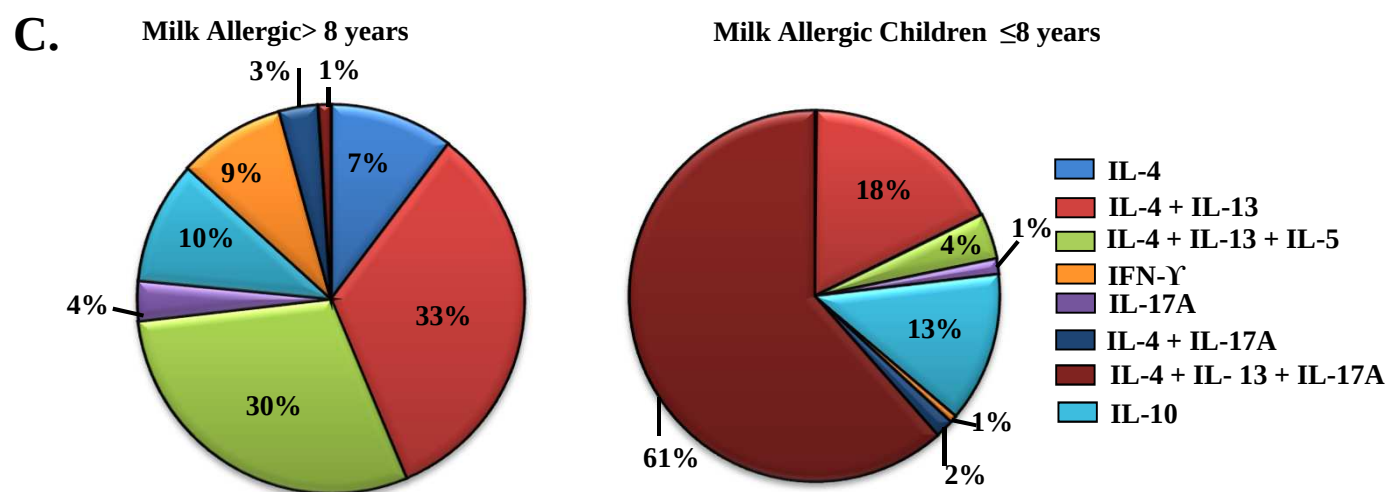
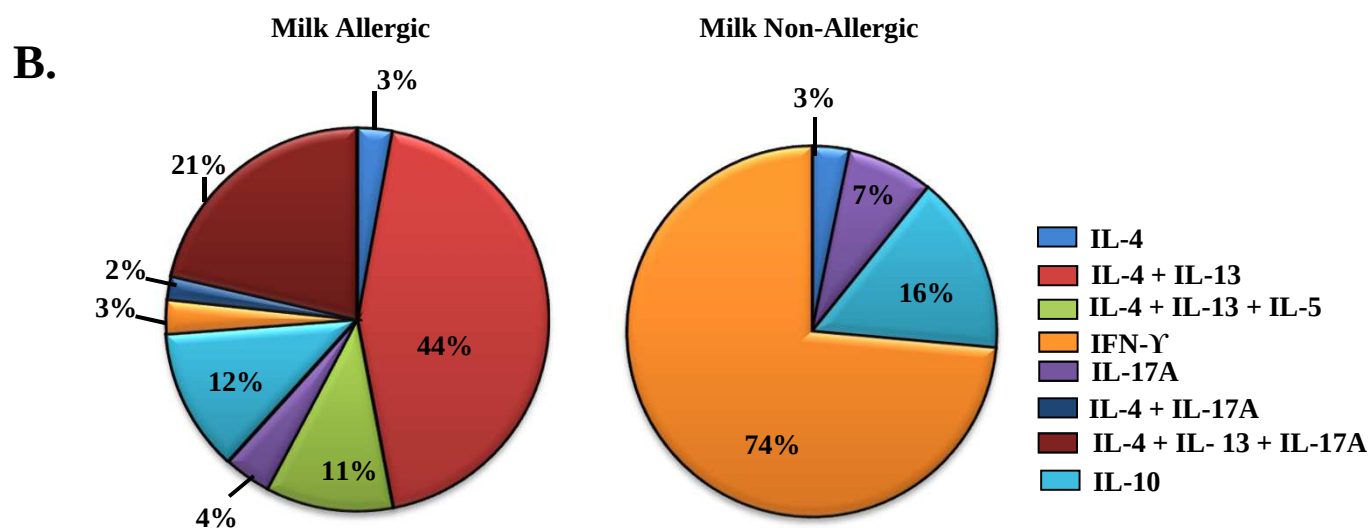
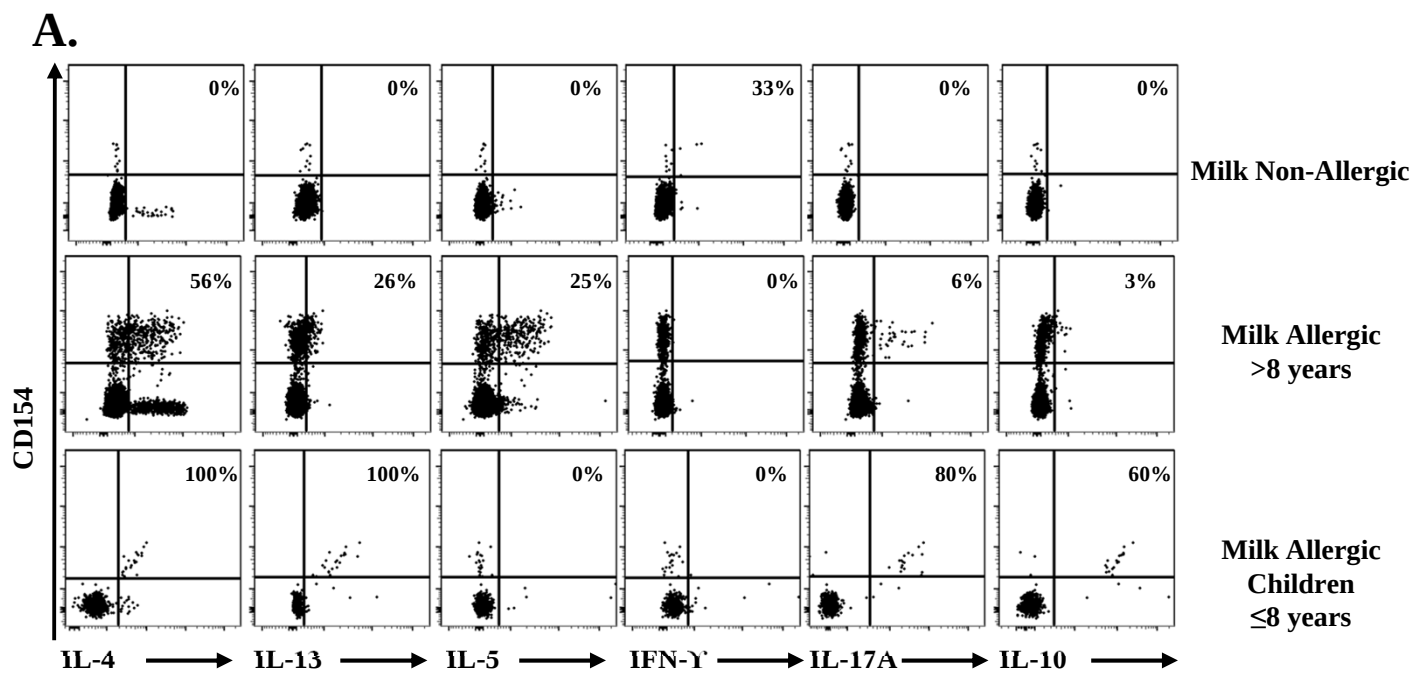


Fig. 2

METHODS

Human Subjects

Subjects were recruited from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute with informed consent and institutional review board approval (IRB title “Allergen and T-cell reagent resources for the study of allergic diseases”; approval number IRB7109). A total of 26 subjects with a documented record of milk allergy and a positive ImmunoCAP score for milk extract (>0.35 kU/L) (Phadia AB, Uppsala, Sweden), were recruited for this study. All patients had onset of cow milk allergy in infancy, typically with first known exposure to cow milk with symptoms consisting typically of urticaria and gastrointestinal discomfort and vomiting. Diagnosis of cow milk allergy was made by specific IgE testing to cow milk, either skin prick testing or serum IgE testing and history of presentation of clinical symptoms. Most recent serum cow milk specific IgE levels are shown in **Table E1**. 18 patients (12 > 8 -year-old and 6 ≤ 8 -year-old) do not tolerate any form of milk and 8 patients (6 > 8 -year-old and 2 ≤ 8 -year-old) tolerate baked form of milk protein. Patients with baked milk intolerance had either accidental ingestion-induced symptoms or failed challenge to baked milk. The 8 patients tolerating baked milk had accidental ingestion of fresh milk-induced symptoms, but passed a baked milk challenge.

13 non-atopic and 5 atopic subjects with no clinical symptoms to milk, a negative ImmunoCAP score and HLA(Human histocompatibility leukocyte antigen)-matched were also recruited as controls for this study. The features of these subjects are shown in **Table E1**. DNA samples were HLA-typed using Dynal UnitraxTM SSP Kits (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

23

24 **Tetramer guided epitope mapping (TGEM)**

25 Peptide libraries were generated based on β -lactoglobulin, α _S1-casein, α _S2-casein, β -casein and
26 κ -casein sequences. The libraries consisted of overlapping peptides spanning the entire allergen,
27 which were 20 amino acids in length with a 12 amino acid overlap synthesized by Mimotopes
28 (Clayton, Australia). Peptide-loaded HLA-DR and HLA-DQ proteins were generated, as
29 previously described^(E1;E2). The tetramer-guided epitope-mapping procedure was conducted as
30 previously described^(E3). Briefly, PBMC from CMA subjects were stimulated for 2 weeks with
31 pool peptides which consisted of 5 overlapping peptides spanning the entire allergen. After 2
32 weeks, PBMC were screened with pMHC-II tetramers loaded with the pool of peptides that
33 corresponded to the stimulated well, those with a positive signal were re-screened again but this
34 time with pMHC-II tetramers loaded with single peptides.

35

36 ***Ex-vivo* analysis of milk-specific CD4⁺ T-cells**

37 CD154⁺ detection assay was carried out as previously described^(E4). Briefly, for detection of
38 CD154⁺-reactive T-cells, 15-35 million of freshly isolated PBMC (at 7×10^6 cells/mL) in
39 culture medium (RPMI 1640 (Gibco) + 10% pooled human serum + 1% PenStrep) were
40 stimulated with 5 μ g/mL of synthesized peptide pools (at a final concentration of 47.5 μ M for
41 Bos d 5, 60 μ M for Bos d 9, 62.5 μ M for Bos d 10, 62.5 μ M for Bos d 11 and 50 μ M for Bos d
42 12), and 1 μ g/ml anti-CD40 (Miltenyi Biotec, Auburn, CA) for 3 hours (for frequency) and 6
43 hours (for ICS) at 37°C. Cells were also mock stimulated with DMSO (0.05% final concentration)
44 as negative control. After stimulation, cells were stained with PE (phycoerythrin)-conjugated

CD154 (Miltenyi Biotec, Auburn, CA) and labeled with anti-PE magnetic beads (Miltenyi Biotec, Auburn, CA) for 20 minutes at 4°C. A 1/100 fraction of cells was saved for analysis. The other fraction was passed through a Miltenyi magnetic column; magnetically enriched cells were next stained with a panel of antibodies of interest for 20 minutes at room temperature. After staining, cells were stained again with Via-probe⁺ (BD Biosciences, East Rutherford, NJ) for 10 minutes at 4°C before flow-cytometry. Data acquisition was performed using a FACSCanto flow cytometer and data were analyzed utilizing FlowJo (Tree Star, Ashland, Ore). Frequency was calculated as previously described for tetramer analysis ^(E5). *Ex vivo* analysis with pMHC-II (Peptide/MHC class II) tetramers was carried out as previously described ^(E5).

Intracellular cytokine staining

For *ex-vivo* intracellular cytokine staining (ICS) combined with CD154 activation assay, BD GolgiStopTM was added during stimulation (BD biosciences, East Rutherford, NJ) according to the manufacturer's instructions. After 10 minutes at room temperature, cells were then fixed with fixation buffer (eBioscience, San Diego, CA) and washed twice with a permeabilization buffer (eBioscience, San Diego, CA). Cells were then stained with a panel of antibodies (eBioscience, San Diego, CA and BD biosciences, East Rutherford, NJ) directed against cytokines of interest for 20 minutes at room temperature; cells were washed and immediately analyzed in FACSCanto flow cytometer.

Statistical analysis

Statistical analysis was performed using the tests indicated in the figure legends utilizing Prism 5.0 software (GraphPad Software, La Jolla, California).

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Table E2. Bos d 5, Bos d 9, Bos d 10, Bos d 11 and Bos d 12 CD4⁺ T-cell epitopes

HLA DRB1 Restriction													
Bos d	Amino acid sequence	01:01	03:01	04:01	07:01	09:01	10:01	11:01	15:01	DRB4	DRB5	DQ6	
*Bos d 5 ₁₇₋₃₆	GTWYSLAMAASDISLLDAQS					1/1 [†] , 0/1 [‡]							
*Bos d 5 ₁₂₉₋₁₄₈	DDEALEKFDKALKALPMHIR									0/4 [†] , 1/11 [‡]	1/3 [†] , 3/3 [‡]		
Bos d 5 ₁₃₇₋₁₅₆	DKALKALPMHIRLSFNPTQL			0/1 [†] , 2/5 [‡]									
±*Bos d 9 ₁₇₋₃₆	NENLLRFFVAPFPEVFGKEK					1/1 [†] , 0/1 [‡]							
±*Bos d 9 ₁₀₅₋₁₂₄	KVPQLEIVPNSAEERLHSMK		1/2 [†] , 2/3 [‡]										
±Bos d 9 ₁₃₇₋₁₅₆	GVNQELAYFYPELFRQFYQL					0/1 [†] , 1/1 [‡]							
*Bos d 9 ₁₄₅₋₁₆₄	FYPELFRQFYQLDAYPSGAW	2/2 [†] , 2/2 [‡]					1/2 [†] , 3/4 [‡]	0/0 [†] , 1/1 [‡]					
±*Bos d 9 ₁₆₉₋₁₈₈	SGAWYYVPLGTQYTDAPSFS	1/2 [†] , 2/2 [‡]					0/2 [†] , 2/4 [‡]	0/0 [†] , 1/1 [‡]		0/4 [†] , 1/11 [‡]			
Bos d 10 ₁₇₋₃₆	QETYKQEKMAINPSKENLC					1/1 [†] , 0/1 [‡]							
Bos d 10 ₁₇₇₋₁₉₆	PQYLKTVYQHQAAMKPWIQP						1/2 [†] , 3/4 [‡]						
Bos d 10 ₁₈₅₋₂₀₄	QHQAAMKPWIQPKTKVIPYV											1/3 [†] , 1/3 [‡]	
Bos d 11 ₂₅₋₄₄	RINKKIEKFQSEEQQQTEDE						0/2 [†] , 2/4 [‡]						
Bos d 11 ₄₁₋₆₀	TEDELQDKIHFAQTQSLVY				0/2 [†] , 1/7 [‡]	1/1 [†] , 0/1 [‡]							
Bos d 11 ₄₉₋₆₈	IHPFAQTQSLVYPFPGPIPN			0/1 [†] , 2/5 [‡]									
Bos d 11 ₆₅₋₈₄	PIPNSLPQNIPPLTQTPVVV			0/1 [†] , 2/5 [‡]									
Bos d 11 ₈₉₋₁₀₈	QPEVMGVSKVKEAMAPKHKE			1/1 [†] , 0/5 [‡]									
Bos d 11 ₁₁₃₋₁₃₂	KYPVEPFTESQSLTLTDVEN				0/2 [†] , 7/7 [‡]								
Bosd 11 ₁₅₃₋₁₇₂	PTVMFPPQSVLSLSQSKVLP				0/2 [†] ,								

			2/7 [‡]	
Bos d 11 ₁₉₃₋₂₁₂	FLLYQEPVLGPVRGPFPIIV		1/2 [†] , 2/7 [‡]	
Bos d 12 ₁₋₂₀	AQEQNQEQPIRCEKDERFFS	0/1 [†] , 1/5 [‡]		1/3 [†] , 0/3 [‡]
Bos d 12 ₅₇₋₇₆	LPYPYYAKPAAVRSPAQILQ			1/3 [†] , 0/3 [‡]
Bos d 12 ₁₀₅₋₁₂₄	SFMAIPPKKNQDKTEIPTIN		2/2 [†] , 0/4 [‡]	
Bos d 12 ₁₅₃₋₁₇₂	PEVIESPPEINTVQVTSTAV	0/1 [†] , 1/5 [‡]		

[‡]Previously identified CD4⁺ T-cell epitopes by Elsayed et al (Molecular Immunology, 2004).

*Previously identified as HLA-II-restricted T-cell epitopes in www.iedb.org. Epitope Bos d 5 ₁₇₋₃₆ (33950, 223605); Bos d 5 ₁₂₉₋₁₄₈ (222188); Bos d 9 ₁₇₋₃₆ (31145, 38207); Bos d 9 ₁₀₅₋₁₂₄ (74689, 51432, 34491, 70444); Bos d 9 ₁₃₇₋₁₅₆ (13715, 13716, 26364, 26341); Bos d 9 ₁₄₅₋₁₆₄ (26341, 45542, 18443); Bos d 9 ₁₆₉₋₁₈₈ (10, 5711, 7690).

[†] # of responders and # of subjects tested with the designated HLA-DR or HLA-DQ with baked milk tolerance.

[‡] # of responders and # of subjects tested with the designated HLA-DR or HLA-DQ with baked milk intolerance.

Table E1. HLA and allergic status of recruited subjects

ID	Age	Sex	HLA (DRB1*)	sIgE milk (f256) kU/L	Symptoms to Milk ingestion	Tolerates baked milk
Milk Allergic Adults						
1	39	F	04:01 , 07:01	10.3	I, II, III, IV, V	No
2	34	M	07:01 , 13:01	0.5	V , VIII	No
3	22	F	04:01 , 10:01	41.2	VIII	No
4	18	M	10:01 , 12:01	4.63	VIII	No
5	30	F	01:01 , 09:01	0.53	VIII	Yes
6	20	M	03:01 , 13:01	3.29	III, IV, V	No
7	20	M	01:01 , 03:01	82.9	I, II	Yes
8	19	M	07:01 , 13:01	41.1	IV, V	No
9	18	M	04:01 , 12:01	26.1	VIII	No
Milk Allergic Teenagers and Children > 8 years of age						
10	15	M	04:01 , 15:01	4.46	I, II, IV	No
11	10	M	13:01 , 15:01	35.1	IV	Yes
12	10	M	03:01 , 12:01	64.7	III, IV, V	No
13	13	F	03:01 , 03:01	64.5	I,II	No
14	9	M	03:01 , 07:01	22.7	II, IV, V	No
15	11	M	07:01 , 15:01	12.1	IV, V	Yes
16	12	F	01:01 , 04:05	100	II, IV, V	No
17	15	F	10:01 , 15:01	1.27	II, IV	Yes
18	13	M	12:01 , 15:01	9.28	II, III, IV, VIII	Yes
Milk Allergic Children under the age of ≤ 8						
19	4	M	10:01 , 15:01	77.2	I, II, IV, V	No
20	3	M	04:01 , 10:01	0.8	III, IV, VII	Yes
21	5	F	07:01 , 11:01	51.7	II, IV , V	No
22	3	M	03:01 , 07:01	22.7	I, II, IV	Yes
23	3	M	03:01 , 07:01	7.3	II, IV	No
24	5	M	09:01 , 10:01	52.2	III, IV	No
25	5	M	01:01 , 15:01	2.86	IV	No
26	8	M	04:01 , 07:01	15.3	II, III, IV, V	No
Non-atopic subjects						
27	29	M	03:01 , 15:01	0	Absent	Yes
28	31	F	01:01 , 15:01	0	Absent	Yes
29	34	F	01:01 , 01:03	0	Absent	Yes
30	34	F	03:01 , 11:01	0	Absent	Yes
31	35	F	04:01 , 14:01	0	Absent	Yes
32	32	F	07:01 , 07:01	0	Absent	Yes
33	38	M	04:01 , 04:01	0	Absent	Yes
34	37	F	03:01 , 07:01	0	Absent	Yes
35	32	M	01:01 , 04:02	0	Absent	Yes
36	35	M	07:01 , 13:02	0	Absent	Yes

37	24	M	07:01 , 13:02	0	Absent	Yes
38	3	M	01:01,	0	Absent	Yes
39	23	F	15:01 , 10:01	0	Absent	Yes
Atopic subjects without milk allergy						
40*	8	M	10:01 , 15:01	0	Absent	Yes
41*	12	M	01:01 , 13:02	0	Absent	Yes
42*	10	F	07:01 , 11:01	0	Absent	Yes
43*	12	M	11:01 , 15:01	0	Absent	Yes
44*	10	F	01:01 , 15:01	0	Absent	Yes

I Itchy mouth, lips and / or pharynx

II Abdominal discomfort and / or diarrhea

III Nausea or vomiting

IV Severe skin itching or hives, acute or angioedema

V Rhinitis and / or conjunctivitis and / or respiratory compromise

VI Dizziness (feeling loss of consciousness)

VII Syncope (loss of consciousness)

VIII Desaturation with respiratory compromise

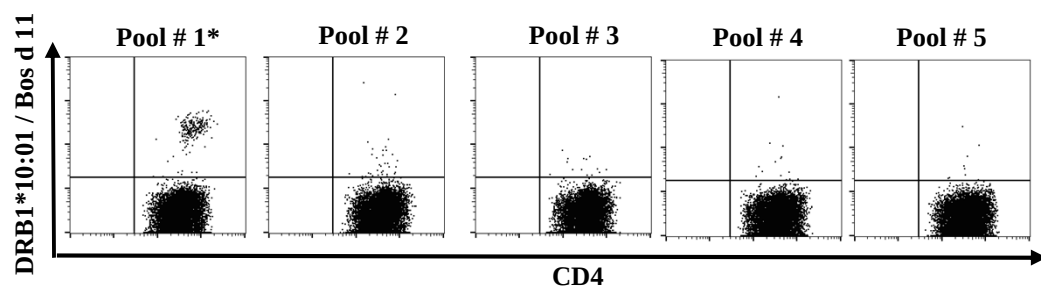
* Subjects also had history of peanut or walnut and positive IgE ImmunoCAP for peanut or walnut

Figure E1. Tetramer Guided Epitope Mapping (TGEM) studies of DRB1*10:01-restricted Bos d-specific CD4⁺ T-cells. **A**, PBMC from a DRB1*10:01 subject with milk allergy were stimulated with 5 pools of Bos d 11 peptides for 2 weeks and subsequently stained with corresponding DRB1*10:01/Bos d 11 pooled peptide tetramers. **B**, Cells that were stimulated with pool #1 were re-stained with individual peptides from the corresponding pool. The staining identified p4 (Bos d 11₂₅₋₄₄) as DRB1*10:01 restricted Bos d 11 T-cell epitopes.

Figure E2. Frequencies of milk allergen reactive CD4⁺ T-cells. **A**, and **B**, Frequencies of β -lactoglobulin-, α _s1-casein-, α _s2-casein-, β -casein- and κ -casein-reactive T-cells in 8 subjects with milk allergy (adults, teenagers and children >8 years of age n=6; children $\leq$ 8 years of age n=2) with CD154 assays after 3 hour Bos d peptide stimulation utilizing freshly isolated PBMC. Each data point represents the frequency of T-cells reactive to each allergen. An ANOVA test (with Bonferroni correction) was used to compare all columns in the statistical analysis. *** P <0.001, NS. Not significant.

Figure E3. Phenotypes of CCR6⁺Bos d-specific T-cells in milk allergic subjects after *ex vivo* pMHC-II tetramer enrichment utilizing freshly isolated PBMC. **A**, and **B**, Tetramer⁺CD45RA⁻ T-cells were gated against CCR4 and CCR6. Each data point represents results for surface expression in tetramer positive T-cells from 23 subjects with milk allergy; 15 adults, teenagers and children >8 years of age (triangles) and 8 children $\leq$ 8 years of age (downside triangles) with CMA. **C**, and **D**, Tetramer positive CD45RA⁻ T-cells were gated against CCR6 and CRTH2. Each data point represents results for surface expression in tetramer positive T-cells from 26 subjects with milk allergy; 18 adults, teenagers and children >8 years of age (triangles) and 8 children $\leq$ 8 years of age (downside triangles) with CMA. A Student *t* test was used in the statistical analysis. **** P <0.0001, * P <0.05, NS. Not significant.

A.



B.

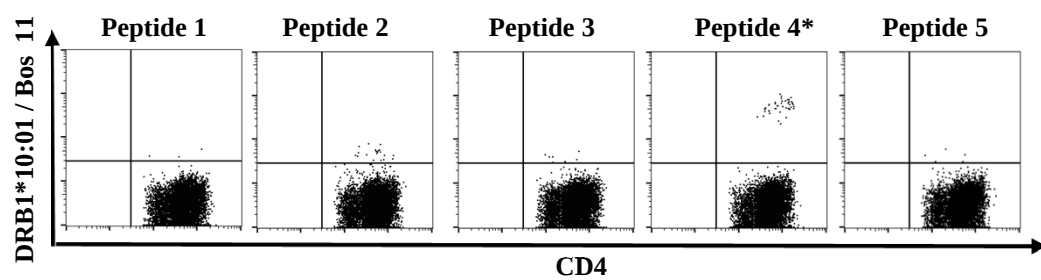
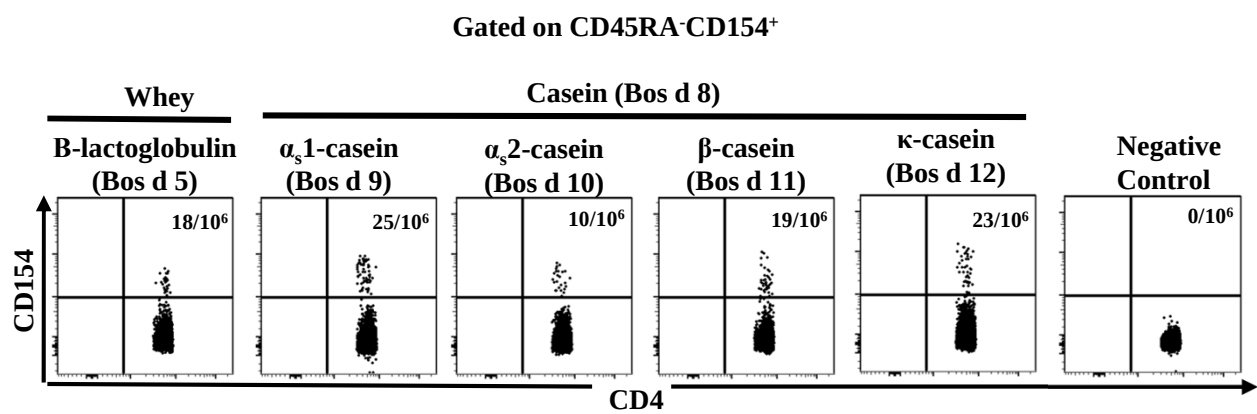


Fig. E1

A.



B.

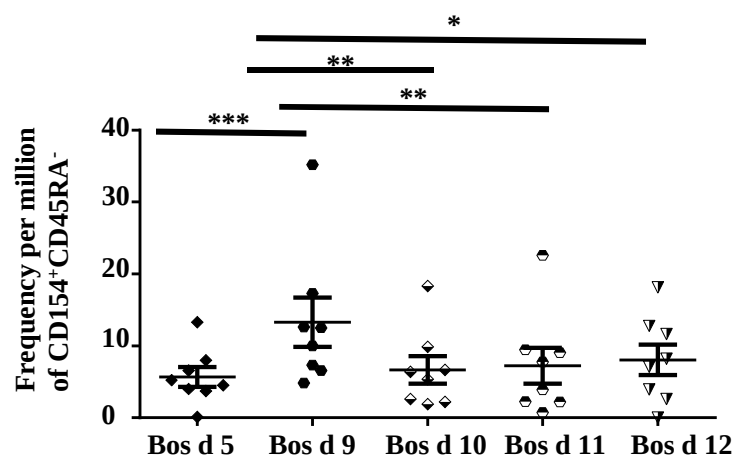


Fig. E2

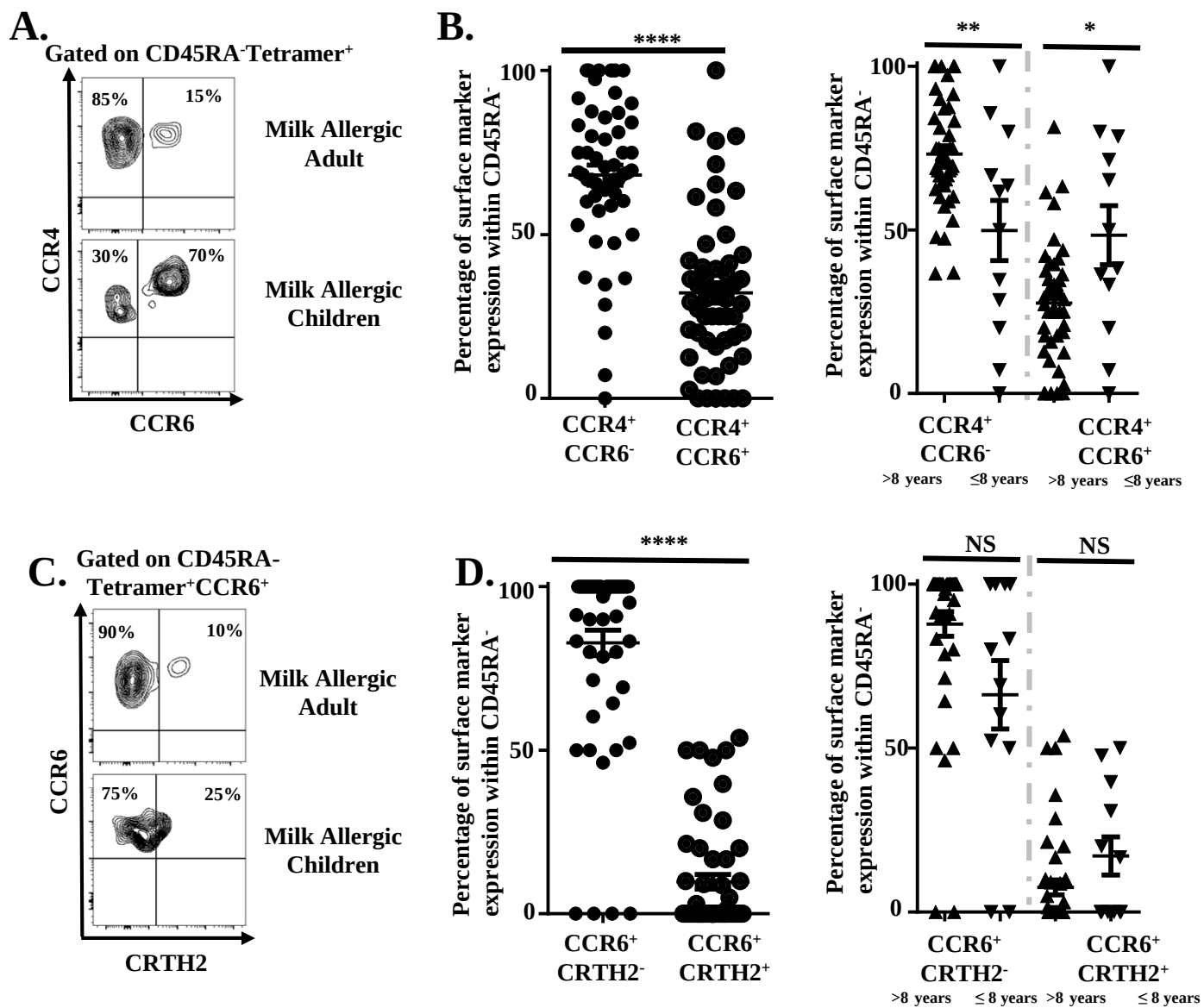


Fig. E3