The role of regulatory B cells in milk oral immunotherapy in children with IgE-mediated cow's milk allergy

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Table of Contents

Abstract

Regulatory B (Breg) cells are new players in the field of immune regulation. They have been implicated in venom immunotherapy and their role is being actively studied in non-IgE-mediated food allergies and autoimmune diseases. To date, no studies have examined the role of Breg cells in IgE-mediated milk allergy, nor has the action of Breg cells been examined in the treatment of food allergies with oral immunotherapy (OIT). Furthermore, there are currently no phenotypic, transcription factor, or lineage markers unique to Breg cells, making it a diverse and challenging focus of research. In my thesis I aimed to assess the change in peripheral Breg cells and milk-specific serum IgE, IgA, and IgG4 in children with IgE-mediated cow's milk allergy (CMA) undergoing milk OIT. Children enrolled in a clinical trial of oral immunotherapy for milk allergy were recruited at the Montreal Children's Hospital (MCH), Hôpital Ste-Justine (HSJ), BC Children's Hospital (BCCH), Chicoutimi Hospital, and the Hospital for Sick Children (SickKids). Seven out of 9 children showed an increase in PD-L1^{+} Breg cells from baseline to the end of escalation phase of OIT. Serum and salivary IgG4 increased significantly for all 3 milk proteins (α-lactalbumin, β-lactoglobulin, and casein) in 11 children from baseline to the end of escalation phase and further increased at 9 months post OIT (9M Post OIT), while casein-specific IgA decreased significantly at the end of escalation phase. Casein-specific IgE showed a trend to decrease at the end of escalation phase and further at 9M Post OIT. The IgG4 to IgE ratio increased significantly for β-lactoglobulin at the end of escalation phase and for casein and β-lactoglobulin at 9M Post OIT. My findings reveal that PD-L1^{+} Breg cells increase during milk OIT and may be part of the mechanism of successful desensitization in children. This population of Breg cells could play a role in other allergic diseases as well and future studies exploring Breg cells in such diseases are required.

Résumé

Les cellules B régulatoires (Breg) sont une découverte récente dans le domaine de la régulation du système immunitaire. Elles jouent un rôle important dans l'immunothérapie aux venins et leur fonction est un sujet de recherche courante des maladies autoimmunes et des allergies alimentaires non-IgE médiées. À date, aucune étude n'a examiné le rôle des cellules Breg dans les allergies alimentaires IgE, ni leurs fonctions dans le traitement des allergies alimentaires par voie d'immunothérapie orale (ITO). De plus, les cellules Breg sont difficiles à analyser puisqu'il n'y a pas de marqueurs ou phénotype uniques à ces cellules connus actuellement.

L'objectif de ma thèse est d'évaluer le changement du niveau des cellules Breg et des IgE, IgA, et IgG4 spécifiques aux protéines de lait chez les enfants avec une allergie aux protéines de lait de vache qui reçoivent l'ITO au lait. Les participants étaient recrutés à l'hôpital de Montréal pour Enfants, l'Hôpital Ste-Justine, BC Children's Hospital, l'Hôpital de Chicoutimi, et SickKids. Sept des 9 enfants ont subi une haute de cellules PD-L1+ Breg à la fin de la phase d'augmentation de dose d'ITO au lait (200ml) comparé à leur point de base. L'IgG4 dans la salive et le sérum a augmenté significativement aux 3 protéines de lait (α-lactalbumine, β-lactoglobuline, et caséine) chez 11 enfants à la dose de 200ml et a continué d'augmenter significativement 9 mois plus tard pendant la phase d'entretien (9M ITO). L'IgA spécifique à la caséine a diminué significativement à la dose de 200ml et l'IgE spécifique à la caséine a eu une tendance à diminuer à la dose de 200ml, et a continué à diminuer à 9M ITO. Mes résultats montrent que les cellules PD-L1+ Breg augmentent pendant l'ITO au lait et peuvent faire partie du mécanisme de désensibilisation chez les enfants. Cette population de cellules Breg pourrait jouer un rôle important dans d'autres maladies allergiques, nécessitant plus d'études qui explorent leur rôle.

Preface

This thesis discusses the changes in regulatory B cells, serum and salivary IgG4, serum IgA, and serum IgE in children with IgE-mediated cow's milk allergy undergoing milk oral immunotherapy in the first multicentered study in Canada. The introduction touches on the importance of oral tolerance and the immune system, the prevalence of cow's milk allergy in children with a new research treatment with oral immunotherapy, and the novel regulatory role of B cells. Section 2.0 presents a literature review of the mechanisms of oral tolerance and how this may break down, leading to sensitization and IgE-mediated food allergies. It further describes the function of Treg and Breg cells in tolerance and disease, and the humoral response in allergic disease. Sections 2.8 and 2.9 provide details on cow's milk allergy, milk oral immunotherapy trials to date, and the current knowledge of immunological changes with oral immunotherapy. The study objectives and methodology are presented in Section 3.0 and 4.0, respectively; followed by the results in Section 5.0. Finally, Sections 6.0 and 7.0 discuss interpretation of results and the conclusion.

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Contribution of Authors

The idea for the study was developed by Dr. Bruce Mazer and Dr. Ben-Shoshan. The study design and protocol for the milk OIT clinical trial were prepared by Dr. Bruce Mazer, Dr. Ben-Shoshan, and Duncan Lejtenyi. Patient recruitment, clinical assessment, clinical data collection, blood and saliva collection, and oral milk administration as per protocol were conducted by Duncan Lejtenyi, the research nurses at the CIM, Dr. Sarah De Schryver, Dr. Moshe Ben-Shoshan, and Dr. Bruce Mazer at the RI-MUHC. Blood and saliva sample processing, cell culture experiments, ELISAs, and data analysis were performed by Dr. Bahar Torabi, with the help of members of the Mazer Lab.

Abbreviations

ALA – α-lactalbumin
BLG - β-lactoglobulin
BCR – B Cell Receptor
Breg– Regulatory B cells
CMA – Cow's Milk Allergy
DC – Dendritic Cells
EPIT – Epicutaneous Immunotherapy
Fas-L – Fas Ligand
iTregs – Inducible Tregs
IPEX – Immunodeficiency, Polyendocrinopathy, Enteritis, X-linked
ILC2 – Innate Lymphoid Cells 2
ITIM – Immunoreceptor Tyrosine-based Inhibition Motif
MS – Multiple Sclerosis
OIT- Oral Immunotherapy
PD-L1 – Programmed Death Ligand 1
SLIT – Subcutaneous Immunotherapy
SCFA – Short Chained Fatty Acids
SU – Sustained Unresponsiveness
TSLP – Thymic Stromal Lymphopoetin
TIM1 - T cell immunoglobulin and mucin domain 1
Treg– Regulatory T cells

CI – Confidence Interval
IQR – Interquartile Range
NS – Non- Significant

1.0 Introduction

Oral tolerance to food allergens is a complex mechanism involving both immune and non-immune cells of the body, in which the gastrointestinal tract plays a crucial function. The gut is home to trillions of resident bacteria and lymphoid cells and it is the largest body surface exposed to the external environment.[1-3] The immune system must be able to recognize innocuous self and non-self antigens, such as proteins from ingested food or the local flora and differentiate them from pathogenic antigens. A breakdown in any point during this process can lead to inflammatory or allergic diseases, including IgE-mediated food allergies.

An estimated 6-8% of children have a food allergy and up to 0.2-2% have a cow's milk allergy.[4,5] Due to the ubiquitous nature of dairy products in our diets, milk is extremely difficult to avoid. The large burden of this disease requires development of new treatment strategies apart from avoidance to alleviate this problem. Oral immunotherapy (OIT) trials have been studied for different foods, including cow's milk, with successful desensitization rates of 33%-90%.[6] The mechanism of oral immunotherapy is not fully understood, however immunotherapy to food, venom, and aeroallergens has been associated with decreases in antigen-specific IgE and increases in antigen-specific IgG4.[6,7]

B lymphocytes are antibody-secreting cells implicated in immunity against pathogens, autoimmunity, and allergic disease. Although best known as effector cells which produce pro-inflammatory antibodies, B cells also participate in regulatory and anti-inflammatory activity.[8] It has been proposed that naive B cells and memory B cells can produce IL-10, an anti-inflammatory cytokine, depending on the signals they receive.[9,10] B cells have also been shown to suppress inflammation through pathways other than IL-10 production. They can secrete other inhibitory cytokines (TGF-β and IL-35), express Fas ligand (Fas-L), T cell immunoglobulin and

mucin domain (TIM1), and programmed death ligand 1 (PD-L1) on their surface, and secrete the proapoptotic protein granzyme B.[8,11-14] Regulatory B cells (Breg) have been associated with suppression of inflammatory, allergic, and autoimmune diseases in mice and humans.[10-16] No studies have examined the correlation between Breg and IgE-mediated milk allergy and anaphylaxis in humans, nor have the action of Breg been examined in the treatment of life threatening food allergies with oral immunotherapy. Since B cells can make both pathogenic IgE (which requires IL-4 and/or IL-13) and regulatory antibodies IgG4 and IgA (which require IL-10 and TGF-β), understanding the contribution of Breg to milk desensitization is an important facet of clarifying the immunological network that is in play in this disease. Currently, our group has undertaken the first Canadian multi-center randomized controlled trial of milk oral immunotherapy at the RI-MUHC and we are therefore evaluating the clinical and immunological parameters of children undergoing OIT compared to milk allergic control subjects.

2.0 Literature Review

2.1 Antigen Uptake and Presentation

Oral tolerance to ingested food antigens is an active process requiring the GALT (gut-associated lymphoid tissue), namely the mesenteric lymph nodes and Peyer's patches.[17] The uptake of food antigens from the gut lumen can be achieved via paracytosis or transcytosis through epithelial cells, endocytosis by M (microfold) cells, or periscoping by $CD103^+$ dendritic cells (DC).[18] A fourth cell type in the lamina propria involved in antigen uptake are $CX3CR1^+$ cells, likely macrophages, that extend dendrites between epithelial cells to capture antigens. These cells can directly transfer antigens to $CD103^+$ DC via gap junctions, do not express CCR7, do not migrate to mesenteric lymph nodes, and secrete high levels of IL-10.[19-23] On the other

hand, the $CD103^+$ DC express CCR7 and migrate to mesenteric lymph nodes for antigen presentation to naïve T cells.[17] These cells also secrete IL-10, IDO (idoleamine 2,3-dioxygenase), TGF-β, and retinoic acid, thus inducing naïve T cells to become regulatory T cells (Treg) including CD4+CD25+Foxp3+ Treg, TGF-β-producing Th3 cells, and Tr1 cells, thereby promoting tolerance.[17] Retinoic acid produced by $CD103^+$DC in the mesenteric lymph nodes up-regulates the expression of α4β7 integrin and CCR9 receptors on Treg, resulting in their homing from MLN to the lamina propria.[17,24,25] It has also been shown that retinoic acid derived from GALT-associated DC can imprint gut-homing on B cells and direct them towards IgA secretion.[26,27]

2.2 Regulatory T and B Cells

More is known about Treg in oral tolerance and food allergies than Breg. Inducible Treg (iTreg), known to be CD4+CD25+Foxp3+ Treg, and Th3 cells, which produce TGF-β and are $Foxp3^-$ $CD25^-$ are the main Treg at play in oral tolerance.[17] Humans and mice deficient in Foxp3 develop food allergies, among many other features of IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome.[28] In one study, children who outgrew their milk allergy displayed a higher number of CD4+CD25+Foxp3+ Treg after a milk challenge.[29] In peanut-allergic children undergoing oral immunotherapy (OIT), there was an increase in the function of Treg and demethylation of Foxp3 CpG sites compared to allergic controls who did not undergo peanut OIT.[30] Treg help promote tolerance via the secretion of IL-10, TGF-b, IL-35, direct cell targeting with CTLA-4 and PD-L1, cytolysis by granzymes A and B, and metabolic disruption using CD25 and CD73.[31] Treg are categorized in different subgroups, depending on their expression of Foxp3 and cytokine production; however, their

individual roles in oral tolerance are not well known. As mentioned above, Treg are present in the intestine, where their inhibitory effects take place in the mesenteric lymph nodes and the lamina propria.[24-25]

Although a currently active area of research, the role of Breg in oral tolerance is less well-understood. In mice sensitized to milk, mesenteric IL-10 producing CD5+ Breg inhibit IgE-mediated anaphylaxis.[32] Children with atopic dermatitis, because of non-IgE mediated milk allergy, have decreased numbers of IL-10 producing Breg compared to controls.[33] Akdis et al showed an increase in IL-10 producing Breg in patients with bee venom allergy undergoing immunotherapy.[34] The inhibitory mechanisms of Breg are similar to Treg cells, using inhibitory cytokines, direct cell targeting, and cytolysis.

2.3 Intestinal Microbiome

The impact of gut microbiota on oral tolerance is an active area of research. Mouse studies have shown that germ-free mice, antibiotic-treated mice, or mice lacking TLRs do not fully develop tolerance to food antigens.[35] Certain bacterial strains, such as from the Clostridia class, tend to promote a TGF-β and IL-10 positive environment with an increase in IgA production in the intestinal lumen and Treg in the colon.[35,37] Metabolic bi-products of the microbes can also shape the intestinal immune response. Short-chain fatty acids (SCFA) produced by fermentation of dietary fibre can promote tolerogenic DC, increasing IL-10 production and Treg.[38,39] Alterations in the gut microbiome may lead to a loss of tolerance and susceptibility to food allergies in humans. Studies have looked at antibiotic use during pregnancy, in infancy, and the mode of delivery at birth to assess the impact on the intestinal microbiome and food allergies.[40-42] However, there has not been any concrete evidence or

causality in humans thus far. Given the multitude of various factors at play, more robust studies are required in this field. Furthermore, the role of the microbiome on oral tolerance should be extended to the skin as well.[18]

2.4 Sensitization

The initiating factor in the loss of tolerance or failure to achieve tolerance in people with food allergy remains an area of active research. One mechanism postulated is sensitization via cutaneous exposure. Whereas high-dose oral exposure of food allergens seems to promote tolerance, low-dose cutaneous exposure leads to a Th2 response and IgE-mediated food allergy.[43] Epithelial cells are important sources of cytokines and in response to injury, inflammation, and innate immune activation, they secrete IL-25, IL-33, and TSLP (thymic stromal lymphopoietin).[44] These cytokines act on DC, mast cells, basophils, and innate lymphoid cells 2 (ILC2), which in turn promote a Th2 response to food antigens exposed via the skin.[44-46] Antigen presentation by inflammatory DC will induce Th2 cells that secrete IL-4, IL-5, and IL-13.[45] IL-4 and IL-13 induce B-cell class-switching to produce antigen-specific IgE. Once IgE is secreted by plasma cells, it binds to the high affinity FcεRI receptor on mast cells and basophils. Upon antigen re-exposure, bound IgE cross-links IgE receptors and activates the cells, causing degranulation and release of their inflammatory mediators, leading to clinical symptoms such as anaphylaxis.

Impaired skin barrier in humans allows allergen exposure via the inflamed skin and has been shown to be a risk for the development of food allergies. Children with atopic dermatitis (AD), a chronic inflammatory skin disorder, have a higher incidence of allergic diseases, including food allergies. It has been shown that children with a filaggrin gene mutation, causing

a loss of skin barrier function, have an increased risk of peanut allergy.[47] A mutation in SPINK5, a protease affecting epithelial integrity, is associated with severe AD and food allergy.[48]

2.5 Regulatory B cells

Although best known as effector cells which produce pro-inflammatory antibodies and cytokines, B cells also participate in regulatory and anti-inflammatory activity.[8]
A regulatory function of B cells was first suggested in 1974 in the context of delayed hypersensitivity reactions in guinea pigs.[49] It wasn't until two decades later that this notion resurfaced in a mouse model of experimental autoimmune encephalitis.[50] Following this reemergence, other mouse studies linked B cells to IL-10 production and suppression of inflammation.[51-52]

There is increasing interest in defining and harnessing the subsets of B cells that produce regulatory cytokines including IL-10, TGF-β, and IL-35. These Breg have been associated with suppression of inflammatory, allergic, and autoimmune diseases in mice and humans.[10-16] An imbalance in Breg has been shown to be important to the pathogenesis of multiple sclerosis.[53] More recently impaired Breg function was reported to contribute to non IgE-mediated allergic conditions. Noh et al showed that children with eczema and sensitivity to milk had fewer milk-specific Breg cells than children who could drink milk without worsening of their skin symptoms.[33,54] Unlike Treg, there are no specific criteria by which Breg are recognized. There are no phenotypic, transcription factor, or lineage markers unique to regulatory B cells known or agreed upon at this time.[55] The heterogeneity of subsets of Breg currently identified at various stages of B cell development has brought controversy regarding their origin. It is possible that

Breg possess intrinsic plasticity and can be induced towards a regulatory phenotype along the spectrum of their development, given the appropriate signals and microenvironment.

2.5.1 IL-10 Producing Breg Cells

Multiple subsets of IL-10 producing Breg have been identified in humans and mice.[13] Breg were first noted to be higher in patients with multiple sclerosis (MS) infected with helminths and had a better clinical outcome than patients with MS without helminth infection.[56] These $CD19^{+}CD1d^{hi}$ B cells suppressed T cell proliferation and IFN-γ production. Another study found patients with MS have higher IL-10 producing Breg during remission than those experiencing relapse,[57] while IFN-β therapy expanded the subset of $CD24^{hi}CD38^{hi}$ Bregs.[58] In patients with systemic lupus erythematosus (SLE), another autoimmune disease, $CD24^{hi}CD38^{hi}$ Breg have impaired ability to produce IL-10.[59-60] Other Breg phenotypes have been identified in various diseases, such as rheumatoid arthritis, Sjogren syndrome, chronic hepatitis B infection, and transplant rejection.[13,61] There are limited studies of human Breg in allergic diseases.

Akdis et al first observed that bee keepers who develop natural tolerance and patients undergoing been venom immunotherapy have an increase in IL-10 producing Breg,[34] specifically in $CD25^{+}CD71^{+}CD73^{-}$ B cells.[10] Interestingly, these Breg were also the source of IgG4 production in these patients. In patients with allergic asthma, the subset of peripheral $CD24^{+}CD27^{+}$ B cells produce less IL-10 than healthy controls.[62] The subset of IL-10 producing Breg in children with atopic dermatitis and non-IgE-mediated milk allergy expressed CD5 on their surface and intracellular FoxP3.[33] Even $CD19^{+}CD138^{+}$ plasma cells have been shown to act as regulatory cells in allergic airways disease in mice via IL-10 production.[63] Thus, studies in allergic disease have demonstrated a heterogeneous definition of Breg.

2.5.2 IL-10 Independent Breg Cells

Breg have been shown to suppress inflammation through pathways other than IL-10 production. In addition to secreting other inhibitory cytokines (TGF-β and IL-35), they can express Fas-L, TIM-1, and PD-L1 on their surface, and secrete granzyme B.[8,13] Mouse models have provided extensive characterization of these Breg, although there have been fewer studies in humans.

TGF- β is involved in many physiological processes in cells, such as proliferation, cell differentiation, and suppression of inflammation.[8,13] Its role is important in the maturation of Treg and the inhibition of effector Tcells.[8] Mutations in the TGF- β receptor predisposes humans to various allergic diseases like asthma and food allergy.[64] Once TGF-β1 is produced in a cell, it homodimerizes and remains inactive until it is cleaved from the latency activated peptide (LAP). Human B cells have been shown to secrete the active form of TGF-β1, which increases isotype switching to IgA.[65]

Granzyme B-positive B cells have been found in solid tumour infiltrates in humans and express CD25, IL-10, and IDO.[66] They are induced by IL-21 produced by T cells present in the tumour microenvironment.[66]

Our knowledge about the inhibitory pathway through PD-1 and its ligand PD-L1 stems from cancer research and therapeutic targets.[67] PD-1 is a member of the CD28 superfamily and comprises of a single N-terminal immunoglobulin variable region-like domain.[68] It contains an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) on its cytoplasmic tail, which interact with SH2 domain-containing phosphatases.[68] The ligation of PD-1 by PD-L1 has been shown to inhibit the PI3K-Akt and the Ras-MEK-ERK pathways in T cells expressing PD-1. PD-L1 also interacts with B7-1 expressed

on T cells, but has greater affinity for PD-1.[69] PD-1 expression is upregulated after T cell activation, requiring nuclear translocation of NFAT and binding to the promoter region of its gene *PDCD1*, whereas T-bet represses the transcription of the gene.[68,70] Thus, both its activation and repression occur during T cell activation; allowing a finite time for its inhibitory action.[68] PD-L1 is constitutively expressed at low levels on APCs, nonprofessional APCs expressing MHC class I, as well as nonhematopoietic cells, and its expression is upregulated by proinflammatory cytokines such as TNF-α, and type I and II interferons.[68] PD-L2, the other ligand of PD-1 is expressed on activated dendritic cells and macrophages and is induced by the same cytokines as PD-L1. PD-L2 on lung dendritic cells also interacts with repulsive guidance molecule B (RGMB) on alveolar and interstitial macrophages and alveolar epithelial cells, thus promoting pulmonary tolerance.[70,71]

The timing and kinetics of PD-1, PD-L1, and PD-L2 expression after activation of T cells indicate their suppressive role during inflammation and in establishing peripheral tolerance. PD-L1$^+$ Breg have been shown to suppress T follicular helper cells (Tfh) expressing PD-1 in humans.[72] Even after rituximab, an anti-CD20 antibody, treatment ex-vivo, the number of PD-L1$^+$ Breg remained unchanged.[73]

2.6 Induction of Breg Cells

In humans, IL-10 production by B cells has been shown via IL-21, MHCII, and CD40 stimulation in patients with autoimmune diseases.[73] TLR 4 and TLR9 ligands are also important inducers of Breg as seen in asthmatic patients.[6] These Breg inducers, either used alone (such as CpG, a TLR9 ligand) or in combination (such as CpG with anti-CD40) have their effect after 48 or 72 hours in culture, depending on the study.[10,15,55,74] Recently, one group found the highest

production of IL-10 by combining CpG, anti-CD40, and anti-BCR.[75] Others have noted different types of Breg when stimulation is done with IL-21 alone without CD40 ligation. In this case, granzyme-B producing Breg differentiated in response to IL-21.[8] IL-10 transfection of primary human B cells pre-stimulated with CpG, resulting in overexpression of IL-10, ensued an immunoregulatory phenotype with an increased expression of CD25, VEGF, PD-L1, GARP, IgG4 mRNA and a decrease in TNF-α and IL-8, without a change in TGF-β, CD24, or CD1d.[15]

It has been hypothesized that both naïve and memory B cells have evolved an immunosuppressive mechanism when activated in a certain setting.[9] For example, during an infectious process where TLR signaling occurs, or during a vigorous adaptive response where all three TLR stimulation, BCR activation, and CD40 are engaged, B cells have the capacity to produce IL-10 and downregulate the response.[9] Furthermore, during this immunosuppressive process, the prevention or resolution of an autoimmune or inflammatory disease can also occur.[9]

2.7 Humoral response

Antibodies of the IgA and IgG4 classes are known as neutralizing or blocking antibodies and are key biomarkers of success for bee venom and food immunotherapy.[6,7,10] IgG, which includes IgG4, is thought to have an inhibitory role as well, by inhibiting the IgE effect through FcγRIIb receptors.[76] B cells class-switch to a different isotype depending on the cytokine milieu. TGF-β promotes IgA class-switch, while IL-10 promotes IgG4 class-switch. IgE production requires the presence of IL-4 and/or IL-13, commonly referred as Th2 cytokines.

IgA plays an important role in mucosal immunity, by binding bacteria, viruses, toxins, and food allergens, preventing their attachment to epithelial cells, and regulating the gut

microbiome.[77] IgA is mainly secreted as a dimer in mucosal surfaces such as the gut, respiratory tract, salivary glands, tear glands, and breast milk.[77] Its principal role is neutralization, while it very weekly functions as an opsonin and does not activate the complement system.[77] It is found as a monomer in plasma.

IgG4 is the least abundant subclass within IgG, with a molecular weight of 146 kDa.[77] It is secreted as a monomer and is typically found in the bloodstream and in the extracellular spaces in tissues.[77] Like IgA, it functions as a neutralizing antibody and does not induce the complement system or activate cells because of low affinity to C1q and Fc receptors.[78] By acting as a blocking antibody, specific IgG4 can compete against specific IgE for allergen binding in the context of food allergy and immunotherapy.[79] Unlike other isotypes, IgG4 antibodies have been shown to exchange one of their Fab arm with another unrelated IgG4 Fab arm, resulting in bispecific immunoglobulins.[78] In normal human serum, hybrid IgG4, comprised of two heavy chains with one λ light chain and one κ light chain, ranged from 21-33% of the total IgG4 concentration.[80] There is scant literature on IgG in the saliva and none on salivary IgG4 in OIT.

IgE is a monomeric antibody and is the most heavily glycosylated immunoglobulin in mammals.[81] One group found a single glycan at asparagine-394 in human IgE that is absolutely required for binding to FcεRI and eliciting anaphylaxis.[82]

2.8 Cow's Milk Allergy

Cow's Milk Allergy (CMA) is a common problem in children and carries an important nutritional and psychosocial impact. True IgE-mediated CMA affects 0.2 to 2% of children.[4,5] Moreover, IgE- mediated cow's milk allergy is among the most common causes of severe allergic reactions and anaphylaxis presenting to the emergency department in Canada.[83] Due to

the ubiquitous nature of dairy products in our diets, milk is extremely difficult to avoid. Children with CMA are typically allergic to multiple cow's milk proteins, notably casein, α-lactalbumin, and β-lactoglobulin.[84]

The large burden of this disease requires development of new treatment strategies apart from avoidance of this food to alleviate this problem. Oral immunotherapy (OIT) trials have been studied for different foods, including cow's milk, with successful desensitization rates of 33%-90%.[6] However, there is tremendous variability among these research trials and their protocols.[85] The mechanism of desensitization with oral immunotherapy is not fully understood. However, studies show that immunotherapy to food, venom, and aeroallergens has been associated with decreases in antigen-specific IgE and increases in antigen-specific IgG4.[6,7]

2.9 Oral Immunotherapy for Food Allergy

OIT consists of ingesting small amounts of the allergenic food, such as egg or milk, and gradually increasing the dose until the target maintenance dose is reached and continued for months to years.[6] OIT by ingesting the food is the most studied form of immunotherapy for food, with sublingual (SLIT) and epicutaneous (EPIT) immunotherapy also being investigated. With SLIT, the product is placed and held under the tongue while it is absorbed and should not be swallowed. EPIT uses the application of a patch containing the allergen protein directly to the skin. It is known that OIT is more effective and can attain higher doses due to its mode of delivery orally, compared to SLIT or EPIT; however, with greater side effects and reactions.[86] There is considerable variation between OIT protocols, however the basic principle remains the same. Most clinical trials aim for desensitization, which is an increased threshold of the ingested allergen without having an allergic reaction.[85] Since desensitization requires regular consumption

of the food, some trials have investigated sustained unresponsiveness after 4-8 weeks of stopping food consumption and re-challenging after that period. Sustained unresponsiveness (SU) cannot be defined as tolerance, particularly when it is not seen in most desensitized subjects.[6] SU is currently defined as the lack of clinical reaction after regular exposure has been discontinued for a period of time, and it is thought to be dependent on some degree of allergen exposure.[87] The rate of desensitization in OIT for milk, egg, and peanut ranges from 33-90%, while SU is seen in only 10-50% of desensitized subjects.[88] Therefore, it may take months to years of regular consumption before SU is reached and even longer before tolerance is achieved. There remain substantial knowledge gaps regarding the molecular pathways leading to SU and tolerance.

OIT trials to various foods, notably milk, egg, and peanut, have shown active immunologic changes with increases in antigen-specific IgG4, decreases in antigen-specific IgE, decreased diversity in IgE epitope recognition, and somatic mutations of IgG4.[89-93] Skin prick test to the allergen decreases within a few months of oral immunotherapy as does basophil activation.[93] A peanut OIT trial showed increased function in antigen-specific $CD4^+CD25^+FoxP3^+$ Treg and hypomethylation of the FOXP3 gene.[30] Another study showed a decrease in diversity and clonality of peanut-specific T-cell receptors over time during OIT.[94] Data on IgA is not consistent between various OIT trials that have measured serum antigen-specific IgA. One study found a rapid increase in egg-specific IgA during the rush phase on day 1 compared to baseline (ie. prior to OIT), which then decreased at the next measured time point at 3 months.[95] Another egg OIT trial studied IgA, IgA1, and IgA2 to different egg components including egg white, ovalbumin, and ovomucoid by ELISA.[96] They found significant increases in IgA and its subtypes in those receiving egg OIT. Between the responders (defined as passing a double- blind placebo controlled challenge after 4weeks off therapy) and non-responders, only

IgA and IgA2 to egg white were significantly different at 36 and 48 months when comparing the 2 groups.[96] They also studied ratios of IgA, IgA1, and IgA2 to IgE for all 3 egg components between responders and non-responders and found increased ratios in responders.[9] The data on IgA in milk OIT is even more varied and will be reviewed below.

2.9.1 Milk Oral Immunotherapy Trials

There have been several milk OIT trials since 1998, with the first randomized controlled trial in 2007.[97] Currently there is no consensus for an established protocol, as seen with the different trials with varying protocols and a range of maintenance doses, duration of treatment, and age at inclusion that have been studied to date.[6] However, multiple studies have used 200 ml of milk (7g protein) as a maintenance dose with 7-10g for the final oral challenge. [6,98] Only two trials have studied sustained unresponsiveness after successful desensitization in 70% and 90% of subjects, demonstrating 40% SU after 6 weeks off therapy [99] and the other demonstrating 42% after 8 weeks off therapy.[6] The total duration of OIT in these 2 studies was 60 weeks and 24 months, respectively.[6,99] The low success rates of SU in milk OIT mirror similar findings in other food OIT trials,[88] again implying longer maintenance duration is required before SU or tolerance can be reached.

As with other food OIT studies, immunological changes are seen during milk OIT. There is inconsistent data on milk component IgE and IgG4 among these studies, with few studies measuring IgA. Savilahti and colleagues measured whole cow's milk-, casein-, and β-lactogloblubulin (BLG)-specific IgA, IgG4, and IgE by ELISA.[91] They found decreases in whole CM-specific IgE, increases in whole CM-specific and BLG-specific IgA and IgG4, and increases in casein-specific IgG4 in children who achieved desensitization.[91] Another group found

increases in α-lactalbumin (ALA), BLG, and casein-specific IgG4 but no changes in IgE after reaching the maintenance dose of 200 ml.[100] Other studies have used peptide microarray assays to study the changes in epitope binding of IgE and IgG4. Successful desensitization was associated with a decrease in IgE binding to CM peptides and an increase in IgG4 binding.[90] Those who discontinued OIT had a broader diversity of IgE and IgG4 binding to CM peptides, and less epitope binding overlap of the two isotypes.[90] Another study found rapid increases in IgG4 positive peptides to casein and BLG with slow decreases in IgE positive peptides to casein with significance only at 24 months.[89]

2.9.2 Salivary IgA and IgG4

There are very few studies on salivary IgA and IgG4, and none on salivary IgG4 in food allergy or food immunotherapy. Some studies investigated the association between total or allergen-specific salivary IgA and the development of atopy such as asthma, atopic dermatitis (AD), or allergic rhinitis (AR).[101-106] Salivary IgA was higher in non-allergic children when compared to children with AR, AD, or asthma.[101-105] One study found no difference in BLG- and ovalbumin-specific salivary IgA in children with AD who were allergic to egg or CM then those who could consume the food.[105] Sandin et al found higher salivary IgA was associated with decreased late-onset wheezing in IgE-sensitized infants.[101] Increases in dust mite-specific salivary IgA were seen in children undergoing dust mite SLIT.[106]

Salivary IgG4 was studied in non-allergic children and dust mite- allergic children with AR.[104] Interestingly, allergic children had higher levels of dust mite-specific salivary IgG4 than non-allergic children.[104] Currently, there are no studies investigating salivary IgA or IgG4 in food oral immunotherapy.

In conclusion, OIT to milk has been shown to be effective and to induce desensitization in children with cow's milk allergy. However, the immunological changes and the mechanisms behind desensitization remain unclear. Moreover, the role of Breg in food immunotherapy is not well understood as data is lacking. Therefore, understanding the contribution of Breg to milk desensitization is an important facet of clarifying the immunological network that is in play in this disease.

3.0 Study Objectives

3.1 Overall Objectives

Currently, there are no studies that have examined the correlation between Breg and IgE-mediated cow's milk allergy and anaphylaxis in humans, nor have the action of Breg been examined in the treatment of life threatening food allergies with oral immunotherapy. Therefore, I aimed to assess the changes in Breg and milk-specific IgE, IgA, and IgG4 in serum and saliva during milk OIT in children with IgE-mediated cow's milk allergy.

3.2 Primary Objectives

1. To determine if successful OIT for children with cow's milk allergy is associated with increases in milk-specific regulatory B cells.
2. To determine if successful OIT in children with cow's milk allergy is associated with increases in milk- specific IgA and IgG4 and decreases in milk-specific IgE.

3.3 Secondary Objectives

To determine which type of regulatory B cells plays a role in our population of children undergoing milk OIT.

4.0 Study Methodology

4.1 Study Design

Our laboratory is the lead center on a Canada-wide clinical trial of oral immunotherapy in cow milk sensitive individuals aged 6-20 years. The recruitment intended at least 34 children randomized to treatment (17) or observational control (17), for adequate power of the study. To date, 55 subjects have been recruited from Montreal Children's Hospital, and recruitment is ongoing at Sainte-Justine Hospital, Chicoutimi, BC Children's Hospital, and Sick Kids Hospital.

4.1.1 Milk Oral Immunotherapy Clinical Trial

This was a randomized control study with a cross-over design involving 5 Canadian centers. Subjects between 6 to 22 years of age, diagnosed with IgE-mediated cow's milk allergy using strict skin testing, serological, and challenge criteria were recruited for this study. They were matched by age and sex and randomized to either undergo oral immunotherapy or to be followed as natural history controls. The control group were offered to participate in the OIT therapy protocol at the completion of the one-year observation period. The OIT subjects initiated therapy with a 2-day rush desensitization treatment using oral doses of milk in our Centre for Innovative Medicine unit (Appendix 1). They continued the achieved dose of milk at home for two weeks. Subsequently, they returned for weekly increases in doses until a maximum of 200 ml of milk was ingested daily (Appendix 1). The primary clinical outcome was a comparison of

the amount of milk consumed safely on oral challenge performed prior to OIT, when the OIT dose had reached its maximum (200 ml or highest tolerated dose) and after one year of therapy. Blood samples to study immunological parameters such as changes in milk-specific IgE, IgG4 and IgA, and regulatory B-cells and regulatory T-cells were collected at defined time points. These time points included the first visit (baseline), 6ml, 25ml, 125ml and 200ml during the escalation phase, 300ml challenge, then at 1 month, 3 months, 6 months, 9 months, 12 months, and every 6-12 months thereafter of maintenance milk OIT. The immunological parameters were also analyzed in the observational control subjects as well as a group of healthy controls without cow's milk allergy.

4.1.2 Sample Preparation and Storage

15-20 ml of blood was drawn into heparinized tubes (green stopper) and 5-10 ml in red stopper tubes. Saliva was also collected later during the trial from subjects recruited at Montreal Children's Hospital. Serum was separated by centrifugation at 3000 rpm for 10 minutes and frozen at -80°C in 500ul aliquots until processing for milk-specific IgE, IgA and IgG4 levels. Saliva was directly aliquoted without additional processing in 500ul samples and frozen at -80°C until processing for milk-specific IgA and IgG4 levels. Peripheral blood mononuclear cells, including T and B cells, were isolated by Ficoll-Hypaque centrifugation. They were resuspended at a concentration of 5 x 10^6/ ml, and frozen in 90% FBS + 10% DMSO medium in cryotubes and stored in liquid nitrogen until use for measurement of Breg. Treg studies are also ongoing in the laboratory of our co-investigator Dr. C Piccirillo.

4.1.3 Measurement of Regulatory B Cells

Peripheral blood mononuclear cells (PBMCs), which contain both T and B cells, were stimulated via a combination of cytokine and mitogens that enhance the induction of IL-10^+ B-cells. These mitogens include CpG-B (1ug/ml, Cedarlane ODN 2006) with anti-CD40 (1ug/ml) and anti-IgM/G (10ug/ml, Cedarlane F(ab')^2), or anti-CD40 (1ug/ml) with IL-4 (200U/ml, R&D systems) and IL-21 (50ng/ml, Peprotech), which increase B-cell production of IL-10.[53] CpG-B, a TLR 9 ligand, is a known stimulator of IL-10- producing B cells, anti-IgM/IgG activates the B cell receptor, and anti-CD40, IL4, IL21 are used for plasma cell and memory B cell induction.[107,108] The isolated PBMCs were cultured at 10^6/ml/well in a 12-well non-tissue culture treated plate in medium alone, in medium with the above activation conditions, in medium with the above activation conditions plus 100ug/ml casein (Sigma C3400, Toronto, Ontario), 100ug/ml α-lactalabumin (Sigma L6010, Toronto, Ontario), and 100ug/ml β-lactoglobulin (Sigma L3908, Toronto, Ontario), and finally in 100ug/ml of casein, α-lactalabumin, β-lactoglobulin alone (Appendix 2).[31] Initial studies were done to optimize the combination of mitogens and milk proteins to determine optimal IL-10 production with minimal cell toxicity. Following 72 hours of incubation,[10,15] the cells were stained with fluorescent antibodies for CD19 (PE-Cy7, BioLegend), CD27 (efluor 450, eBioscience), CD24 (APC, BioLegend), CD38 (PE Dazzle 594, BioLegend), CD5 (PerCP, BioLegend), PD-L1 (FITC, BD Biosciences), and intracellular IL-10 (PE, BD Biosciences), viability dye (Zombie aqua, BioLegend). Milk protein-induced Breg populations were determined by flow cytometry. Controls included medium alone as a negative control, along with cells from non-milk-allergic controls. The Breg were expressed as a percent of the total $CD19^+$ B cells, and the changes in Breg in response to culture were followed over time, from baseline to the 200ml dose time point.

4.1.4 Measurement of TNF-α and IL-10

Culture supernatant at 24 hours was analyzed for TNF-α (BioLegend), and at 72 hours for IL-10 (BioLegend) by enzyme-linked immunosorbent assay (ELISA). PBMCs from 9 patients were studied at baseline and 200ml time point, along with 3 non-allergic controls and 3 allergic controls not receiving milk OIT.

4.1.5 Measurement of Milk-Specific IgE, IgG4, and IgA

Milk- specific IgE, IgG4, and IgA in the serum were measured by ELISA at baseline, 200ml time point (end of escalation phase), and 9 Months Post OIT (maintenance phase). Milk-specific IgG4 in the saliva was measured by ELISA at baseline and at the 200ml time point. Serum IgE was measured using a goat anti-human capture antibody (Bethyl A80-108A) and biotin-conjugated goat anti-human detection antibody (Bethyl A80-108B). Serum IgA was measured using goat anti-human capture antibody (Bethyl A80-102A) and goat anti-human detection antibody (Bethyl A80-102P). Serum and salivary IgG4 were measured with the ebioscience human IgG4 ELISA kit (50-112-8781). Casein was dissolved in 1M NaOH at a concentration of 25mg/ml on a shaker for 4 hours. α-lactalbumin and β-lactoglobulin were dissolved in ddH_2O at a concentration of 25mg/ml. Casein, α-lactalbumin and β-lactoglobulin were coated at a concentration of 20ug/ml in coating buffer in wells and incubated at 4°C overnight.

4.2 Ethics Approval

All appropriate ethics reviews and approvals were obtained before beginning this study.

The study was approved by the McGill University Health Centre (MUHC) Research Ethics Board, the University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board and the University of Toronto/SickKids Research Ethics Board.

4.3 Ethical Considerations

In the consent form given at study enrolment, participants were advised that they will be randomly assigned to either oral immunotherapy or observation for a one-year period. They were advised that blood samples will be collected at various visits. Participants were aware that their data will be shared only among the OIT study team. Participants in the observation control group who did not receive OIT treatment, were told that they may undergo such therapy after a year of observation and a positive oral food challenge.

4.4. Statistical Analysis

Statistical analysis was performed using Wilcoxon matched-pairs signed rank test when comparing data at 2 time points and 2way ANOVA with Tukey's multiple test for multiple comparisons. Calcuations were done on the GraphPad Prism 7.0 software (GraphPad Software, Inc. San Diego, CA, USA). A p value of <0.05 was considered significant.

5.0 Results

5.1 Clinical Data

From 2013 to 2017, 48 participants were enrolled from MCH; 4 of which did not qualify

due to a negative baseline challenge (Appendix 3). Twenty-three subjects were randomized to the treatment group, 21 subjects to the control group, and after 1 year of observation 12 of the control subjects received milk OIT. Overall, 18 subjects successfully reached the end of escalation phase, while 10 withdrew from the study (6 from the treatment group and 4 from the control group.

5.2 **Milk-Specific Serum IgE, IgA, and IgG4**

Casein-, α-lactalbumin-, β-lactoglobulin- specific serum IgE, IgA, and IgG4 were measured in 11 subjects at baseline and 200ml, and in 6 of the 11 subjects at 9 months maintenance dose after OIT (9M Post OIT) (Figures 1 to 3). At the time these experiments were performed, adequate serum samples were available at baseline and 200ml in only 11 subjects, and at 9M Post OIT in 6 subjects.

5.2.1 Serum IgE

There was a decreasing trend in casein-specific serum IgE from baseline to 200ml (mean difference -12.7ng/ml, 95% CI -24.6, -0.7ng/ml, p= 0.05) and a trend to decrease at 9M Post OIT compared to 200ml (mean difference -11.4ng/ml, 95% CI -14.2, 36.5ng/ml), but not significantly (Figure 1). There were no statistically significant changes in α-lactalbumin- and β-lactoglobulin-specific serum IgE from baseline to 200ml to 9M Post OIT (Appendix 4).

5.2.2 Serum IgA

Casein-specific IgA decreased significantly from baseline to 200ml (mean difference -22.6ng/ml, 95% CI -44.9, -0.4ng/ml, p 0.008, and did not significantly decrease at 9M Post OIT

compared to 200ml (mean difference -1.4ng/ml, 95% CI -9.7, 12.6) (Figure 2). There were no statistically significant changes in α-lactalbumin- and β-lactoglobulin-specific serum IgA from baseline to 200ml to 9M Post OIT (Appendix 5).

5.2.3 Serum IgG4

Serum IgG4 increased significantly from baseline to 200ml and from 200ml to 9M Post OIT for all three milk proteins (Figure 3). Casein-specific IgG4 increased significantly from baseline to 200ml (mean difference 972ug/ml, 95% CI 19-1942ug/ml, p 0.049), from baseline to 9M Post OIT (mean difference 2813ug/ml, 95% CI 786-4840ug/ml, p 0.0009), and from 200ml to 9M Post OIT (mean difference 2009ug/ml, 95% CI 376-3234ug/ml, p 0.009).

α-lactalbumin-specific IgG4 increased significantly from baseline to 200ml (mean difference 1275ug/ml, 95% CI 285-2266ug/ml, p 0.014), from baseline to 9M Post OIT (mean difference 2933ug/ml, 95% CI 1277-4588ug/ml, p 0.002), and from 200ml to 9M Post OIT (mean difference 1763ug/ml, 95% CI 107-3419ug/ml, p 0.037).

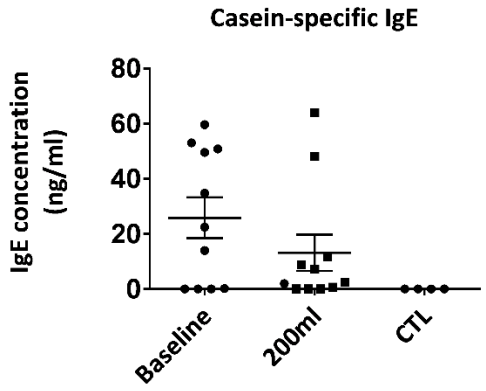
β-lactoglobulin-specific IgG4 increased significantly from baseline to 200 ml (mean difference 1510ug/ml, 95% CI 646-2375ug/ml, p 0.004), from baseline to 9M Post OIT (mean difference 2931ug/ml, 95% CI 1520-4342ug/ml, p 0.0005), and from 200ml to 9M Post OIT (mean difference 1612ug/ml, 95% CI 201-3023ug/ml, p 0.026).

5.2.4. Non-Allergic Controls

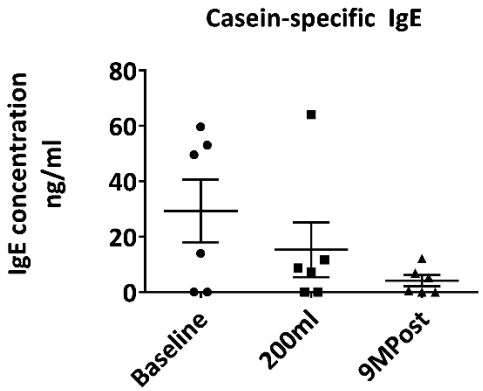
The casein-, α-lactalbumin-, and β-lactoglobulin- specific serum IgE of 4 non-milk-allergic controls were 0ng/ml. The casein-, α-lactalbumin-, and β-lactoglobulin- specific serum IgA of 3 non- milk-allergic controls ranged from 2.3-12.3ng/ml, 1.3-17.9ng/ml, 4.2-9.7ng/ml,

respectively. The casein-, α-lactalbumin, and β -lactoglobulin- specific serum IgG4 of 3 healthy non-milk-allergic controls ranged from 15.4-1677.4ug/ml, 95.3- 445.6ug/ml, and 18.3-1188.5ug/ml.

Figure 1. Casein-specific serum IgE at baseline, 200ml, and 9 Months Post OIT time-points. Error bars represent the mean and SEM. P-value NS (non-significant).

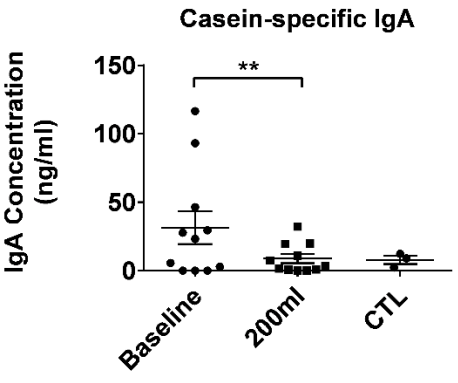


N=11

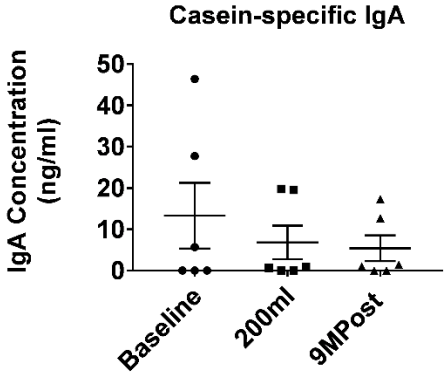


N=6

Figure 2. Casein-specific serum IgA at baseline, 200ml, and 9 Months Post OIT time-points. ** p= 0.008



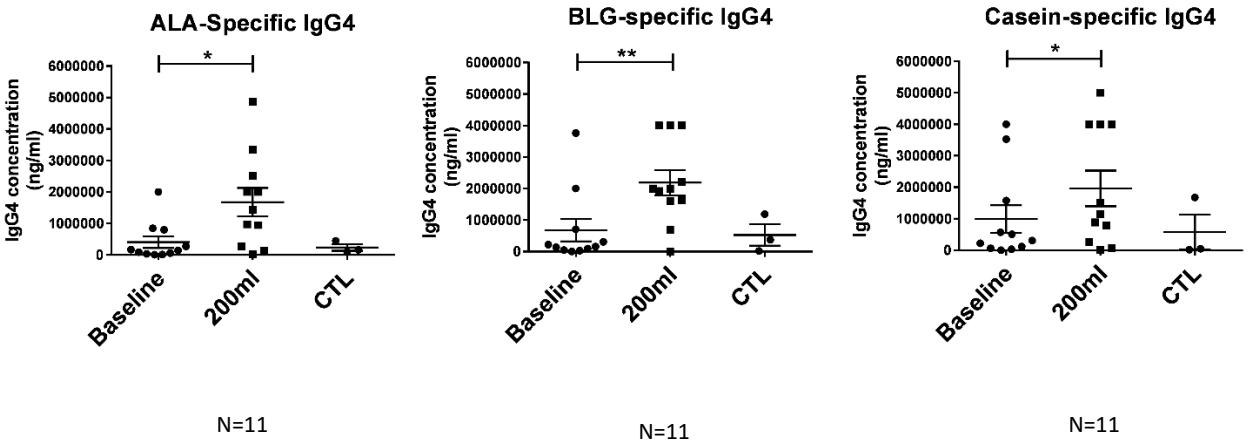
N=11



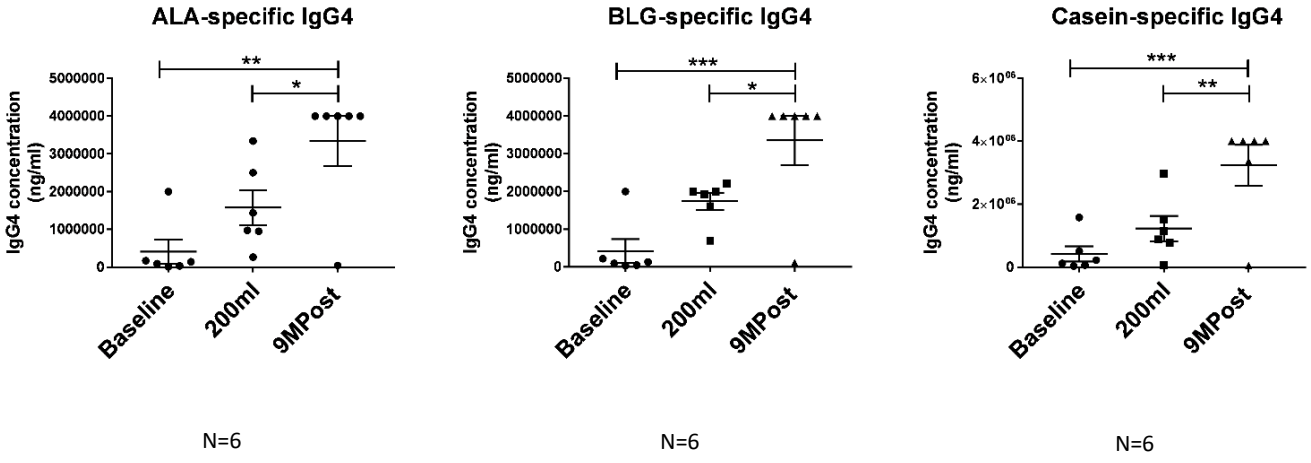
N=6

Figure 3. Milk-specific serum IgG4 at baseline, 200ml, and 9 Months Post OIT time-points. **A.** Serum ALA, BLG, casein-specific IgG4 at baseline and 200ml for 11 patients and 3 non-allergic controls. **B.** Serum ALA, BLG, casein-specific IgG4 at baseline, 200ml, and 9 Months Post OIT for 6 patients. * p< 0.05, ** p<0.01, *** p<0.001.

A.



B.



5.2.5 Serum IgG4/IgE and IgA/IgE Ratio

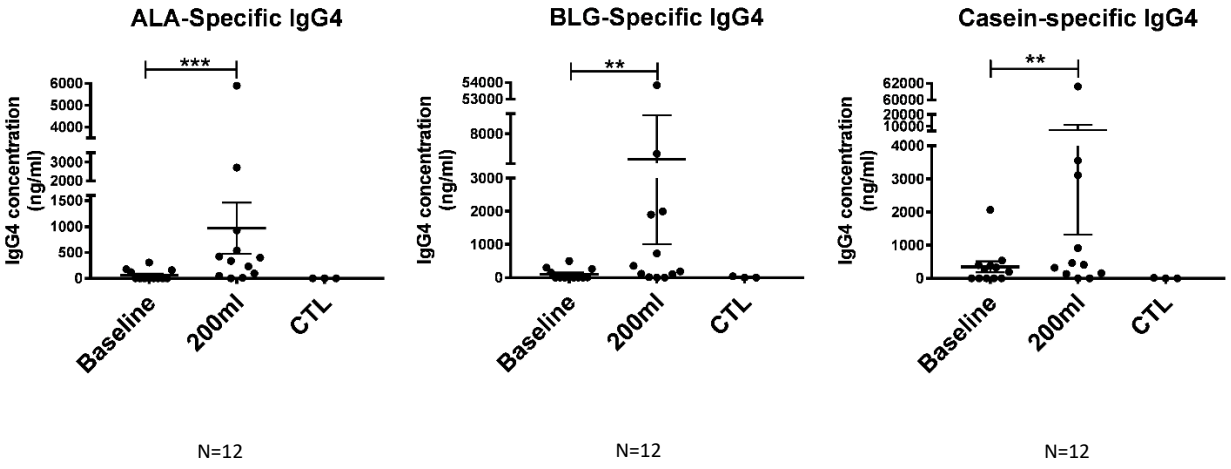
The serum ratio of IgG4 to IgE was significantly increased for β-lactoglobulin at the 200ml time point compared to baseline (p= 0.005) (Appendix 6). The serum ratio of IgG4 to IgE was significantly increased for β-lactoglobulin and casein at 9 Months Post compared to baseline (p= 0.03). There was no significant difference in the α-lactalbumin-specific serum IgG4/IgE ratio at 200ml and 9 Months Post compared to baseline, nor in the serum IgA/IgE ratio for all three milk proteins at the end of escalation phase.

5.2.6 Salivary IgG4

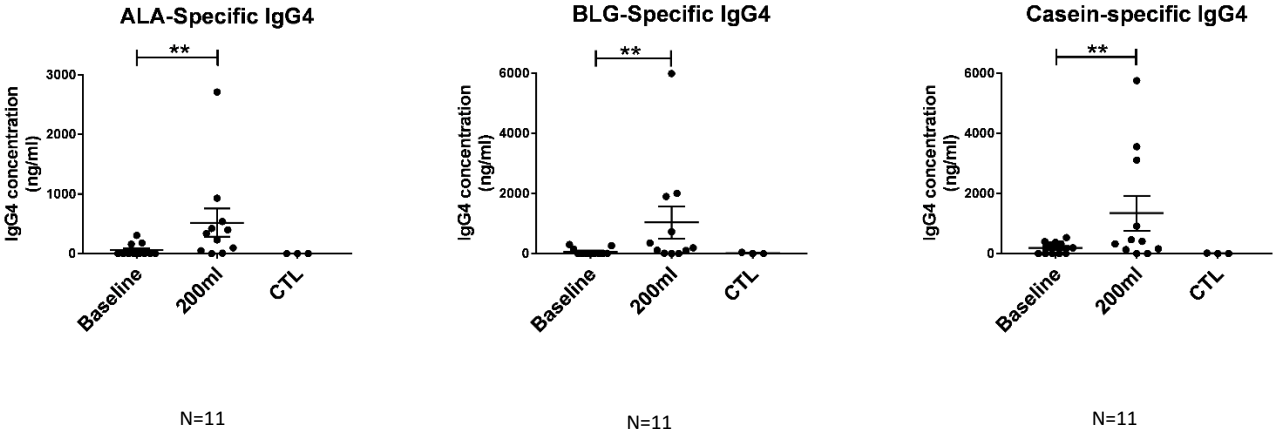
Salivary IgG4 was measured in 12 patients at baseline and the 200 ml time point, and in 3 non-allergic controls. Salivary IgG4 significantly increased from baseline to 200ml for all three milk proteins (Figure 4). The mean salivary IgG4 concentration at baseline was 63.5 ng/ml (SEM 29.7ng/ml), 102.7 ng/ml (SEM 48.5ng/ml), and 349.2 ng/ml (SEM 165.7 ng/ml) for α-lactalbumin, β-lactoglobulin, and casein respectively. The mean salivary IgG4 concentration at the 200ml dose of escalation phase was 967.4 ng/ml (SEM 496.0 ng/ml), 5435.0 ng/ml (SEM 4428.0 ng/ml), and 6369.0 ng/ml (SEM 5050 ng/ml) for α-lactalbumin, β-lactoglobulin, and casein respectively. The salivary IgG4 of 3 non-allergic controls were undetectable for all 3 milk proteins (Figure 4A).

Figure 4. Milk-specific salivary IgG4 at baseline and 200ml time-points.
A. Salivary ALA, BLG, casein-specific IgG4 at baseline and 200ml for 12 patients and 3 non-allergic controls. **B**. By removing the one outlier, the difference remains statistically significant for all three milk proteins. ** p<0.005, *** p=0.001.

A.



B.



5.3 PD-L1^{+} Breg

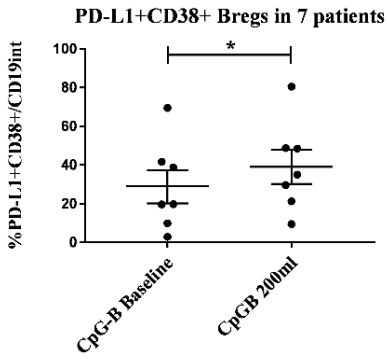
Breg were analyzed in 9 patients at baseline and end of escalation phase (200ml time point). The more recent samples with adequate PBMC counts were chosen. There was a significant increase in 7 out of 9 patients in the percentage of CD19^{int}PD-L1^{+} and CD19^{int}PD-L1^{+}CD38^{+} populations at the end of escalation phase (200ml) in 3 conditions: CpG-B/anti-IgM/IgG/anti-CD40, αCD40/IL4/IL21, and αCD40/IL4/IL21 plus milk proteins (Figure 5A). The total CD19^{int}PD-L1^{+} and CD19^{int}PD-L1^{+}CD38^{+} cells were significantly increased in the same 7 patients at the 200ml time point in the αCD40/IL4/IL21 condition only (Figure 5B). The majority (89.16%, 95% CI 81.21-95.56%) of the CD19^{int}PD-L1^{+} cell populations in all conditions were CD38^{+} cells (Figure 6). The PD-L1 population was not seen in CD19^{hi} B cells, except for one subject who also had an increase at 200ml compared to baseline in this population.

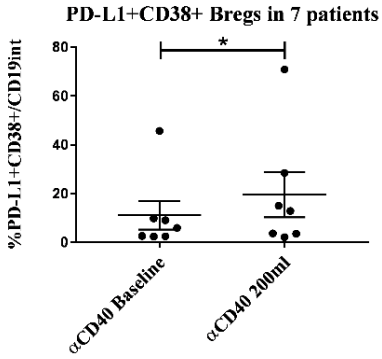
The 2 subjects demonstrating a decrease in the PD-L1 population had a higher baseline percentage in all conditions. They also ingested the highest volume of milk during their challenge compared to the 7 other patients (Table 1).

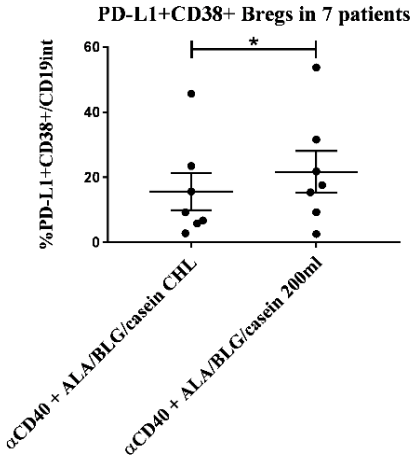
The median percentage difference between the end of escalation phase and baseline in all 9 patients was 7.1% (IQR -6.1, 10.5%), 1.22% (IQR -4.1, 13.9%), 2.72% (IQR -14.2, 4.1%) and 3.5% (IQR -13.6, 8.1%) for the CpG-B/anti-IgM/IgG/αCD40, αCD40/IL4/IL21, CpG-B/anti-IgM/IgG/αCD40 plus milk proteins, and αCD40/IL4/IL21 plus milk proteins conditions, respectively (Figure 7).

Figure 5. CD19^{int}PD-L1^{+}CD38^{+} at baseline and 200ml time points in 7 subjects.
A. The percentage of CD19^{int}PD-L1^{+}CD38^{+} in 3 conditions. **B.** The total cell number of CD19^{int}PD-L1^{+}CD38^{+} in the αCD40/IL4/IL21 condition. * p<0.05

A.







B.

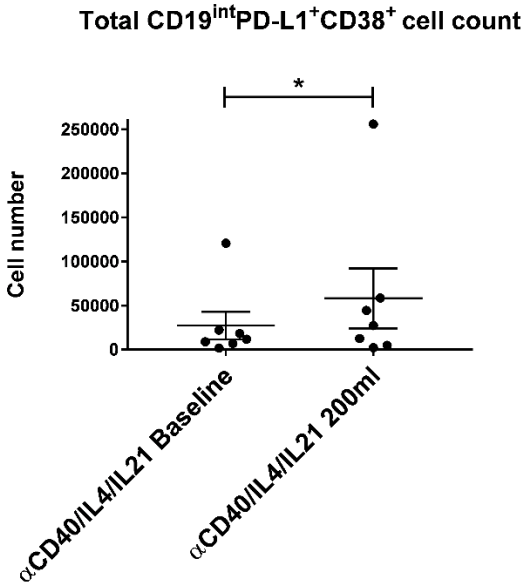
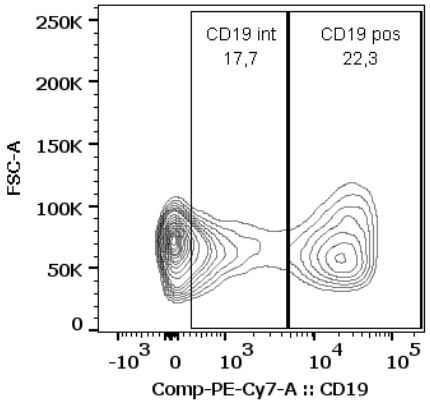
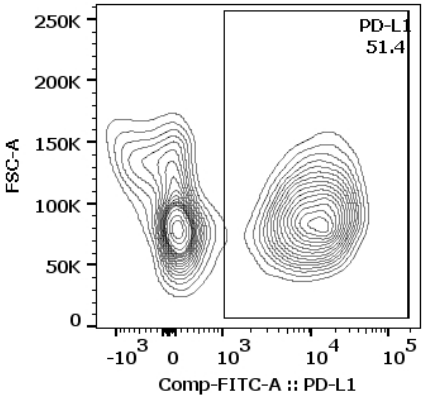


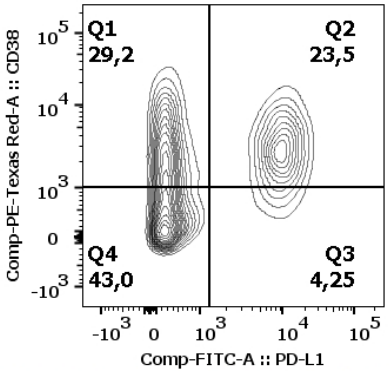
Figure 6. CD19^{int}, PD-L1^{+} and PD-L1^{+}CD38^{+} populations.
A. Gating strategy for CD19^{int} and CD19^{int}PD-L1^{+} populations. **B.** CD19^{int}PD-L1^{+} CD38^{+} population at baseline and 200ml in one subject.

A.





B. **Baseline** **200ml**



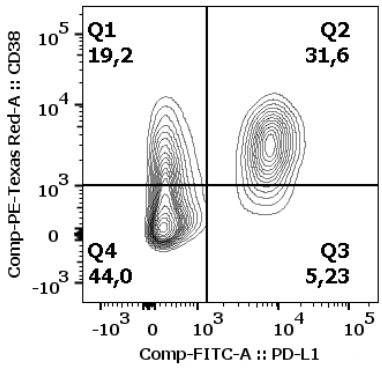
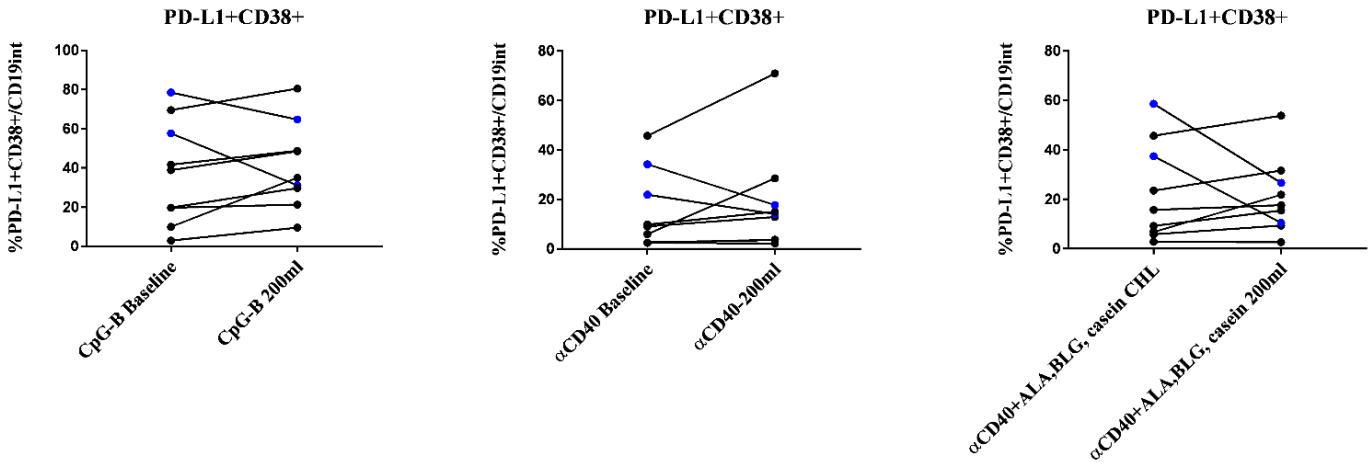


Table 1. Clinical data on 9 patients who successfully completed the escalation phase of milk OIT and 2 allergic controls and non- allergic controls. Subjects 4 and 8 (in red) are the two who showed a decrease in the PD-L1 populations. The non-allergic controls had a negative challenge to milk.

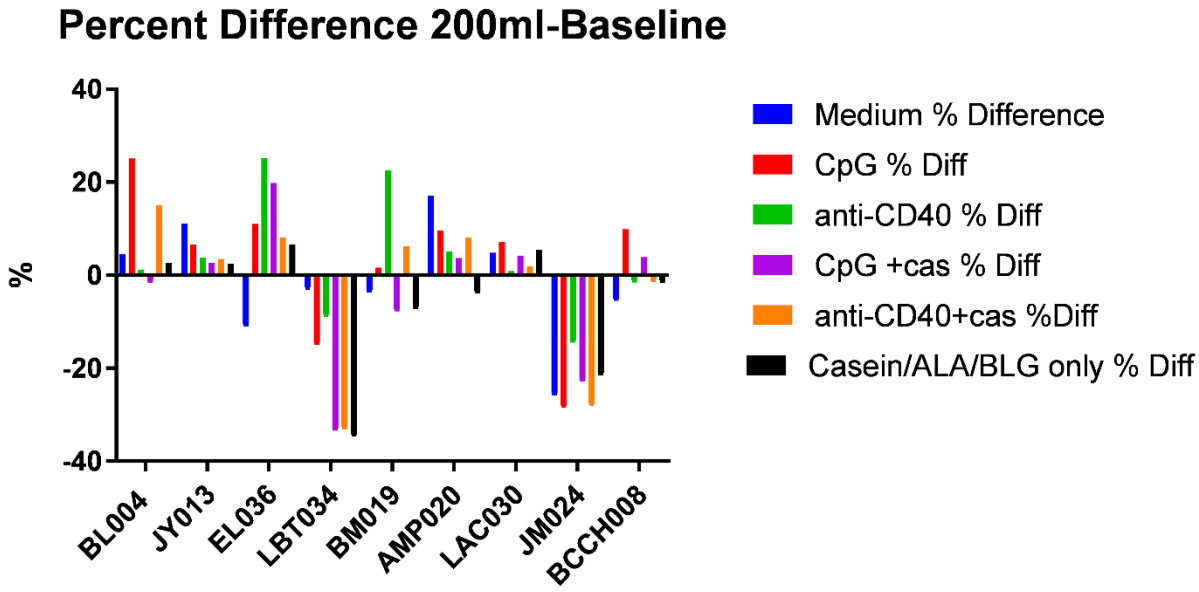
Subject	Gender	Age (years)	Duration of escalation phase (weeks)	Cumulative volume of milk at challenge (ml)	Baseline $CD19^{int}$PD-L1CD38 CpG/antiIgM/G/αCD40 (%)	Baseline $CD19^{int}$PD-L1CD38 αCD40/IL4/IL21 (%)
1	M	19	19.6	44.4	9.93	2.46
2	F	8	26.2	0.1	2.92	9.11
3	M	6.5	28	0.1	69.5	45.7
4	F	14	29.1	89.4	78.5	21.9
5	M	17	20.1	14.4	19.7	5.99
6	F	16.5	19.5	44.4	38.9	9.81
7	F	12	72.1	1.4	41.7	2.65
8	M	16	23.3	149.4	54.8	28.4
9	F	9	29.2	14.4	19.8	2.5
Mean	-	13.1	29.7	38.9	37.31	14.3
Median (95% CI)	-	14 (8, 17)	26.2 (19.6, 29.2)	14.4 (0.1, 89.4)	38.9 (9.9, 69.5)	9.1 (2.5, 28.4)
Allergic CTL 1	F	6	-	44.4	21.7	13.6
Allergic CTL 2	F	7	-	14.4	19.5	5.4
Non-allergic CTL 1	F	15	-	149.4	83.1	51
Non-allergic CTL 2	M	9	-	149.4	92.5	68.5
Non-allergic CTL 3	F	15	-	149.4	2.7	2.7

Figure 7. Percentage CD19^{int}PD-L1^{+}CD38^{+} at baseline and 200ml time points.
A. The percent change of CD19^{int}PD-L1^{+}CD38^{+} in 9 patients in 3 conditions: CpG-B/anti-IgM/IgG/anti-CD40, anti-CD40/IL4/IL21, and anti-CD40/IL4/IL21 plus ALA, BLG, casein. 2 patients with decreasing percentage in blue. **B**. The percent difference in CD19^{int}PD-L1^{+}CD38^{+} in 9 patients in all conditions at 200ml compared to baseline. P-value NS.

A.



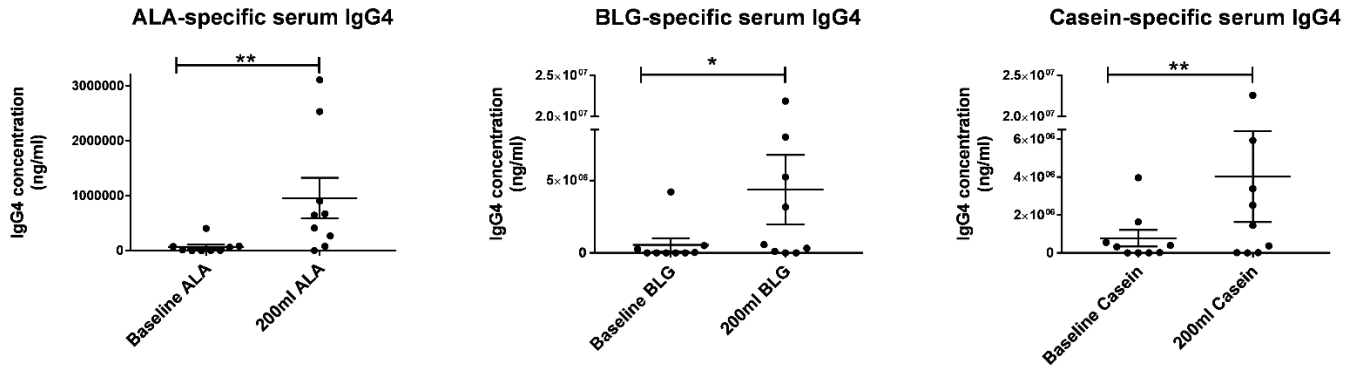
B.



5.3.1 Serum IgG4

Serum IgG4 to α-lactalbumin, β-lactoglobulin, and casein was measured in these 9 patients at baseline and 200ml. There was a significant increase in IgG4 for all three milk proteins (Figure 8).

Figure 8. Serum IgG4 to three milk proteins at baseline and 200ml in 9 patients.
* p <0.05, ** p< 0.01.



5.4 IL-10 Producing Breg

A significant difference in the IL-10 positive B cell populations was not seen in 8 patients at baseline and 200ml in the six conditions (Figure 9). The populations with IL-10 production were $CD19^+$ (Figure 10A), $CD19^+CD27^+$, $CD19^+CD38^+$, $CD19^+CD27^+CD38^+$, and $CD19^{int}CD38^+$ cells. The total IL-10 producing $CD19^+$ Breg cell count did not significantly increase at 200ml compared to baseline for all six conditions (Figure 10B).

Similarly, significant differences were not found in the IL-10 concentration in supernatants at 72 hours and the TNF-α concentration at 24 hours in all six conditions in 9

patients (Figures 11 and 12). The IL-10/ TNF-α ratio did not show a significant difference between baseline and the 200ml time point (Figure 13).

One of the 2 subjects who had PD-L1 decrease at 200ml showed an increase in the percentage of IL-10 production at 200ml in most conditions and Breg subtypes, as well as an increase in total IL10-producing $CD19^+$ Breg cell count (Figure 14). The other subject showed a smaller increase in the percentage of IL-10 production and total cell number in some conditions in the $CD19^+$ Breg population only.

Figure 9. Percentage of IL-10 producing Breg cells in 4 different subtypes. P-value NS.

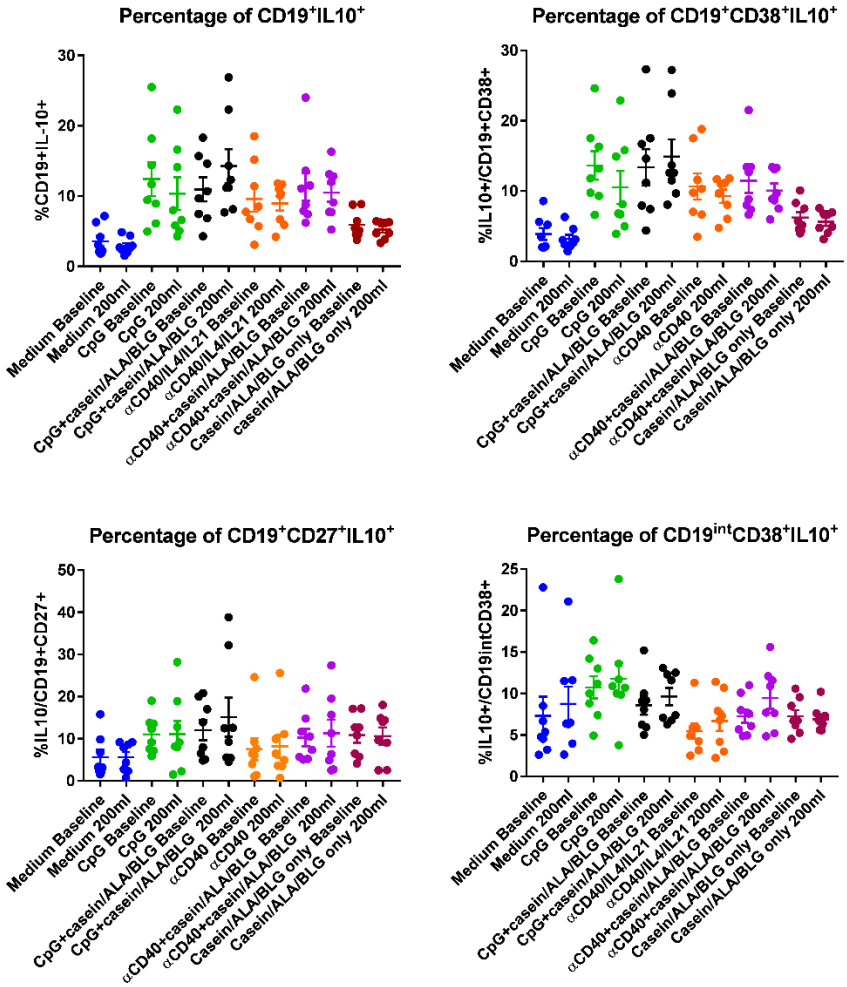
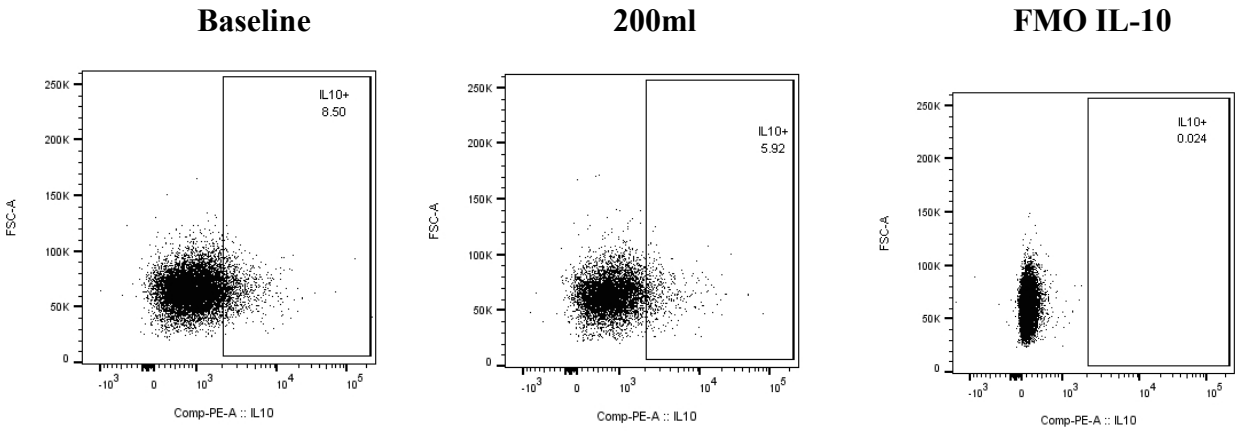


Figure 10. IL-10 population and cell count in CD19^{+} Breg.
A. IL-10 population in CD19^{+} Breg cells in the αCD40/IL4/IL21 condition at baseline and 200ml in one patient. **B.** Total IL-10 producing CD19+ Breg cell count. P-value NS.

A.



B.

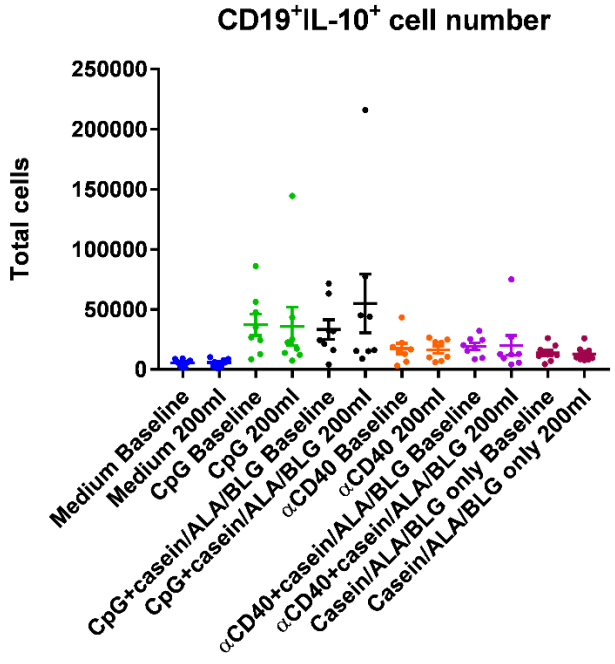


Figure 11. IL-10 concentration in supernatant at baseline and 200 ml at 72 hours. N=9. P-value NS.

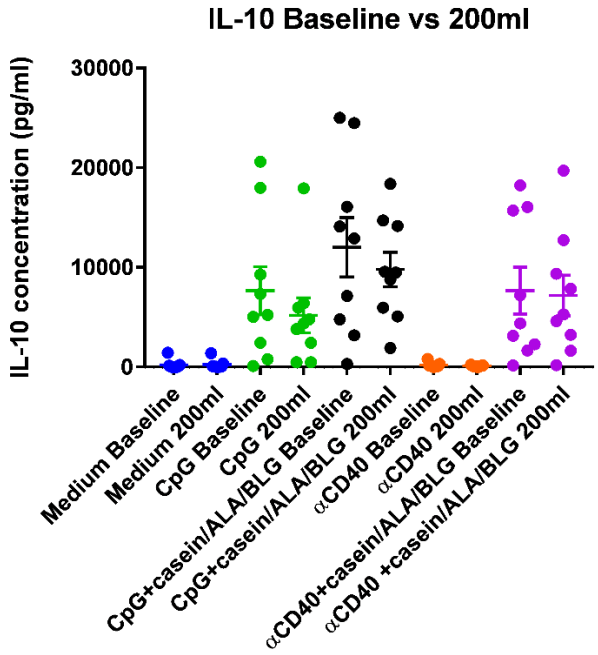


Figure 12. TNFα concentration in supernatant at baseline and 200ml at 24 hours. N=9. P-value NS.

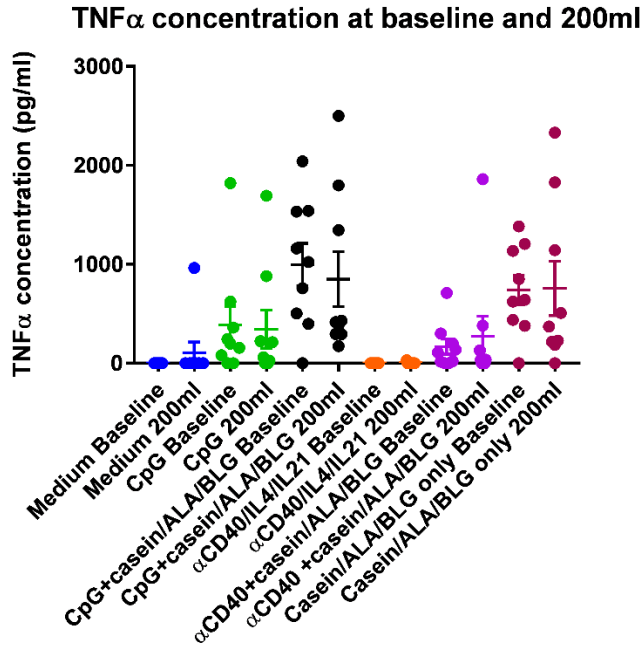


Figure 13. IL-10/TNFα ratio at baseline and 200ml. Ratio presented in 4 conditions in 9 patients. Two non-allergic controls and 3 allergic controls results shown for CpG/anti-IgM/G/αCD40 (purple).

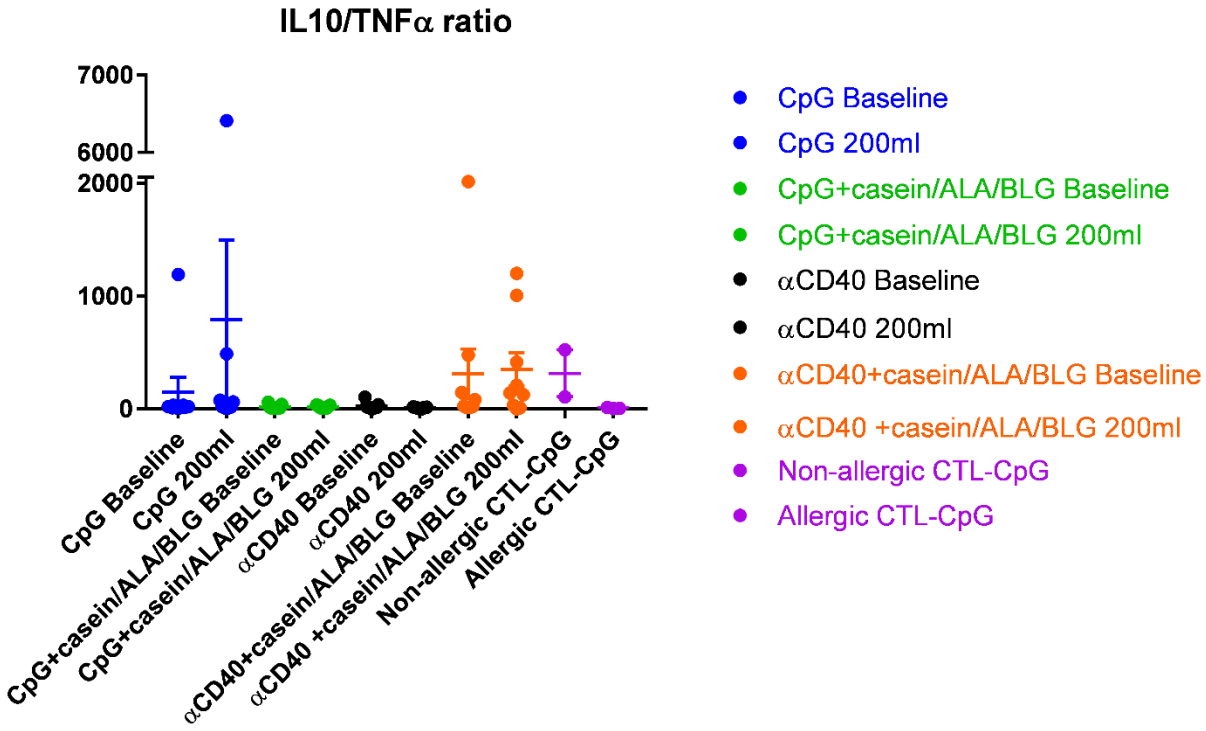
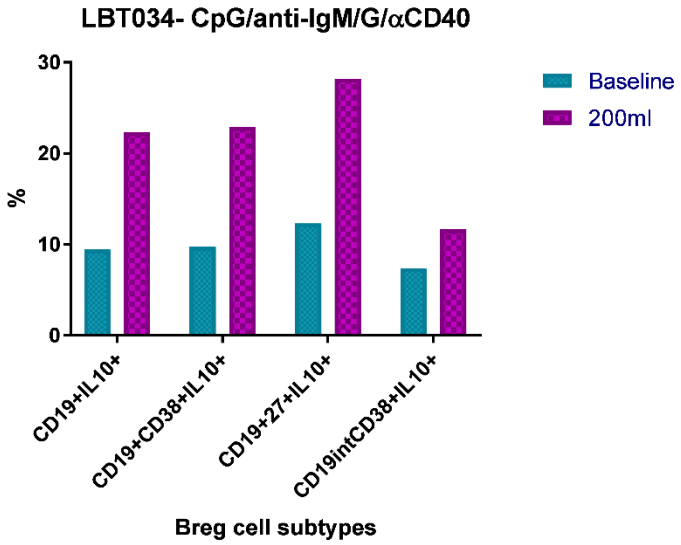
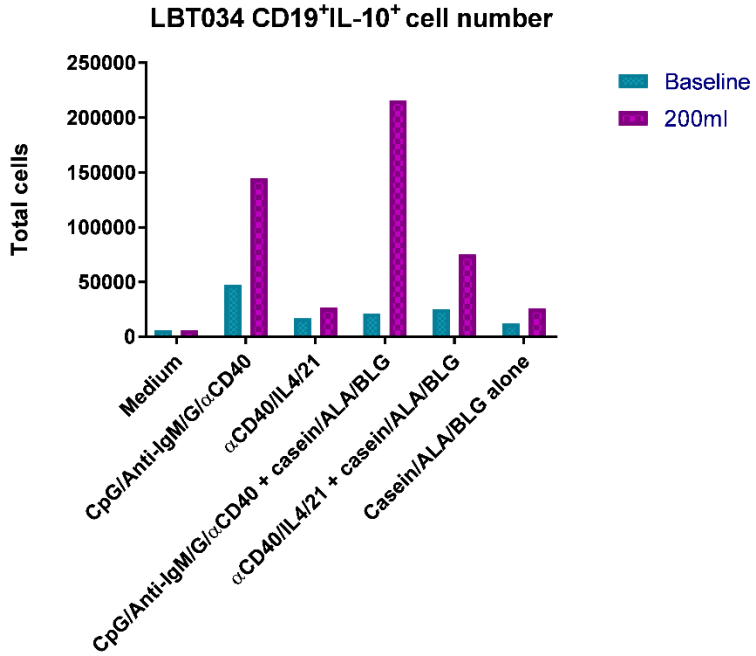


Figure 14. IL-10 percentage and cell count at baseline and 200ml in one patient. A. Subject LBT034 IL-10 percentage in 4 Breg cell subtypes for condition CpG/Anti-IgM/G/αCD40. **B.** Total cell number of IL-10 producing $CD19^{+}$ Breg cells in all conditions for LBT034.

A.



B.



6.0 Discussion

We have conducted the first study assessing the role of regulatory B cells in children with IgE-mediated cow's milk allergy undergoing milk OIT. Our study is the first to demonstrate the presence of PD-L1^{+} Breg in food allergy and OIT. Successful desensitization was associated with an increase in PD-L1^{+} Breg in a subset of children studied. However, IL-10 producing Breg were not increased in our population. We found that children who successfully reached the end of escalation phase of milk OIT, at 200ml, had a significant increase in serum and salivary IgG4 to α-lactalbumin, β-lactoglobulin, and casein. Interestingly, we showed a significant decrease in casein-specific serum IgA at 200ml compared to baseline, and a decreasing trend for casein-specific serum IgE at 200ml. The IgG4/IgE ratio was significantly increased for β-lactoglobulin at the end of escalation phase and for both casein and β-lactoglobulin at 9M Post OIT.

In 7/9 (77.8%) of subjects, PD-L1^{+} Breg increased at 200ml in the CD19^{int} population, of which the majority was CD38^{+}. This is the first time PD-L1^{+} Breg have been assessed in the context of food OIT. These cells may be transitioning B cells or plasmablasts, given their lower CD19 surface expression and their CD38 expression.[61,109] Khan et al showed their PD-L1^{+} B cells were resistant to rituximab, an anti-CD20 antibody, which suggests they were plasma cells.[12-13,110] During B cell differentiation and maturation, CD38 is highly expressed on germinal centre B cells and plasmablasts; the latter also expressing lower BCR levels in tonsils and lymph nodes as they progress towards end stage differentiation to become plasma cells.[110] Tissue plasma cells express varying levels of CD19 and no longer express CD20 and CD22.[110] Long-lived plasma cells home to the bone marrow or GALT, where they secrete large quantities of high affinity isotype-switched immunoglobulins.[111] The population of Breg in our study could be plasmablasts given the intermediate expression of CD19 and high CD38.

The regulatory mechanism of the PD-1/PD-L1 pathway has been extensively studied and targeted in clinical oncology with successful treatment of advanced cancers and even hematologic malignancies.[112-113] Under physiologic conditions, PD-1 expressed on activated T cells inhibits further signaling when ligated with PD-L1 expressed on antigen presenting cells, including B cells.[112] This inhibitory mechanism helps with tolerance (to self and innocuous antigens) and prevents overactivation during an infection.[112] This immune checkpoint is exploited by cancer cells to escape recognition and clearance by the immune system. In Hodgkin Lymphoma, for example, malignant B cells express PD-L1, as well as PD-1, while tumor-infiltrating lymphocytes express PD-1 on their surface.[112-113] Blocking PD-1 with a monoclonal antibody used as treatment of certain cancers, will allow the activation of T cells in the microenvironment and successful tumour clearance.[114] Outside of cancer immunology, a recent study in the field of allergy in humans showed IL-10 producing B cells with PD-L1 expression on their surface when co-cultured with $CD40L^+$ ILC3s.[115] If our population of $PD\text{-}L1^+$ Breg are plasmablasts making their way towards the bone marrow or GALT, they could act as regulators of other B cells expressing PD-1. If they are transitioning B cells, they may play an inhibitory role in the germinal centre of lymph nodes via PD-L1, where they are surrounded by follicular T_H cells, antigen presenting cells, and other B cells expressing PD-1.
These potential mechanisms of inhibition by PD-L1 in milk OIT need to be investigated in future studies.

2 subjects did not demonstrate an increase in the PD-L1 population. They had higher baseline percentage and were able to ingest a higher cumulative volume of milk at challenge compared to the median of the group (Table 1). In addition, both were older than the average age

of this cohort. A larger sample size is needed to determine if these clinical observations have a real effect in desensitization and Breg.

The IL-10 producing Breg did not increase significantly in this sample population, however they may with a larger sample size, and could be significant players in the desensitization process. Interestingly, one of the 2 subjects who did not show an increase in PD-L1^{+} Breg had an increase in IL-10^{+} Breg in most of the conditions and subtypes (Figure 14). It is possible that both types of Breg have complementary functions in the development of desensitization and hence it is sufficient to have one of these increases to achieve desensitization successfully. The timing of the highest proportion of these cells can also differ and may not be at the end of escalation phase, but later during maintenance phase, as seen with the increasing serum IgG4 concentration, which requires IL-10 for class-switching.[77] Furthermore, Breg, like Treg [27] may likely be situated in the intestinal tract rather than the peripheral blood and perhaps their numbers differ dramatically in the gut.[26]

The increase in serum IgG4 to α-lactalbumin, β-lactoglobulin, and casein at the end of escalation phase is consistent with other studies,[99] while serum IgG4 at 9 months of maintenance phase has not been rigorously assessed by other studies. Our finding of salivary IgG4 has never been reported in allergic diseases or OIT to our knowledge. Our findings suggest that salivary IgG4 may be used as an additional non-invasive tool with serum IgG4 in assessing patients undergoing OIT. Contrary to our hypothesis, casein-specific serum IgA decreased significantly at the end of escalation phase. This finding was also seen in two studies; one in egg OIT and the other in bee venom immunotherapy.[34,95] It may be that serum IgA increases very early during OIT, perhaps even as early as day 1 of the rush phase, as shown by Sugimoto et al.[95] Casein-specific serum IgE showed a decreasing trend at 200ml and 9M Post, while the IgG4/IgE ratio

significantly increased for β-lactoglobulin at the end of escalation phase and for both casein and β-lactoglobulin at 9M Post OIT. Some studies in milk OIT did not find a significant change in serum IgE, while another found a decrease only in whole CM and not in casein or β-lactoglobulin-specific IgE.[91,116] Our findings support the neutralizing role of IgG4 in regard to total number, however its inhibitory function may also play a role. The impact of its inhibitory role is currently not known in our population. Whether the ratio of IgG4 to IgE is more important [117] than the total concentration of milk-specific IgG4 is difficult to interpret. Our results show a significant increase of IgG4 for all three milk proteins, but only a significant increase in the ratio of IgG4 to IgE for β-lactoglobulin at the end of escalation and for casein and β-lactoglobulin at 9M Post OIT.

Nine months into maintenance phase of OIT, the serum IgG4 continued to increase significantly for all 3 milk proteins, while casein-specific IgE continued to show a trend to decrease and casein-specific serum IgA was unchanged. This suggests that the immune system is still actively changing during this phase, therefore OIT should not be stopped to assess sustained unresponsiveness as it will most likely halt the process. Long-term follow up is needed to assess the time when serum and salivary IgG4 concentrations will plateau and when they will decrease, which may be the critical switch from desensitization to immune tolerance. As shown with non-allergic controls, the level of IgG4 in the serum and saliva are very low or undetectable, as is serum IgE and IgA.

Our study was limited by the small number of subjects in our serum, saliva, and cellular data. At the time of the ELISA experiments, adequate serum samples were available in only 11 subjects who reached the end of escalation phase. And after a long process of optimizing the

cellular experiments, I was able to perform them on 9 subject samples who completed the end of escalation phase with the necessary number of cells at baseline and 200ml, along with allergic and non-allergic controls. An increase in number of samples may show significant differences not currently found with serum IgE, PD-L1^{+} Breg and IL-10-producing Breg, as humans demonstrate a large variation and our sample size did not show a normal distribution. In addition, we assessed the number and concentration of antibodies and Breg, but not their function. This should be assessed in future experiments. The supernatant analysis of IL-10 and TNFα were not B cell-specific as they included all PBMCs. The IL-10 production by B cells might play a significant part locally, although their numbers in the peripheral circulation were not significantly changed. Our study analyzed peripheral blood as it is less invasive than intestinal biopsies. Additionally, other cells could play an important role, such as dendritic cells, and should be included in future studies.

7.0 Conclusion

This is the first study assessing Breg in milk OIT in children. Successful escalation phase was associated with a decrease in casein-specific serum IgA and an increase in serum IgG4 and salivary IgG4 to all three milk proteins, which further continued to increase at 9 months post OIT. A population of Breg positive for PD-L1 increased in a subset of children who successfully reached escalation phase. Further studies evaluating the role of this subset of Breg in milk OIT and other food OIT will enhance our understanding of the inhibitory mechanism at play. Ongoing longitudinal studies in milk OIT will help clarify the pathway of desensitization, SU, and tolerance.

8.0 References

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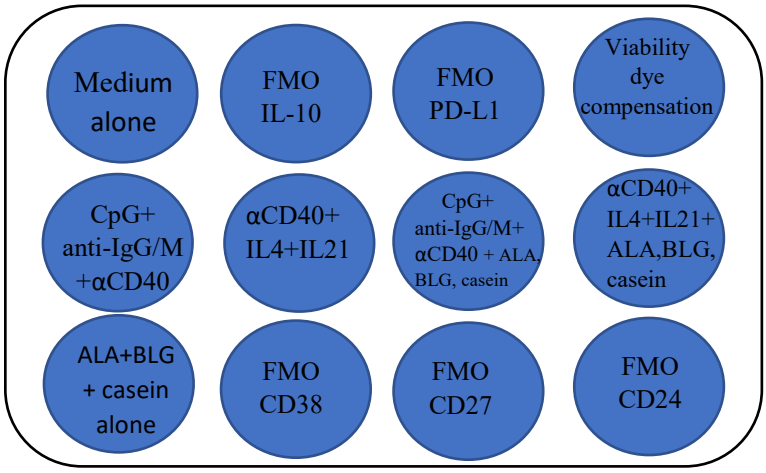
9.0 Appendix

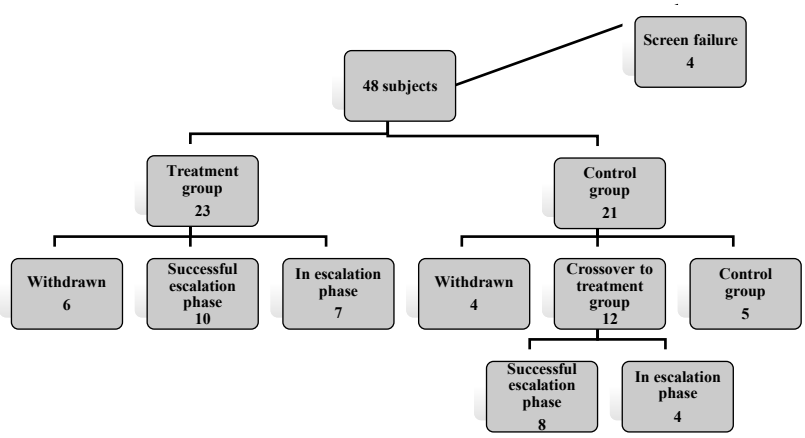
Appendix 1. Milk OIT Protocol

Study Time Point	Milk Dilution	Volume (ml)
Day 1	1/100	1
	1/100	2
	1/100	4
	1/100	8
	1/10	1.6
Day 2	1/10	1.6
	1/10	3.2
	1/10	6.4
	1/10	12
	Undiluted	2.5
Week 2	Undiluted	4
Week 3	Undiluted	6
Week 4	Undiluted	8
Week 5	Undiluted	10
Week 6	Undiluted	12
Week 7	Undiluted	15
Week 8	Undiluted	20
Week 9	Undiluted	25
Week 10	Undiluted	30
Week 11	Undiluted	40
Week 12	Undiluted	50
Week 13	Undiluted	75
Week 14	Undiluted	100
Week 15	Undiluted	125
Week 16	Undiluted	150
Weeks 17-52	Undiluted	200

Appendix 2. Cell culture conditions in cell culture plate.

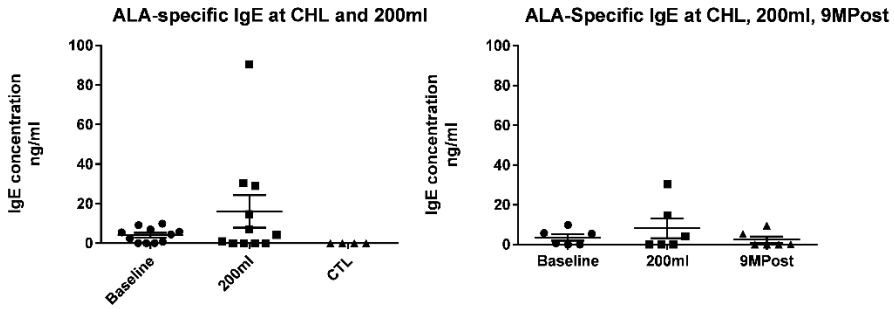


Appendix 3. Number of subjects enrolled and randomized to treatment and control group.

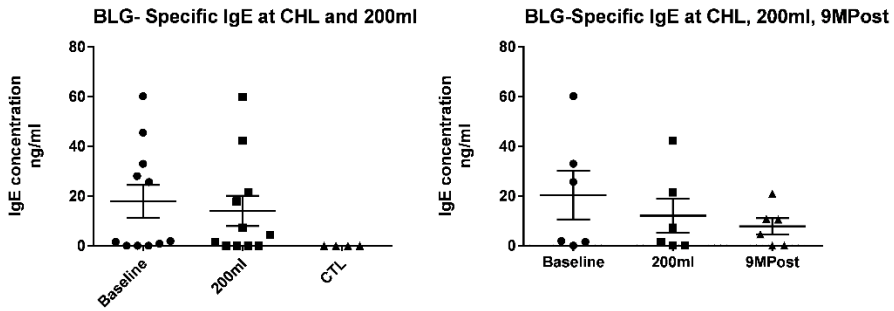


Appendix 4. ALA-specific and BLG-specific serum IgE at baseline, 200ml, and 9 Months Post OIT. **A**. ALA-specific serum IgE. **B**. BLG-specific serum IgE. P-value NS.

A.



B.

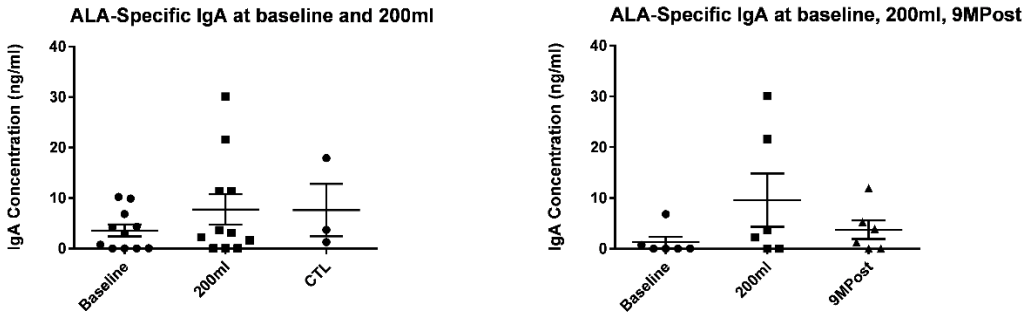


N=11

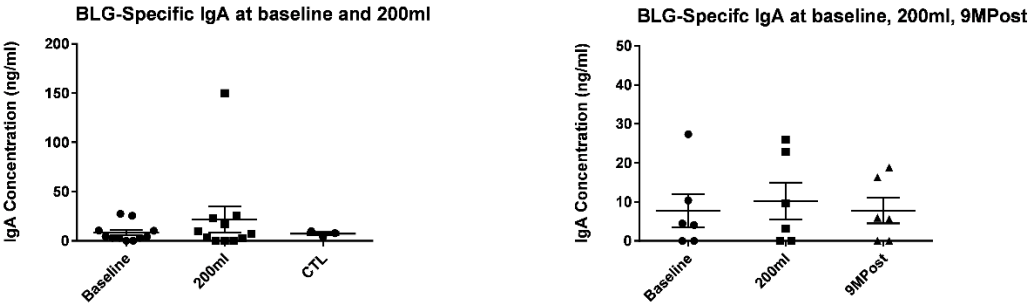
N=6

Appendix 5. ALA-specific and BLG-specific serum IgA at baseline, 200ml, and 9 Months Post OIT. **A**. ALA-specific serum IgA. **B**. BLG-specific serum IgA. P-value NS.

A.

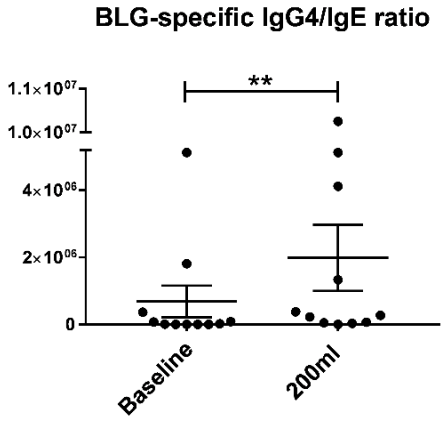


B.



N=11 N=6

Appendix 6. BLG-specific IgG4/IgE ratio at baseline and 200ml. ** p = 0.0049



N=11