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 $\leq$ 8-year-old) do not tolerate any form of milk and 8 patients (6> 8-year-old and 2 $\leq$ 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 Unitray^{TM} SSP Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Tetramer guided epitope mapping (TGEM)

Peptide libraries were generated based on β-lactoglobulin, α_S1-casein, α_S2-casein, β-casein and κ-casein sequences. The libraries consisted of overlapping peptides spanning the entire allergen, which were 20 amino acids in length with a 12 amino acid overlap synthetized by Mimotopes (Clayton, Australia). Peptide-loaded HLA-DR and HLA-DQ proteins were generated, as previously described[E1;E2]. The tetramer-guided epitope-mapping procedure was conducted as previously described [E3]. Briefly, PBMC from CMA subjects were stimulated for 2 weeks with pool peptides which consisted of 5 overlapping peptides spanning the entire allergen. After 2 weeks, PBMC were screened with pMHC-II tetramers loaded with the pool of peptides that corresponded to the stimulated well, those with a positive signal were re-screened again but this time with pMHC-II tetramers loaded with single peptides.

Ex-vivo analysis of milk-specific $CD4^+$ T-cells

$CD154^+$ detection assay was carried out as previously described [E4]. Briefly, for detection of $CD154^+$-reactive T-cells, 15-35 million of freshly isolated PBMC (at 7 x 10^6 cells/mL) in culture medium (RPMI 1640 (Gibco) + 10% pooled human serum + 1% PenStrep) were stimulated with 5µg/mL of synthesized peptide pools (at a final concentration of 47.5 µM for Bos d 5, 60 µM for Bos d 9, 62.5 µM for Bos d 10, 62.5 µM for Bos d 11 and 50 µM for Bos d 12), and 1 µg/ml anti-CD40 (Miltenyi Biotec, Auburn, CA) for 3 hours (for frequency) and 6 hours (for ICS) at 37°C. Cells were also mock stimulated with DMSO (0.05% final concentration) 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 GolgiStop^{TM} 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).

REFERENCES

(E1) Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. J Clin Invest 1999; 104(12):R63-R67.

(E2) Kwok WW, Liu AW, Novak EJ, Gebe JA, Ettinger RA, Nepom GT et al. HLA-DQ tetramers identify epitope-specific T cells in peripheral blood of herpes simplex virus type 2-infected individuals: direct detection of immunodominant antigen-responsive cells. J Immunol 2000; 164(8):4244-9.

(E3) Novak EJ, Liu AW, Gebe JA, Falk BA, Nepom GT, Koelle DM et al. Tetramer-guided epitope mapping: rapid identification and characterization of immunodominant CD4+ T cell epitopes from complex antigens. J Immunol 2001; 166(11):6665-70.

(E4) Archila LD, Jeong D, Pascal M, Bartra J, Juan M, Robinson D et al. Jug r 2-reactive CD4 T cells have a dominant immune role in walnut allergy. J Allergy Clin Immunol 2015.

(E5) Kwok WW, Roti M, Delong JH, Tan V, Wambre E, James EA et al. Direct ex vivo analysis of allergen-specific CD4+ T cells. J Allergy Clin Immunol 2010; 125(6):1407-9.

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** $_{17\text{-}36}$	GTWYSLAMAASDISLLDAQS					1/1†, 0/1‡						
***Bos d 5** $_{129\text{-}148}$	DDEALEKFDKALKALPMHIR									0/4†, 1/11‡	1/3†, 3/3‡	
Bos d 5 $_{137\text{-}156}$	DKALKALPMHIRLSFNPTQL			0/1†, 2/5‡								
$^{\pm *}$**Bos d 9** $_{17\text{-}36}$	NENLLRFFVAPFPEVFGKEK					1/1†, 0/1‡						
$^{\pm *}$**Bos d 9** $_{105\text{-}124}$	KVPQLEIVPNSAEERLHSMK		1/2†, 2/3‡									
$^{\pm}$**Bos d 9** $_{137\text{-}156}$	GVNQELAYFYPELFRQFYQL					0/1†, 1/1‡						
***Bos d 9** $_{145\text{-}164}$	FYPELFRQFYQLDAYPSGAW	2/2†, 2/2‡					1/2†, 3/4‡	0/0†, 1/1‡				
$^{\pm *}$**Bos d 9** $_{169\text{-}188}$	SGAWYYVPLGTQYTDAPSFS	1/2†, 2/2‡					0/2†, 2/4‡	0/0†, 1/1‡		0/4†, 1/11‡		
Bos d 10 $_{17\text{-}36}$	QETYKQEKNMAINPSKENLC					1/1†, 0/1‡						
Bos d 10 $_{177\text{-}196}$	PQYLKTVYQHQKAMKPWIQP						1/2†, 3/4‡					
Bos d 10 $_{185\text{-}204}$	QHQKAMKPWIQPKTKVIPYV											1/3†, 1/3‡
Bos d 11 $_{25\text{-}44}$	RINKKIEKFQSEEQQQTEDE						0/2†, 2/4‡					
Bos d 11 $_{41\text{-}60}$	TEDELQDKIHPFAQTQSLVY				0/2†, 1/7‡	1/1†, 0/1‡						
Bos d 11 $_{49\text{-}68}$	IHPFAQTQSLVYPFPGPIPN			0/1†, 2/5‡								
Bos d 11 $_{65\text{-}84}$	PIPNSLPQNIPPLTQTPVVV			0/1†, 2/5‡								
Bos d 11 $_{89\text{-}108}$	QPEVMGVSKVKEAMAPKHKE			1/1†, 0/5‡								
Bos d 11 $_{113\text{-}132}$	KYPVEPFTESQSLTLTDVEN				0/2†, 7/7‡							
Bosd 11 $_{153\text{-}172}$	PTVMFPPQSVLSLSQSKVLP				0/2†,							

			2/7‡			
Bos d 11 $_{193\text{-}212}$	FLLYQEPVLGPVRGPFPIIV		1/2†, 2/7‡			
Bos d 12 $_{1\text{-}20}$	AQEQNQEQPIRCEKDERFFS	0/1†, 1/5‡		1/3†, 0/3‡		
Bos d 12 $_{57\text{-}76}$	LPYPYYAKPAAVRSPAQILQ					1/3†, 0/3‡
Bos d 12 $_{105\text{-}124}$	SFMAIPPKKNQDKTEIPTIN			2/2†, 0/4‡		
Bos d 12 $_{153\text{-}172}$	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 $_{17\text{-}36}$ (33950, 223605); Bos d 5 $_{129\text{-}148}$ (222188); Bos d 9 $_{17\text{-}36}$ (31145, 38207); Bos d 9 $_{105\text{-}124}$ (74689, 51432, 34491, 70444); Bos d 9 $_{137\text{-}156}$ (13715, 13716, 26364, 26341); Bos d 9 $_{145\text{-}164}$ (26341, 45542, 18443); Bos d 9 $_{169\text{-}188}$ (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

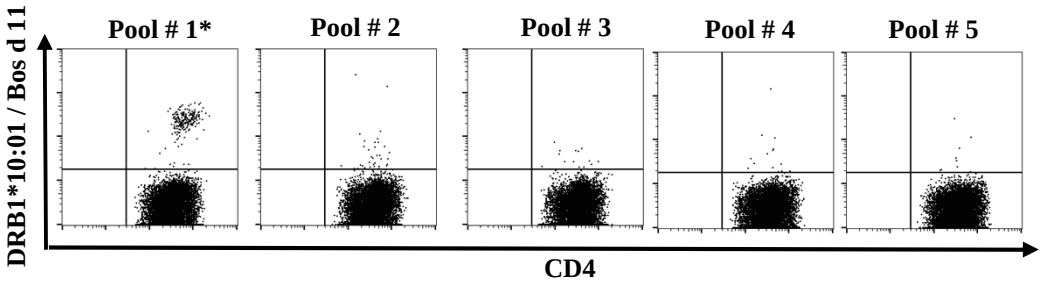
* Subjects also had history of peanut or walnut and positive IgE ImmunoCAP for peanut or walnut

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 $11_{25\text{-}44}$) 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 ≤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 ≤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 ≤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.


Pool # 1*
Pool # 2
Pool # 3
Pool # 4
Pool # 5
DRB1*10:01 / Bos d 11
CD4

B.

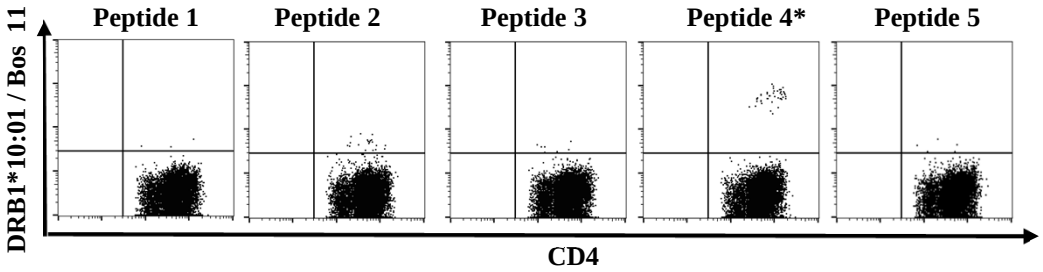
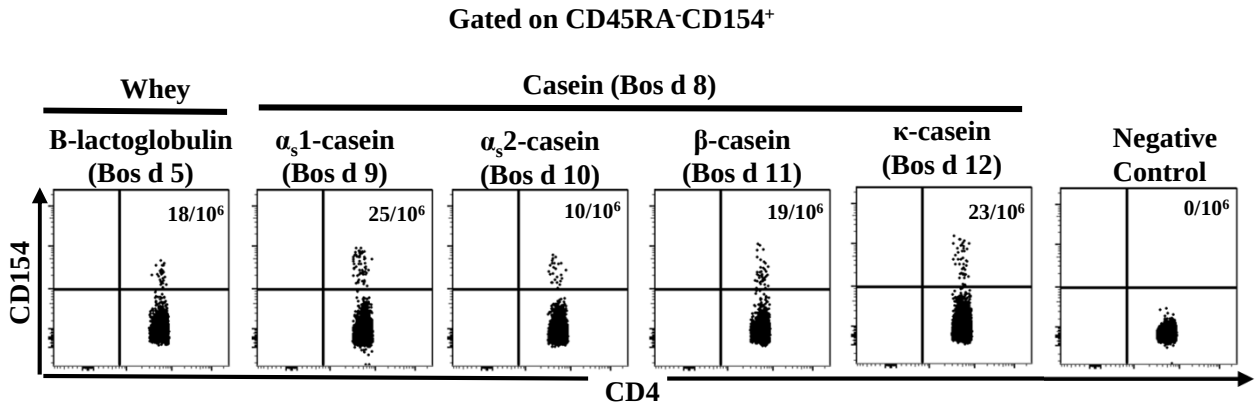

Peptide 1
Peptide 2
Peptide 3
Peptide 4*
Peptide 5
DRB1*10:01 / Bos 11
CD4

Fig. E1

A.


Gated on CD45RA-CD154+
Whey
Casein (Bos d 8)
B-lactoglobulin (Bos d 5)
αs1-casein (Bos d 9)
αs2-casein (Bos d 10)
β-casein (Bos d 11)
κ-casein (Bos d 12)
Negative Control
18/10^6
25/10^6
10/10^6
19/10^6
23/10^6
0/10^6
CD154
CD4

B.

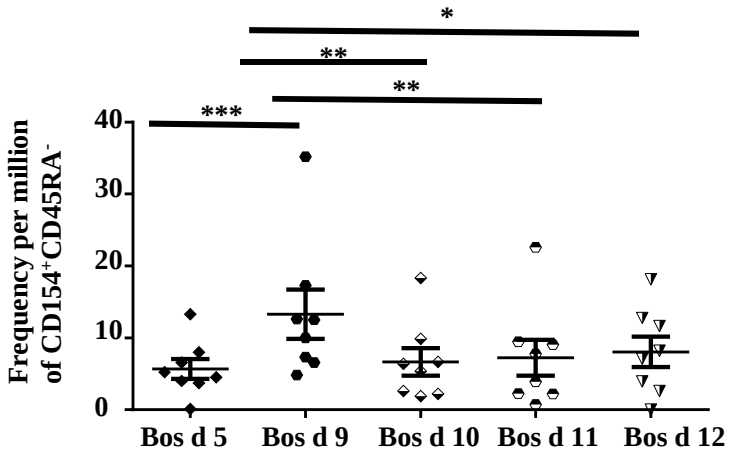

*
**
**
***
Frequency per million of CD154+CD45RA-
40
30
20
10
0
Bos d 5
Bos d 9
Bos d 10
Bos d 11
Bos d 12

Fig. E2

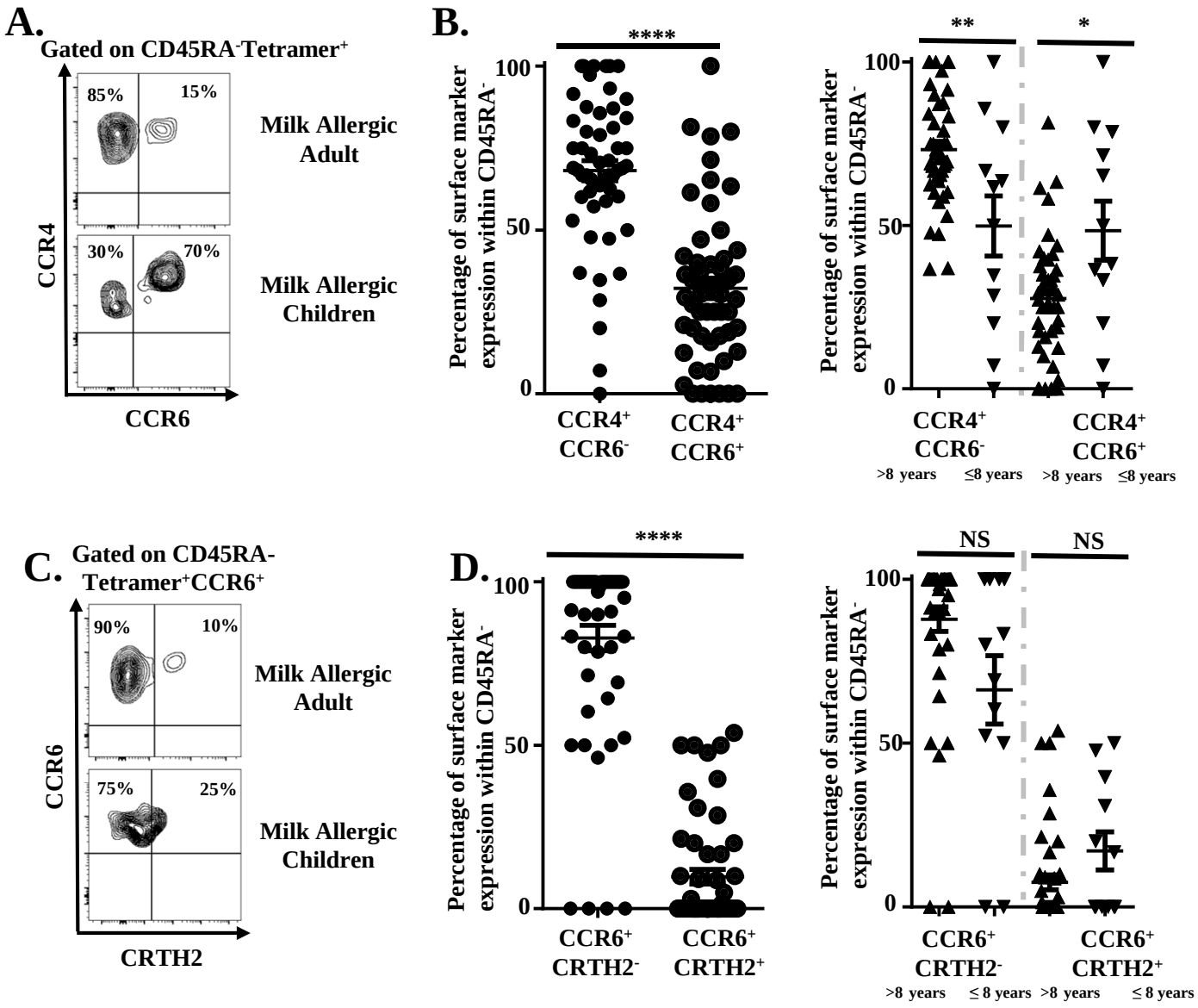

A.
Gated on CD45RA-Tetramer+
85%
15%
30%
70%
CCR4
CCR6
Milk Allergic Adult
Milk Allergic Children
B.
****
**
*
Percentage of surface marker expression within CD45RA-
100
50
0
CCR4+ CCR6-
CCR4+ CCR6+
>8 years
≤8 years
C.
Gated on CD45RA- Tetramer+CCR6+
90%
10%
75%
25%
CCR6
CRTH2
Milk Allergic Adult
Milk Allergic Children
D.
****
NS
NS
CCR6+ CRTH2-
CCR6+ CRTH2+
≤ 8 years

Fig. E3