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# $\alpha_{S1}$-casein Elucidate Major T Cell Responses in Cow's Milk Allergy

**L. Diego Archila, PhD**[a], **Fatima S. Khan, MD**[a], **Nupur Bhatnagar, PhD**[a], **David Robinson, MD**[b], **Mary L. Farrington, MD**[b], and **William W. Kwok, PhD**[a,c]

[a]Benaroya Research Institute at Virginia Mason

[b]The Virginia Mason Medical Center, Seattle, Wash

[c]The Department of Medicine, University of Washington

## Short summary

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

### Keywords

Food allergy; Cow's milk allergy; Bos d; αS1-casein; T-cells; MHC class II tetramers; epitopes

## 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: $\alpha_S$1-casein, $\alpha_S$2-casein, β-casein and κ-casein[(3)]. IgE sensitization is particularly frequent against $\alpha_S$1-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 $\alpha_S$1-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.



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A total of 26 allergic subjects, including 18 CM allergic subjects >8 years of age and 8 CM allergic subjects ≤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 $\alpha_S$1-casein-(including 4 previously identified[6]), 3 $\alpha_S$2-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, $\alpha_S$1-casein, $\alpha_S$2-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 $\alpha_S$1-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 $\alpha_S$1-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 $\alpha_S$1-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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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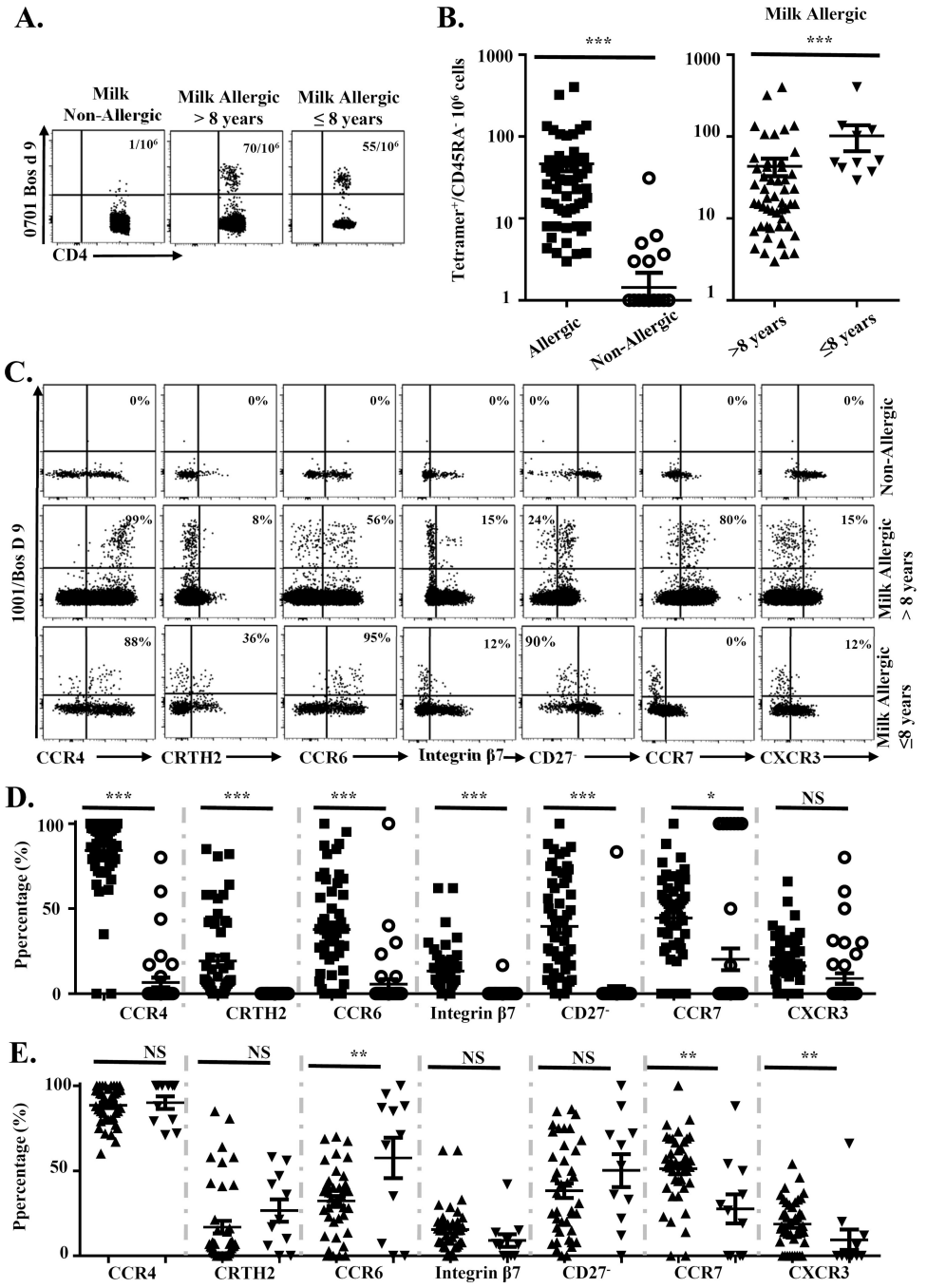


**Figure 1. Frequency and phenotype of Bos d-epitope-specific CD4$^+$ T-cells**

**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 $9_{145\text{-}164}$ 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.

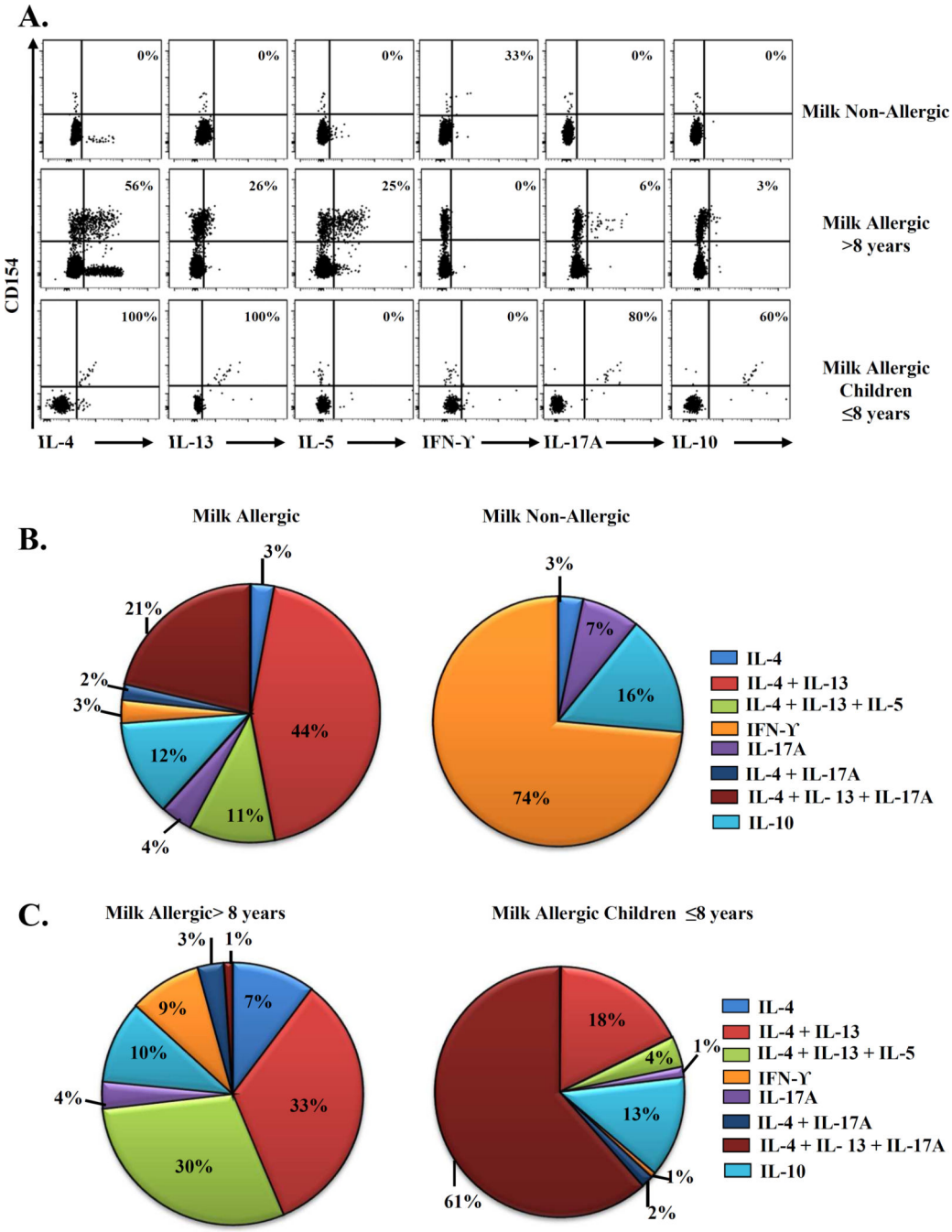


**Figure 2. Cytokine profiles of Bos d-reactive T-cells**

**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.