Identification of potential inflammation markers for outgrowth of cow's milk allergy

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Running title: Immune markers for outgrowth of CMA

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Abstract

Background: Immunoglobulin E (IgE)-mediated cow's milk allergy (CMA) is an immune-mediated reaction to cow's milk (CM). Non-IgE-mediated CMA resolves in most children in the first years of life, whereas IgE-mediated CMA outgrowth is often later or not at all. The exact mechanisms underlying resolution of IgE-mediated CMA are not fully understood.

Methods: We aim to gain insight in the mechanisms underlying resolution of IgE-mediated CMA by analysing saliva samples of allergic infants using protein data from the Olink® Target 96 Inflammation panel. Twenty-four children who outgrew their CMA after 12 months were compared to 15 with persistent CMA.

Results: Persistent CMA was accompanied by an increase in interleukin-15 receptor subunit alpha in the first 6 months, followed by a decrease, hinting towards an initial increased T cell response. At the same time caspase-8 was increased and IL-7 was decreased in persistent CMA. For CMA resolution, we found elevated levels of delta and notch-like epidermal growth factor-related receptor. Furthermore, adenosine deaminase (ADA) increased significantly between 0 and 12 months in resolved CMA, but not in persistent CMA. KEGG pathway analysis suggests mainly the TNF signaling pathway to be important in the resolution of CM allergy.

Conclusion: Olink® Target 96 Inflammation panel analysis of saliva samples can reveal potential immunological markers and mechanisms involved in CMA resolution.

KEYWORDS

cow’s milk allergy, infants, immunoglobulin E, saliva, Olink, allergy resolution, persistent allergy

1. BACKGROUND

Cow's milk allergy (CMA) occurs when the immune system overreacts to proteins found in cow's milk (CM). Immune-mediated reactions to CM are categorized as immunoglobulin E (IgE)-mediated, non-IgE-mediated or mixed[1]. Most infants that did not outgrow their allergy within 1 year after diagnosis have IgE-mediated CMA[2]. IgE-mediated CMA occurs when after exposure to CM proteins via the skin, gastrointestinal tract or respiratory system, people develop IgE antibodies against CM. During the sensitization phase, antigens are processed by antigen presenting cells and presented to naïve T cells, which further differentiate into T helper (Th) 2 cells. The latter then interact with B cells, resulting in an immunoglobulin class switch and the production of CM protein-specific IgE antibodies, which bind to mast cells. During the effector phase, re-exposure to the allergen causes cross-linking of the mast cell-bound IgE and thus induce the degranulation of mast cells, and the release of inflammatory mediators such as histamine, causing allergic reactions within a few hours after exposure[3].
Resolution of IgE-mediated CMA is in most cases achieved within the first 3 years of life[4]. The exact mechanisms are not known, but there is an agreement that individuals reaching higher CM-specific IgE levels have a lower probability to outgrow their CMA[5]. Furthermore atopic dermatitis, other food allergy and respiratory allergy have been identified as predictive for persistent CMA[5]. Infant formula supplemented with a probiotic, prebiotic or synbiotic blend have been proposed to accelerate resolution of IgE-mediated CMA[6].
In this study, we aimed to get a better understanding of the mechanisms underlying resolution of IgE-mediated CMA by analysing saliva samples from infants with IgE-mediated CMA using the Olink® Target 96 Inflammation panel, measuring 92 proteins related to inflammation. We studied the changes of these proteins in children who outgrew CMA after 12 months compared to those with persistent CMA. Moreover, we compared Olink® inflammation panel proteins between infants who received a standard amino acid-based formula (AAF) and those who received an amino acid-based formula supplemented with a synbiotic blend (AAF-syn).

2. METHODS

2.1 Sample collection and experimental design

Saliva samples from 39 infants who participated in the PRESTO clinical trial (NTR3725)[7] were collected in the clinic at least 1 hour after feeding using the SalivaBio Children's Swab method (Salimetrics, Carlsbad, USA). Samples were immediately frozen at -80°C and transported on dry-ice (solid CO2) to Danone Nutricia Research. Samples were aliquoted, transported on dry-ice to Radboudumc Nijmegen and stored at -80°C until analysis.
In the PRESTO study, infants diagnosed with IgE-mediated CMA received specialized infant formula for the dietary management of their allergy (AAF or AAF-syn). The synbiotic blend consisted of probiotic *Bifidobacterium breve* M-16V and prebiotic oligosaccharides (oligofructose, inulin)(dosage described by Chatchatee et al[7]). For both formula, the acquisition of tolerance to CM was similar and in line with natural outgrowth of CMA[7].
IgE-mediated CMA was determined as described elsewhere[7]. For more detail, see Supplementary Material, section 1. Resolution of CMA after 12 months of the start of the study was demonstrated as previously described[7] as provided in the Supplementary Material, section 2.

The infants were selected as described elsewhere[8] and samples were collected at 3 visits (baseline – after CMA diagnosis, 6 and 12 months after entering the study), with the exception of one infant with persistent CMA for whom only samples at baseline and visit 6 months were collected.
At the start of the study, all infants were aged 3-13 months. Twelve months after entering the study, twenty-four infants (10 AAF, 14 AAF-syn) resolved their CMA, while 15 (6 AAF, 9 AAF-syn) were still allergic to CM. Detailed clinical characteristics per allergy group and per treatment group are provided elsewhere[8,9].

2.2 Ethical approval

Ethical approval was obtained as described elsewhere[7]. In the Supplementary Material (Table S1), more details are provided, including an overview of all ethics committees.

2.3 Preprocessing and quality control

Ninety-two proteins related to inflammation and immune response were measured with the Olink® Target 96 Inflammation panel (Olink Proteomics, Uppsala, Sweden). An overview is provided on the Olink® website (https://olink.com/content/uploads/2021/09/1029-v1.3-inflammation-panel-content.pdf). The aliquoted saliva samples were directly added to the plates, and were randomly distributed over two plates to avoid batch effects. The Olink® Target 96 Inflammation panel uses the proximity extension assay (PEA)[10] protocol with quantitative PCR (qPCR) readout. For more detail, see Supplementary Material, section 3.
Threshold cycle (Ct) values from the qPCR were transformed to Normalized Protein Expression (NPX) values and normalized between plates as described in the Supplementary Material, section 4. Quality control (QC) was performed as described in the Supplementary Material, section 5.
Proteins below the limit of detection (LOD) in more than 20% of the samples were removed from the data set and not considered for further analysis.
The results of the quality control and filtering are provided in the Supplementary Material, section 6 and Figure S1. Fifty-eight proteins, listed in Table S2 were included in the statistical analysis.

2.4 Statistical analysis

Linear mixed models (LMM) analysis was performed using R version 4.2.1[11] and the R package OlinkAnalyze version 3.3.1[12] to determine differences between allergy groups (resolution of CMA at 12 months (yes/no)) or treatment groups (AAF/AAF-syn) within visits, and between visits within allergy or treatment groups. For more details, see Supplementary Material, section 7.
To identify (KEGG)[13] pathways which show statistically significant, concordant differences between allergy or treatment groups within visits, and between visits within allergy or treatment groups, enrichment analysis was performed in ConsensusPathDB[14]. For more details, see Supplementary Material, section 8.

3. RESULTS

3.1 Significant differences between visits within allergy groups

In persistent CMA, interleukin-15 receptor subunit alpha (IL-15RA) showed an increase between baseline and 6 months, followed by a decrease between 6 and 12 months (see Table 1 , Table S3, and boxplots in Figure S2). In contrast, no significant changes in IL-15RA were observed in the saliva of infants who did outgrow their CMA (Tables 1 and S3, Figure S2). For oncostatin-M (OSM), cystatin D (CST5) and hepatocyte growth factor (HGF) similar changes between visits were observed in both allergy groups (Tables 1 and S3, Figure S2). Levels of tumor necrosis factor ligand superfamily member 14 (TNFSF14) and protein S100-A12 (EN-RAGE) increased over visits in both allergy groups (Tables 1 and S3, Figure S2). However, in resolved CMA the increase was already significant between 0 and 6 months, while for persistent CMA significance was only observed after 12 months (Tables 1 and S3, Figure S2). Interleukin-7 (IL-7) and caspase-8 (CASP-8) significantly decreased and increased respectively between 0 and 12 months in persistent CMA, but not in resolved CMA (Tables 1 and S3, Figure S2). Urokinase-type plasminogen activator (uPA), delta and notch-like epidermal growth factor-related receptor (DNER), interleukin-8 (IL-8) and adenosine deaminase (ADA) increased significantly between 0 and 12 months in resolved CMA, but not in persistent CMA (Tables 1 and S3, Figure S2). No significant differences between allergy groups were found within visits (Table S4).

3.2 Significant differences between visits within treatment groups

For OSM, EN-RAGE, TNFSF14, CST5, DNER, IL-8 and HGF similar changes were found between visits in both treatment groups (see Table 2, Table S5 and boxplots in Figure S3). In AAF, CASP-8 increased significantly between 0 and 6 months, while at 12 months the CASP-8 level was not significantly different from the baseline level anymore (Tables 2 and S5, Figure S3). In contrast, in AAF-syn, there was no significant difference in CASP-8 between 0 and 6 months, but CASP-8 levels were significantly higher at 12 months compared to baseline (Tables 2 and S5, Figure S3). In AAF, IL-7 did not change significantly over visits (Tables 2 and S5, Figure S3). In AAF-syn, IL-7 levels were significantly higher at 6 and 12 months compared to baseline (Tables 2 and S5, Figure S3). ADA, tumor necrosis factor receptor superfamily member 9 (TNFRSF9) and uPA did not change significantly over visits in AAF, but significantly increased between 0 and 12 months in AAF-syn (Tables 2 and S5, Figure S3). IL-15RA did not change significantly over visits in AAF but significantly decreased between 6 and 12 months in AAF-syn (Tables 2 and S5, Figure S3). No significant differences between treatment groups were found within visits (Table S6).

3.3 Significantly different enriched pathways between visits within allergy groups

KEGG pathway analysis showed that cytokine-cytokine receptor interaction and TNF signaling pathways significantly changed between 6 and 12 months and between baseline and 12 months, with most of the proteins being increased while others decreased (Tables 3 and S7). IL-17 and Toll-like receptor signaling pathways significantly changed between baseline and 12 months, with most of the proteins being increased while others decreased (Tables 3 and S7).

Table S8 displays all KEGG pathways for the comparisons between allergy groups within visits. None of the results were significant (all q-values > 0.05).

3.4 Significantly different enriched pathways between visits within treatment groups

Cytokine-cytokine receptor interaction and several signaling pathways (IL-17, Toll-like receptor, TNF) were higher at visits 6 and 12 months compared to baseline, but did not differ between visits 6 and 12 months in AAF (Tables 4 and S9). In contrast, AAF-syn showed a significant increase in these pathways between 6 and 12 months, but no significant change from baseline at 6 and 12 months (Tables 4 and Table S9) .

3.5 Significantly different enriched pathways between treatment groups within visits

At visit 6 months, cytokine-cytokine receptor interaction, as well as several signaling pathways (IL-17, TNF, Toll-like receptor) were lower in AAF-syn than in AAF (see Tables 5 and S10).

4. DISCUSSION

In this study, the Olink® Target 96 Inflammation panel was applied on saliva samples from infants allergic to CM at baseline, of which some outgrew their CMA, while for others the CMA was persistent. Saliva is a non-invasive source and easy to collect especially in young children. In addition, salivary constituents are known to reflect local and systemic diseases[15].

During the study, infants received AAF or AAF-syn. LMM and KEGG pathway enrichment analysis were applied to identify changes between visits within allergy (persistent CMA versus resolved CMA) or within treatment (AAF versus AAF-syn) groups .

In infants with persistent CMA, we found a significant increase of IL-15RA between baseline and 6 months, followed by a significant decrease between 6 and 12 months. IL-15RA binds interleukin 15 (IL-15)[16], and could thereby increase T-cell activity[17]. IL-15 has been reported to have anti-inflammatory effects in mice exposed to airway allergens[18]. This suggests that the decrease in IL-15RA between 6 and 12 months could reduce IL-15 levels, which in its turn could enhance the allergic response.

A significant increase in TNFSF14 and EN-RAGE (S100A12) was observed earlier in resolved CMA than in persistent CMA. Remarkably, previous studies have found that TNFα, a cytokine related to TNFSF14, and TNFRSF25, a TNFSF14 receptor, might enhance allergic inflammation[19,20]. Moreover, TNFSF14 can induce T cell activation and proliferation[21]. Furthermore, S100A12 has been reported to induce mast cell activation[22] and enhance allergic inflammation and asthma[22]. While these findings suggest a pro-inflammatory role for TNFSF14 and EN-RAGE, our results suggest that these markers instead seem to protect against allergic inflammation and induce outgrowth of CMA.

Remarkably, while IL-1alpha has been reported as linked to T cell activation[23] and being pro-inflammatory[24], we showed a significant increase for IL-1alpha between baseline and 6 months only in resolved CMA. Moreover, Willart *et al.* showed that IL-1R knockout mice could not mount a Th2 response to house dust mite[25].

In persistent CMA, IL-7 decreased significantly between baseline and 12 months. A decrease in IL-7 has been related to increased severity of atopic dermatitis in mice through up-regulation of the Th2 immune response[26].

Our finding that CASP-8 significantly increases between baseline and 12 months in persistent CMA contradicts with a previous finding in a study on allergic contact dermatitis, where reduced CASP-8 was related to increased severity of allergy[27]. In contrast, Teh *et al.* reported that CASP-8 reduces the number of Tregs[28], which are known to inhibit allergy development[29].

Our results suggest an increase in uPA, DNER, IL-8 and ADA between baseline and 12 months in resolved CMA. The increase in uPA corresponds with a recent study on outgrowth of CMA in peripheral blood mononuclear cells which also used the Olink® Target 96 Inflammation panel[30]. Furthermore, uPA has been reported as a marker associated with oesophageal allergic responses to food[31].

DNER is involved in the regulation of interferon-gamma (IFN-γ)[32], a suppressor of allergic response[33]. To the best of our knowledge, a direct relationship between DNER and allergy has not been reported earlier. Remarkably, in contrast to our results, elevated levels of IL-8 have been related to mast cell activation[34] and more severe allergy in asthma[35]. ADA degrades adenosine to inosine, thereby inhibiting T cell activity[36]. It has been suggested that elevated levels of adenosine in ADA-deficient mice are related to allergic airway inflammation[37–39]. The potential role of ADA in asthma has also been demonstrated in humans[40].

We observed that the majority of the proteins belonging to the TNF, toll-like receptor (TLR) and IL-17 signaling pathways significantly increased between 0 and 12 months in infants who have resolved CMA, but not in those with persistent CMA. TNF signaling can both induce and protect against allergic inflammation[41]. Our results suggest that TNF signaling contributes to resolution of CMA by protecting against allergic inflammation. Also, the TLR signaling pathway has been reported to be protective against inflammation[42].

Remarkably, IL-17 signaling, reported as a pathway involved in allergic inflammation[43] significantly increased over visits in the group that has resolved CMA. However, for IL-17 signaling, 9 of the 11 measured proteins overlap with TNF signaling (Table S8). Therefore, the overall increase in the members of the IL-17 pathway can be explained by the large overlap with the TNF signaling.

Based on our results we hypothesize the following mechanisms for persistence and resolution of IgE-mediated CMA (Figure 1). In persistent CMA, an increase of IL-15RA, which binds IL-15, possibly results in increased T cell activity. The increase of CASP-8 and decrease in IL-7 possibly reduce the number of Tregs and enhances the Th2 immune response. This could lead to the increased production of IgE antibodies, which bind to mast cells. This could result in increased degranulation of mast cells and release of inflammatory mediators after re-exposure to CM antigens. In resolved CMA, the Th2 response is possibly disrupted by an increase in DNER, which facilitates the IFN-γ production by T cells. Tregs generate adenosine[44], and the increase in ADA suggests increased degradation of the adenosine produced by Tregs to inosine, which can further inhibit T cell activity and allergic response.

A significant increase in CASP-8 was observed earlier in AAF than in AAF-syn. In AAF-syn, a significant decrease in IL-7 between baseline and the later visits was found. Increased CASP-8 and decreased IL-7 were both reported to reduce the severity of allergy (see above). This suggests that intake of AAF or AAF-syn could diminish allergy severity.

Our results suggest a significant increase of ADA, TNFRSF9 and uPA between baseline and 12 months in AAF-syn. This is in contradiction with previous studies suggesting that synbiotics reduce levels of TNFα, a cytokine related to TNFRSF9[45,46]. Increases of ADA, TNFRSF9 and uPA have been associated with resolution of CMA[30]. To the best of our knowledge, a relationship between synbiotics and levels of CASP-8, IL-7, ADA and uPA has not been reported earlier.

Furthermore, a significant decrease in IL-15RA, which was related to enhanced allergic response (see above) was observed between 6 and 12 months in AAF-syn, but not in AAF.

The results mentioned above suggests that the synbiotic blend contributes to both pro- and anti-inflammatory immune response. This could probably explain why no significant effect of the synbiotic blend on resolution of CMA could be demonstrated by Chatchatee et al[7].

At visit 6 months, the majority of the proteins of the IL-17, TNF and TLR signaling pathways were lower in AAF-syn than in AAF. In the group receiving the AAF, a significant increase in the proteins of these signaling pathways could already be observed between baseline and 6 months, while in the group receiving the AAF-syn, the increase was observed later (between 6 and 12 months). Lower levels of TNF-α after intake of synbiotics have been reported earlier[45]. To the best of our knowledge, no relationship between IL-17 signaling has been demonstrated. Similar as in the comparison to allergy groups, the relationship between synbiotics and IL-17 signaling can probably be explained by the large overlap with TNF signaling. TLRs may have a role in immunotolerance mediated by probiotics[47].

Taken together, the results for the food intervention suggest the following mechanism for the synbiotic blend. A decrease in IL-7 and an increase of ADA, TNFRSF9 and uPA after intake of the synbiotic could have a positive effect on the anti-inflammatory immune response. However, this positive effect is possibly compensated by a decrease of IL-15RA, known to enhance the allergic response.

The current study has some limitations that should be mentioned. First, as all infants were allergic at baseline and therefore were not exposed to CM proteins except for the CM challenge, it is difficult to relate the effects suggested by the results to reduction of antigen presentation, therefore our hypotheses have to be taken with caution. Second, the immune system consists of a large number of interactions between cells and immune factors, which together determine immune fitness. As a consequence, an association between a single salivary immune marker and immune fitness will be moderate at best[48]. Third, no significant differences were observed in biomarker NPX values when comparing children that outgrew their CMA to those with persistent CMA within visits. This may be caused by the small sample size. Future studies should include a larger sample size. Fourth, several biomarkers could not be (reliably) detected in saliva, and preferably, the biomarker assessments should also be conducted in blood[49]. However, for this study, there was limited availability of serum samples, and serum samples were not collected at visit 6 months.

In summary, infants who resolved their CMA were characterized by increased levels of IL-7, DNER and ADA over visits resulting in a reduced allergic response to CM. In contrast, in infants with persistent CMA, immune factors associated with accelerated response to CM allergens, mainly IL-15RA and CASP8, increased over visits and IL-7 decreased. KEGG pathway analysis suggests mainly the TNF signaling pathway to be important in the resolution of CM allergy. Our results show that analyzing data from the Olink® Target 96 Inflammation panel on saliva samples can reveal potential immunological markers and mechanisms associated with the resolution or persistence of CMA.

AUTHOR CONTRIBUTIONS

Diana M Hendrickx: Formal Analysis, Investigation, Supervision, Writing – Original Draft Preparation

Mengyichen Long: Formal Analysis, Investigation

PRESTO study team: Resources

Harm Wopereis: Project Administration, Writing – Review & Editing

Renate G van der Molen: Resources, Supervision, Writing – Review & Editing

Clara Belzer: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing

APPENDIX

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CONFLICT OF INTEREST STATEMENT

Harm Wopereis is an employee of Danone-Nutricia Research. The project is part of a partnership programme between NWO-TTW and Danone-Nutricia Research. The other authors declare that they have no known conflicts of interest.

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DATA AVAILABILITY STATEMENT

The Olink® Target 96 Inflammation panel data and R code have been deposited in Gitlab (https://git.wur.nl/afsg-microbiology/publication-supplementary-materials/2024-hendrickx-et-al-earlyfit-presto-immune-study). Clinical data are available from Danone Nutricia research upon reasonable request (contact: Harm Wopereis: Harm.Wopereis@danone.com).

KEY MESSAGE

Immunoglobulin E (IgE) mediated cow's milk allergy (CMA) has been reported to resolve in the first three years of life in most children, but the underlying mechanisms are not fully understood. This study aims to gain more insight into such mechanisms by analysing proteins measured with the Olink® Target 96 Inflammation panel in saliva samples from allergic children. The results reveal potential immunological markers and mechanisms for both persistence and resolution of CMA.

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TABLES

Table 1. Significant differences between visits within allergy groups as determined by linear mixed models and post hoc analysis.

Protein	**Persistent CMA at visit 12M**			**Resolved CMA at visit 12M**		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
IL-15RA	sign.↑	sign.↓	non-sign.	non-sign.	non-sign.	non-sign.
OSM	sign.↑	non-sign.	sign.↑	sign.↑	non-sign.	sign.↑
CST5	sign.↓	non-sign.	sign.↓	sign.↓	non-sign.	sign.↓
TNFSF14	non-sign.	non-sign.	sign.↑	sign.↑	non-sign.	sign.↑
EN-RAGE	non-sign.	non-sign.	sign.↑	sign.↑	non-sign.	sign.↑
IL-1 alpha	non-sign.	non-sign.	non-sign.	sign.↑	non-sign.	non-sign.
HGF	non-sign.	non-sign.	sign.↑	non-sign.	non-sign.	sign.↑
IL-7	non-sign.	non-sign.	sign.↓	non-sign.	non-sign.	non-sign.
CASP-8	non-sign.	non-sign.	sign.↑	non-sign.	non-sign.	non-sign.
uPA	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
DNER	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
IL-8	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
ADA	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑

Note: Abbreviations: Sign.: significant; non-sign.: not significant; IL-15RA: interleukin-15 receptor subunit alpha; OSM: oncostatin-M; CST5: cystatin D; TNFSF14: tumor necrosis factor ligand superfamily member 14; EN-RAGE: protein S100-A12; IL-1 alpha: interleukin-1 alpha; HGF: hepatocyte growth factor; IL-7: interleukin-7; CASP-8: caspase-8; uPA: urokinase-type plasminogen activator; DNER: delta and notch-like epidermal growth factor-related receptor; IL-8: interleukin-8; ADA: adenosine deaminase.

Table 2. Significant differences between visits within treatment groups as determined by linear mixed models and post hoc analysis.

Protein	Standard amino acid formula (AAF)			Amino acid formula with synbiotic (AAF-syn)		
	0M to 6M	6M to 12M	0M to 12M	0M to 6M	6M to 12M	0M to 12M
OSM	sign.↑	non-sign.	sign.↑	sign.↑	non-sign.	sign.↑
EN-RAGE	sign.↑	non-sign.	sign.↑	sign.↑	non-sign.	sign.↑
TNFSF14	sign.↑	non-sign.	sign.↑	sign.↑	non-sign.	sign.↑
CST5	sign.↓	non-sign.	sign.↓	sign.↓	non-sign.	sign.↓
CASP-8	sign.↑	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
IL-7	non-sign.	non-sign.	non-sign.	sign.↓	non-sign.	sign.↓
DNER	non-sign.	non-sign.	sign.↑	non-sign.	non-sign.	sign.↑
IL-8	non-sign.	non-sign.	sign.↑	non-sign.	non-sign.	sign.↑
HGF	non-sign.	non-sign.	sign.↑	non-sign.	non-sign.	sign.↑
ADA	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
TNFRSF9	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
uPA	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign.↑
IL-15RA	non-sign.	non-sign.	non-sign.	non-sign.	sign.↓	non-sign.

Note: Abbreviations: Sign.: significant; non-sign.: not significant; OSM: oncostatin-M; EN-RAGE: protein S100-A12; TNFSF14: tumor necrosis factor ligand superfamily member 14; CST5: cystatin D; CASP-8: caspase-8; IL7: interleukin-7; DNER: delta and notch-like epidermal growth factor-related receptor; IL8: interleukin-8; HGF: hepatocyte growth factor; ADA: adenosine deaminase; TNFRSF9: tumor necrosis factor receptor superfamily member 9; uPA: urokinase-type plasminogen activator; IL-15RA: interleukin-15 receptor subunit alpha.

Table 3. Significantly different enriched KEGG pathways between visits within allergy groups (at least 4 measured proteins, q-value ≤ 0.05, excluding pathways of which less than 10% of their members were measured and KEGG disease pathways) as determined with ConsensusPathDB. Between brackets the total number of measured proteins. In case of significant differences, it is also mentioned how many proteins increased or decreased.

pathway	Persistent CMA at visit 12M			Resolved CMA at visit 12M		
	0M to 6M	6M to 12M	0M to 12M	0M to 6M	6M to 12M	0M to 12M
Cytokine-cytokine receptor interaction (39)	non-sign.	non-sign.	non-sign.	non-sign.	sign. 34↑, 5↓	sign. 28↑, 11↓
TNF signaling pathway (13)	non-sign.	non-sign.	non-sign.	non-sign.	sign. 12↑, 1↓	sign. 11↑, 2↓
IL-17 signaling pathway (11)	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign. 10↑, 1↓
Toll-like receptor signaling pathway (12)	non-sign.	non-sign.	non-sign.	non-sign.	non-sign.	sign. 11↑, 1↓

Note: Abbreviations: Sign.: significant; non-sign.: not significant; TNF: tumor necrosis factor; IL-17: interleukin-17.

Table 4. Significantly different enriched KEGG pathways between visits within treatment groups (at least 4 measured proteins, q-value ≤ 0.05, excluding of which less than 10% of their members were measured and KEGG disease pathways) as determined with ConsensusPathDB. Between brackets the total number of measured proteins. In case of significant differences, it is also mentioned how many proteins increased or decreased.

pathway	**Standard amino acid formula (AAF)**			**Amino acid formula with synbiotic (AAF-syn)**		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
Cytokine-cytokine receptor interaction (39)	sign. 29↑, 10↓	non-sign.	sign. 28↑, 11↓	non-sign.	sign. 33↑,6↓	non-sign.
IL-17 signaling pathway (11)	sign. 10↑, 1↓	non-sign.	sign. 10↑, 1↓	non-sign.	sign. 11↑	non-sign.
Toll-like receptor signaling pathway (12)	sign. 11↑, 1↓	non-sign.	sign. 11↑, 1↓	non-sign.	sign. 12↑	non-sign.
TNF signaling pathway (13)	sign. 10↑, 3↓	non-sign.	sign. 11↑, 2↓	non-sign.	sign. 11↑, 2↓	non-sign.

Note: Abbreviations: Sign.: significant; non-sign.: not significant; IL-17: interleukin-17; TNF: tumor necrosis factor.

Table 5. Significantly different enriched KEGG pathways between treatments within visits (at least 4 measured proteins, q-value ≤ 0.05, excluding of which less than 10% of their members were measured and KEGG disease pathways) as determined with ConsensusPathDB. Between brackets the total number of measured proteins. In case of significant differences, it is also mentioned how many proteins increased or decreased.

pathway	**0M**	**6M**	**12M**
	AAF-syn vs AAF	**AAF-syn vs AAF**	**AAF-syn vs AAF**
Cytokine-cytokine receptor interaction (39)	non-sign.	sign. 11↑, 28↓	non-sign.
IL-17 signaling pathway (11)	non-sign.	sign. 11↓	non-sign.
TNF signaling pathway (13)	non-sign.	sign. 11↓, 2↑	non-sign.
Toll-like receptor signaling pathway (12)	non-sign.	10↓, 2↑	non-sign.

Note: Abbreviations: Sign.: significant; non-sign.: not significant; IL-17: interleukin-17; TNF: tumor necrosis factor.

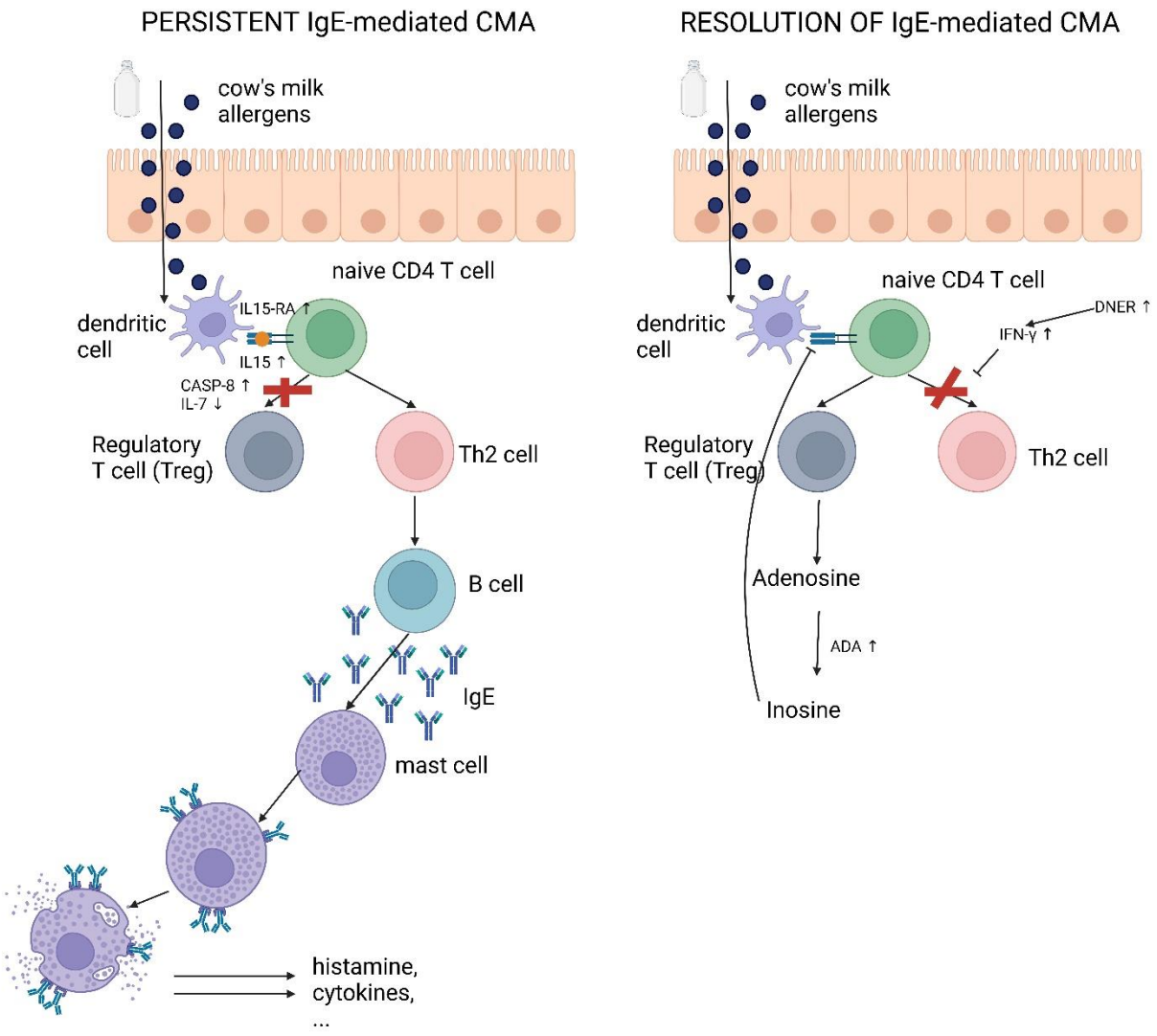

Figure 1. Potential mechanisms for persistent CMA (left) and resolution of CMA (right) revealed by our study. In infants with persistent CMA an increase of IL-15RA, which binds IL-15, possibly results in increased T cell activity. An increase of CASP-8 and a decrease of IL-7 could reduce the number of regulatory T cells (Tregs), and enhance the Th2 immune response, leading to the production of IgE antibodies which bind to mast cells. This could result in increased mast cell degranulation and release of inflammatory mediators after re-exposure to CM antigens. In resolved CMA, the Th2 response is possibly disrupted by an increase in DNER, which facilitates the IFN-γ production by T cells. Tregs generate adenosine which can further inhibit T cell activity and allergic response. Created with BioRender.com