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Article type : Letter to the Editor

PIPE-cloned human IgE and IgG4 antibodies: New tools for investigating cow's milk allergy and tolerance

Short title: PIPE-cloned human antibodies against bovine beta-lactoglobulin

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Keywords: antibodies, Basophil activation test (BAT), beta-lactoglobulin (BLG), Cow's milk allergy (CMA), PIPE cloning

List of Abbreviations

AIT	allergen immunotherapy
BAT	basophil activation test
BLG	beta-lactoglobulin
CM	cow's milk
CMA	cow's milk allergy
D1	name of engineered antibodies due to their published, variable regions
ELISA	enzyme-linked immunosorbent assay
FA-AIT	food allergen-immunotherapy
IL	interleukin
ISAC	Immuno-Solid phase Allergy Chip
OIT	oral immunotherapy
PIPE cloning	polymerase incomplete primer extension cloning
PBMC	peripheral blood mononuclear cells
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-T	TBS-Tween
UHT milk	ultra-high temperature processed milk

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Main text

To the Editor,

Cow's milk (CM) allergy (CMA) is defined as an immune-mediated adverse response to CM proteins. 2 - 3 % of children are suffering from CMA, but many develop natural tolerance after 3 - 4 years.[1,S10] Food allergen-immunotherapy (FA-AIT) applying increasing antigen doses (oral immunotherapy, OIT) can contribute to improvement of CMA,[S11,S12] with variable clinical efficacy[2,S13], but immunologically often resulting in decreased specific IgE levels and increased specific IgG4 levels[S14,S15]. IgG4 is i) anti-inflammatory as it does not activate the complement system; ii) bi-specific due to fab-arm exchange and, thus, it has less crosslinking capacity than IgE, and has iii) blocking capacity.[3] The interplay of IgE and IgG4 may thus be decisive for the immune balance in CMA.

Antibody of different isotypes with the same variable region could be essential in molecular studies of the mechanism underlying CMA and might shed light onto the function of specific IgG4 antibodies during the acquisition of tolerance.

Polymerase Incomplete Prime Extension (PIPE) cloning is a cutting-edge technique to engineer antibodies against various targets and facilitates simple exchange of the constant regions to increase the range of subtypes of antibodies.[4–6] In this study, we combined variable regions from the high-affinity IgE antibody "D1" [7] (KD = 1.4×10^{-9} M; $k_{on} = 1.4 \times 10^{6}$, $k_{off} = 1.54 \times 10^{-3}$)[S16] specific for the major CM allergen beta-lactoglobulin (BLG), with the constant domains of IgE or IgG4 antibodies (Figure 1A). Correct antibody assembly was confirmed by SDS-PAGE in comparison to commercial antibodies of the same isotype (Figure 1B). The exclusive specificity of D1 antibodies were proven in ELISA (Figure 1C), in ISAC112 microarray (Figure 1D and E) and in ImmunoCAP (data not shown). Antibodies were furthermore species-specific to bovine BLG when testing milk samples from various animal species and showed a sensitivity comparable to a commercial anti-BLG-ELISA (Figure S1).

We next applied our antibodies for the quantification of BLG-specific IgE and IgG4 antibodies in serum of milk allergic patients (n=9) and sensitised but tolerant patients (n=8). Allergic children had significantly-higher levels of IgE ($p < 0.01$), but no differences in the IgG4 levels were seen between the groups (Figure S2), in accordance with previous studies.[8] Our data suggest that the PIPE-cloned antibodies may be useful for a precise quantification of allergen-specific IgE and IgG4. This is advantageous, as specific IgE is usually quantified by comparing the reactivity to IgE absorbed to a solid phase, likely leading to an underestimation of specific IgE and semi-quantitative results.

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Basophil activation test (BAT) is a reliable method to evaluate functional capacities of antibodies, allowing at least an approximation to the real-life situation of an allergic patient. When IgE-stripped basophils from healthy donors were sensitised with D1 IgE antibodies and triggered with BLG, basophil activation levels comparable to primary basophils from milk allergic patients in other studies were achieved.[9] Activation of the basophils, measured by the surface expression of the activation marker CD63, was specifically seen after stimulation with BLG, but not with the control milk allergen casein ($p < 0.05$) (Figure 2A) and this was associated with increased production of the intracellular Th_2 cytokines IL-4 ($p= 0.0187$) and IL-13 ($p= 0.0048$) (Figure 2B). Overall, the results propose that our IgE antibodies are functional and may be useful tools to standardise BAT assays to achieve comparability between different laboratories.

The blocking capacity of D1 IgG4 antibodies, was investigated in an ELISA. As expected, the D1 IgG4, harbouring the same variable region, could decrease D1 IgE binding to plate-bound BLG in a concentration-dependent manner (Figure 2C). In accordance with these results, D1 IgG4 inhibited the activation of IgE-stripped, IL-3 primed, D1 IgE-sensitised and BLG-stimulated basophils from healthy donors in a dose-dependent manner. The addition of 50 nM ($p= 0.0402$) or 100 nM D1 IgG4 ($p= 0.0029$) significantly decreased the percentage of $CD63^+$ basophils (Figure 2D). In agreement with others, our findings confirmed the importance of the IgG4:IgE-ratio, as no inhibition was achieved with the lowest concentration of D1 IgG4 (10 nM). In line with our D1 IgE/D1 IgG4-model, overlapping epitope-binding of IgG4 and IgE antibodies may play an essential role in acquiring tolerance.[8] At the same time, this is also the limitation of our molecular model: The situation in allergic patients with polyclonal antibodies, binding to various epitopes on the allergen, is more complex. However, in a parallel study, with a PIPE-cloned monoclonal IgG4 to the major birch pollen allergen Bet v 1, we were able to demonstrate its blocking capacity using the sera from birch allergic patients.[S17]

Whether the IgG4 antibodies could be applied as passive immunotherapy in cow milk allergic patients depends on a thorough risk-benefit analysis, but studies in cat allergy promise that this might be possible in the future.[S18]

Our antibodies have so far proven valuable not only for the quantification of BLG and specific human serum antibodies against it, but also for functional studies. With these tools some questions could be addressed in the future: Are IgG4 antibodies involved in the establishment of *persisting* tolerance, or in primary tolerance to milk? Contrasting results about the efficacy of oral immunotherapies for CMA may be explained by differences in the methods regarding the duration of the intervention, dosages of the allergen or the composition of the milk. The PIPE-cloned antibodies presented here may add to the

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comparability of such studies between laboratories. Another application of the D1 antibodies may be in studies on the effect of milk processing on immune responses. Food processing can massively influence the structure of the allergens and therefore alter their allergenicity.[S19,S20] Especially during heat processing, important BLG epitopes are lost, but IgE binding is significantly increased after pasteurisation.[S21] Overall, the generated antibodies may shed light on milk allergenicity, bring critical insight into the development of CMA and help developing prevention strategies.

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Conflicts of Interest

EJJ is founder and shareholder of Biomedical Int. R + D and business partner of Bencard Allergie, Germany as well as AllergyTherapeutics, UK, but has no COI in relation to the presented work. SNK is founder and shareholder of Epsilogen Ltd. and holds patents on IgE antibodies for cancer therapy. AF is conducting research supported by Danone SA and Hipp GmbH. He has been in the advisory boards of Abbott SA and Hipp GmbH over the last two years. The other authors have no potential conflicts of interest to disclose.

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Figure Legends

Figure 1: Correct assembly and BLG-specific binding of PIPE-cloned D1 IgE and IgG4 antibodies.

(A) Schematic overview of the pVitro1 antibody constructs for the production of D1 IgE and IgG4. **(B)** SDS-PAGE of D1 antibodies in comparison with commercial isotype antibodies under non-reducing (-) and reducing condition (+). **(C)** Specificity of D1 anti-BLG antibodies in an ELISA. 3 µg/ml of BLG or Bet v 1 (control) were coated onto ELISA plates, and binding of respective D1 antibodies at different concentrations was determined. Data represent the mean of three independent experiments, error bars indicate SD. **(D)** Overview of ISAC112 microarray layout. **(E)** Specific binding of D1 IgE to BLG (Bos d 5) in ISAC112.

Figure 2: Functionality testing of D1 IgE in passively sensitised basophils: CD63 upregulation and intracellular cytokine production and blocking capacity testing of D1 IgG4: blocking of IgE binding in ELISA and inhibition of IgE crosslinking in BAT in a concentration-dependent manner.

(A) Stripped and IL-3 primed basophils from six healthy donors, native or passively sensitized with 5 nM D1 IgE were triggered with BLG, or casein control, both at 1000 ng/ml. Control experiments were performed in absence of IgE. Normal distribution was confirmed by Shapiro-Wilk normality test and differences between the groups assessed with repeated measures ANOVA with Greenhouse-Geisser correction and Sidak multiple comparison test: $F (1.022, 5.110) = 24.01$, $p= 0.0041$. Gating strategy: See Figure S3, supplement.

(B) IgE-stripped and IL-3 primed basophils of healthy donors, native or passively sensitized with 5 nM D1 IgE, were stimulated with 1000 ng/ml BLG or medium control. Y-axis: percentage of $CD63^+$ cells, x-axis: levels of intracellularly expressed cytokines. Differences in the groups were statistically analysed using two-way ANOVA with Greenhouse-Geisser correction: $F (1.276, 6.382) = 45.27$, $p= 0.0003$ and a Bonferroni multiple comparison test. *P < .05, **P < .01, and ***P < .001. Gating strategy: See Figure S3, supplement.

(C) BLG was coated onto ELISA plates, D1 IgG4 added in increasing concentrations (see colour code for white to black columns), and then D1 IgE added in various concentration as shown on x-axis. Bound IgE was detected by HRP-labelled anti-human-IgE antibody. Data represents the mean ± SD of three independent experiments.

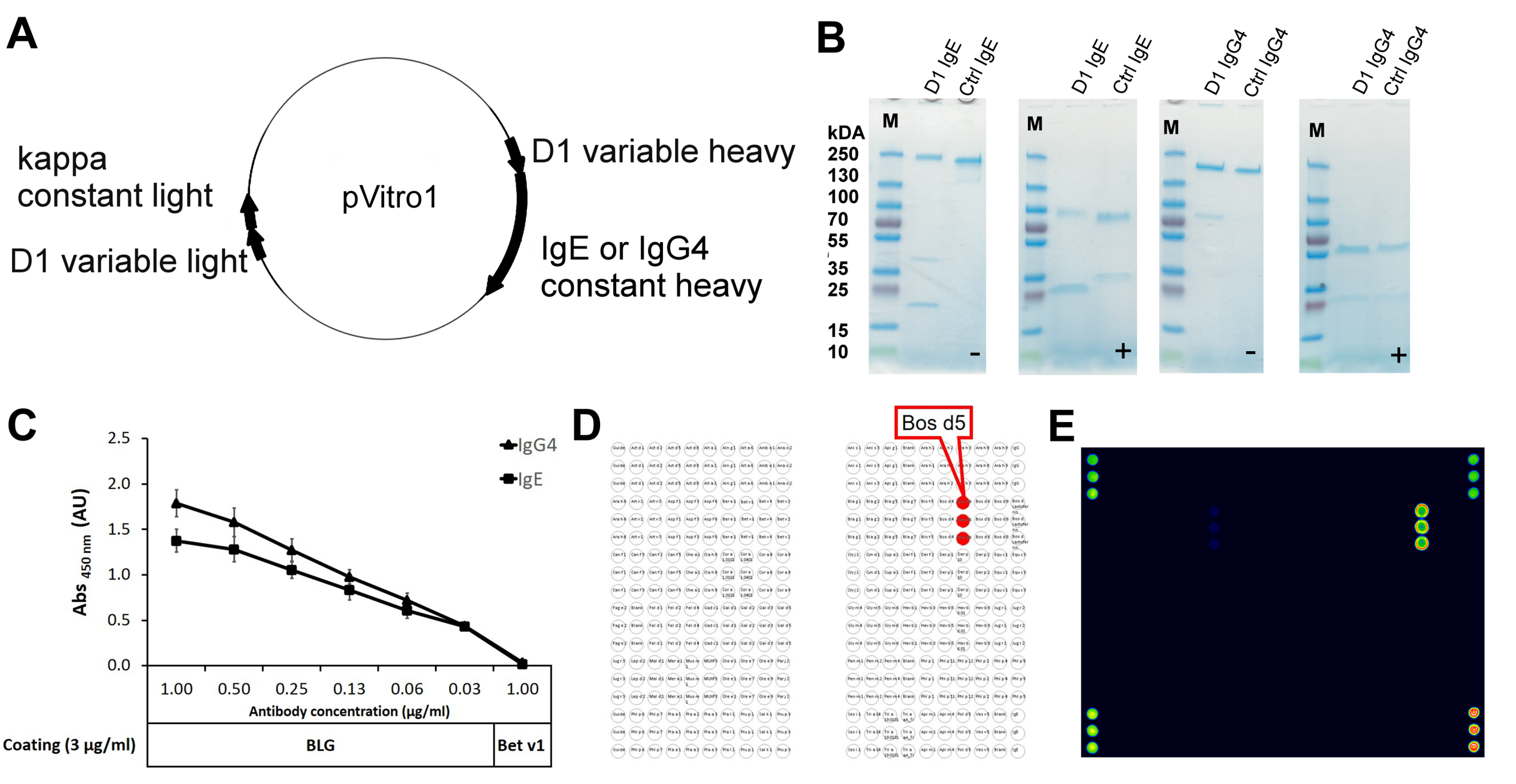
(D) Percentage of $CD63^+$ activated basophils in eight healthy donors (y-axis) after IgE-stripping, IL-3 priming, sensitising with 5 nM BLG-specific D1 IgE and subsequent stimulation with 1000 ng/ml BLG alone, or BLG mixed with 0 – 100 nM D1 IgG4 antibodies, as described in the x-axis. Significant differences were

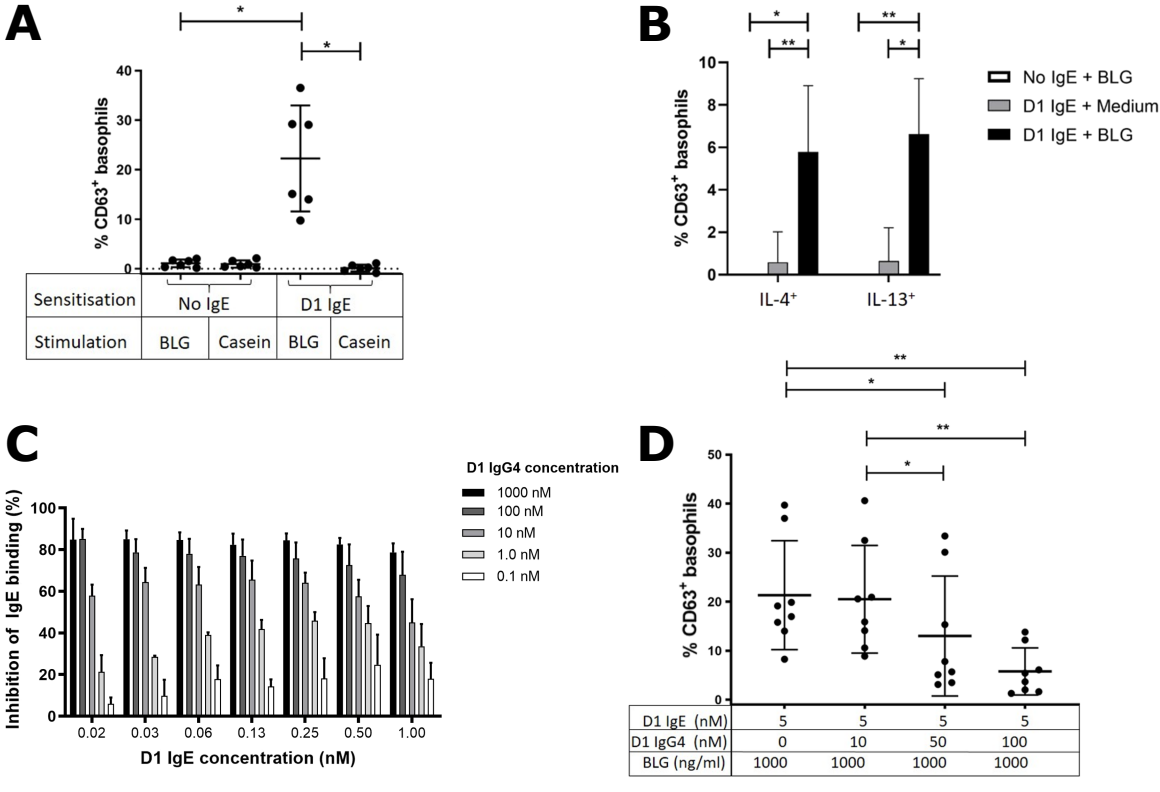
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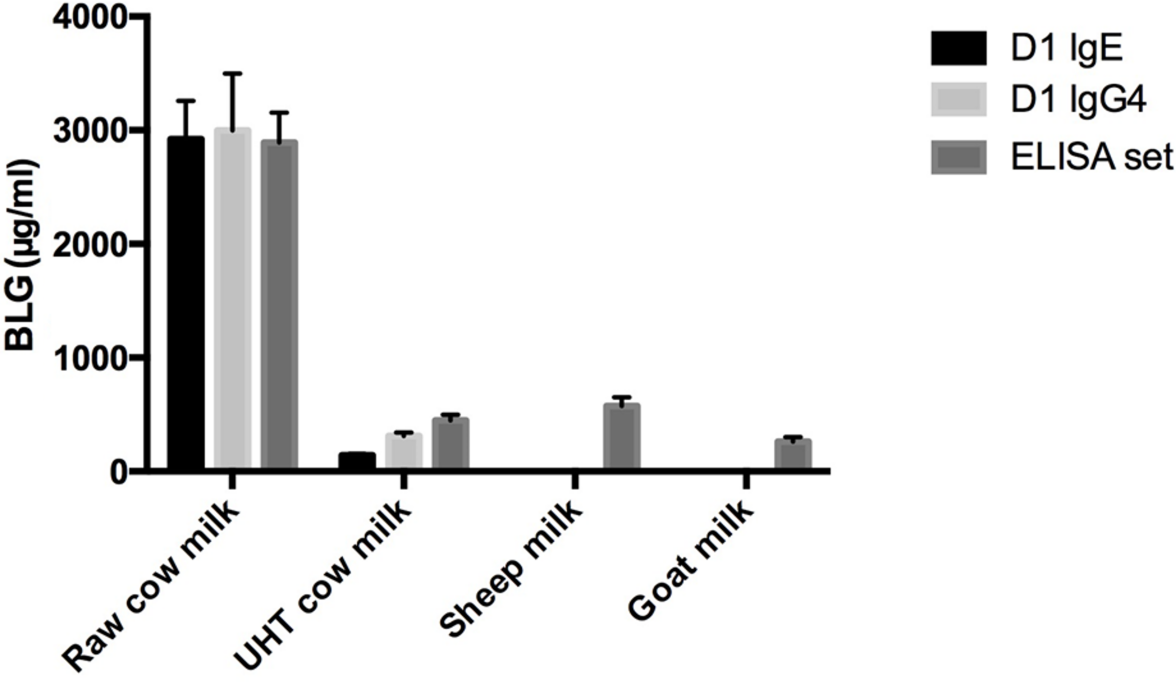
assessed with the Friedmann-Test: $\chi^2(4) = 19.80$, $p = 0.0002$, $n = 8$ and Dunn's multiple comparison test *P < .05, **P < .01, and ***P < .001. Gating strategy: see Fig. S3, supplement.

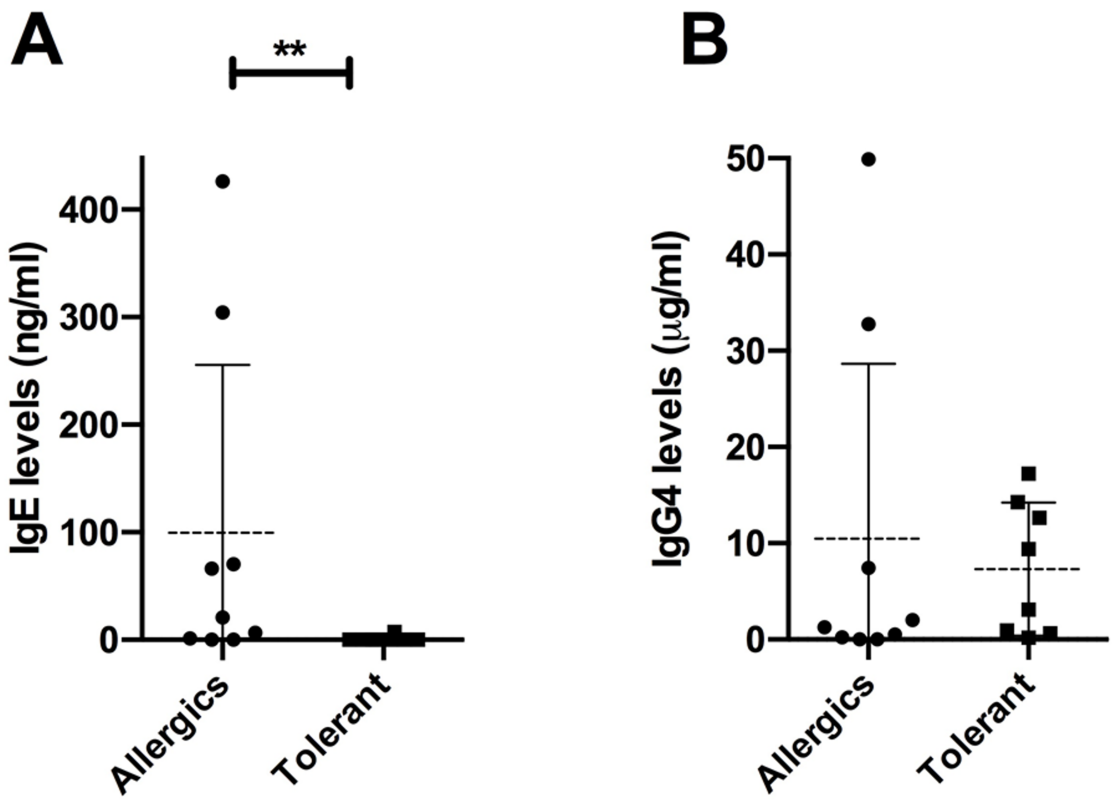
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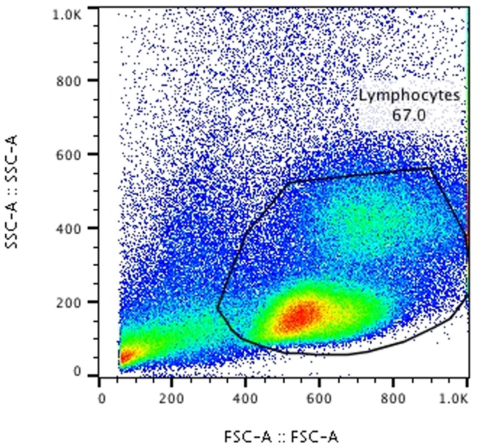

A
kappa
constant light
D1 variable light
pVitro1
D1 variable heavy
IgE or IgG4
constant heavy
B
D1 IgE
Ctrl IgE
D1 IgE
Ctrl IgE
D1 IgG4
Ctrl IgG4
D1 IgG4
Ctrl IgG4
kDA
M
250
130
100
70
55
35
25
15
10
-
+
-
+
C
IgG4
IgE
Abs 450 nm (AU)
2.5
2.0
1.5
1.0
0.5
0.0
1.00
0.50
0.25
0.13
0.06
0.03
1.00
Antibody concentration (µg/ml)
Coating (3 µg/ml)
BLG
Bet v1
D
Bos d5
E


A
% CD63+ basophils
40
30
20
10
0
*
*
Sensitisation
No IgE
D1 IgE
Stimulation
BLG
Casein
BLG
Casein
B
% CD63+ basophils
10
8
6
4
2
0
*
**
**
*
IL-4+
IL-13+
No IgE + BLG
D1 IgE + Medium
D1 IgE + BLG
C
Inhibition of IgE binding (%)
100
80
60
40
20
0
0.02
0.03
0.06
0.13
0.25
0.50
1.00
D1 IgE concentration (nM)
D1 IgG4 concentration
1000 nM
100 nM
10 nM
1.0 nM
0.1 nM
D
% CD63+ basophils
50
40
30
20
10
0
**
*
**
*
D1 IgE (nM)
5
5
5
5
D1 IgG4 (nM)
0
10
50
100
BLG (ng/ml)
1000
1000
1000
1000

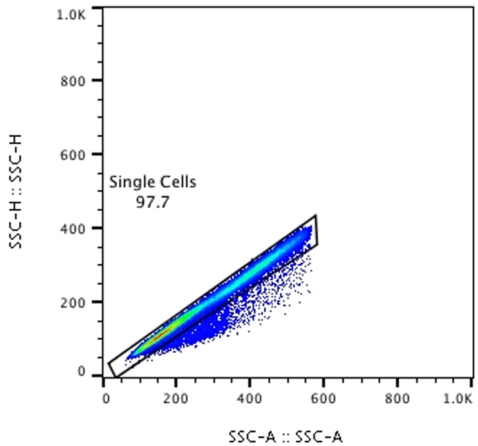

4000
3000
2000
1000
0
BLG (µg/ml)
D1 IgE
D1 IgG4
ELISA set
Raw cow milk
UHT cow milk
Sheep milk
Goat milk


A
**
IgE levels (ng/ml)
400
300
200
100
0
Allergics
Tolerant
B
IgG4 levels (µg/ml)
50
40
30
20
10
0
Allergics
Tolerant

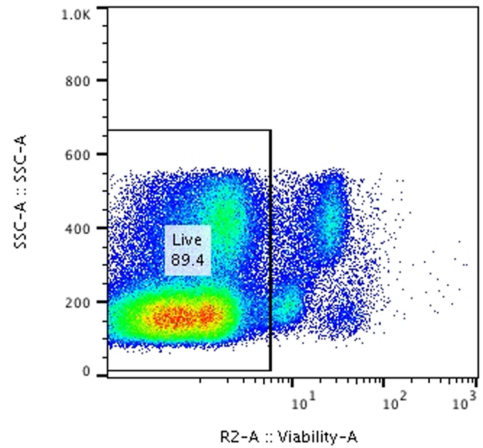
Main population



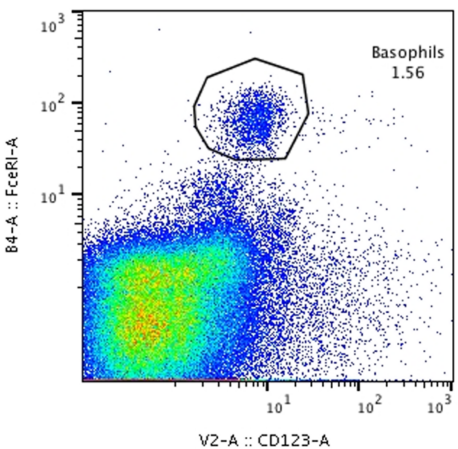
single cells



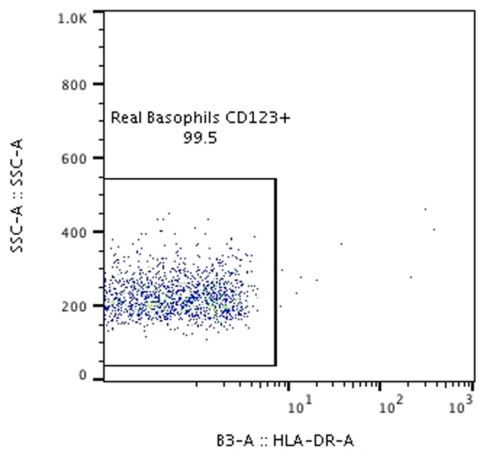
live cells



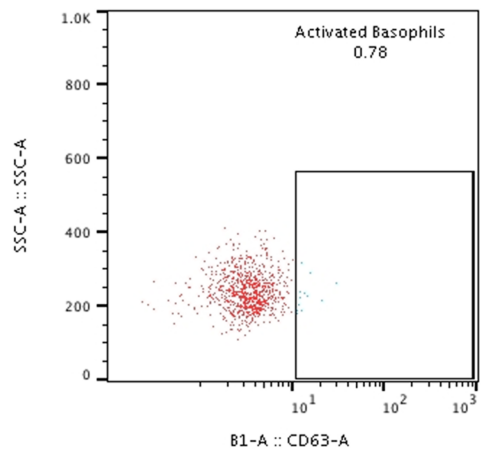
Basophils CD123+FcERI+



Basophils HLA-DR-



Basophils CD63+



Supplementary Material

Cell cultivation and maintenance

Expi293F cells (#A14527, Thermo Fisher Scientific) were cultivated in 30 ml Expi293 Expression Medium (#A1435101, Gibco, Thermo Fisher Scientific) at 37 °C, 8 % CO_2, according to the manufacturer's instructions. PBMCs of healthy patients were incubated in complete Iscove's Modified Dulbecco Medium (#12440-053, Gibco, Thermo Fisher Scientific); supplemented with 1 % GlutaMAX™ (#35050-038, Gibco, Thermo Fisher Scientific) and 10 % AB serum (# 20726, Male AB Serum, Innovative Research, Novi, MI, USA).

PIPE cloning and antibody production

Published variable region sequences (Table S1) of an IgE antibody specific for BLG[7] were combined with the human ε and γ4 constant regions to create different subtypes of antibodies binding to the same allergen epitope. pUC57 plasmids with an ampicillin resistance gene carrying the heavy and light chain of the variable region were purchased from BioCat GmbH (Heidelberg, Germany); vectors with the heavy and light chains of the constant regions for IgE and IgG4 (pVitro1, hygromycin resistance) were provided by King's college, London, UK. Variable and constant regions were amplified in four separate PIPE PCRs as described previously[4,5].

Briefly, 1 µl template DNA (10 ng/µl) was mixed with 2.5 µl of suitable forward and reverse primer (10 µM), 25 µl 2x Phusion Flash High-Fidelity PCR master mix (# F548S, Thermo Fisher Scientific) and 19 µl nuclease-free water. The reaction was started as initial denaturation at 98 °C for 30 s and then 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 15 s, extension at 72 °C for the time indicated in table S2, followed by a cool down to 4 °C.

Table S1. Light chain and heavy chain sequences of D1 IgE and IgG4

Light chain sequence: *Italic: human leader,* **bold: D1 V_L sequence,** underlined: hu κ equence, ***bold italic: stop codon***	*ATGTTGCCATCACAACTCATTGGGTTTCTGCTGCTCTGGGTTCCAGCTAGCCGCGGT***GACATCGTGATGACACAGAGCCCT AGCAGCCTGTCTGCCAGCGTGGGAGACAGAGTGACCATCACCTGTAGAGCCAGCCAGGGCATCAGCTCTAGACTGGCC TGGTATCAGCAGAAGCCTGGCAAGGCCCCTAAGCTGCTGATCTATGCCGCTAGCTCTCTGCAGTCTGGCGTGCCCTCTAG ATTTTCTGGCAGCGGCTCTGGCACCGAGTTCACCCTGACCATATCTAGCCTGCAGCCTGAGGACTTCGCCACCTACTACTG CCAGCAGTACCACAGCTACCCCTGGACATTTGGCCAGGGCACCAAGGTGGAAATC**AAGCGTACGGTGGCGGCGCCATCT GTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCA GAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACA GCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGC GAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGT***TGA***
IgE heavy chain sequence: *Italic: human leader,* **bold: D1 V_H sequence,** underlined: hu ε equence, ***bold italic: stop codon***	*ATGGACTGGACCTGGAGGATCCTCTTCTTGGTGGCGGCCGCCACAGGCGCGCACTCC***CAGGTGTCCCTGAGAGAATCTGG CGGAGGACTGGTGCAGCCTGGCAGATCTCTGAGACTGAGCTGTACCGCCAGCGGCTTCACCTTTAGACACCACGGCATG ACCTGGGTCCGACAGGCTCCTGGAAAAGGCCTGGAATGGGTCGCCTCTCTGTCTGGCTCTGGCACCAAGACACACTTCG CCGACTCTGTGAAGGGCAGATTCACCATCAGCCGGGACAACAGCAACAACACCCTGTACCTGCAGATGGACAACGTGC GCGACGAGGACACCGCCATCTACTATTGTGCCAAGGCCAAGAGAGTGGGCGCCACCGGCTATTTCGATCTGTGGGGAA GAGGCACCCTGGTCACCGTTTCTTCT**GCTAGCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCC CTCCAATGCCACCTCCGTGACTCTGGGCTGCCTGGCCACGGGCTACTTCCCGGAGCCGGTGATGGTGACCTGGGACACAG GCTCCCTCAACGGGACAACTATGACCTTACCAGCCACCACCCTCACGCTCTCTGGTCACTATGCCACCATCAGCTTGCTGAC CGTCTCGGGTGCGTGGGCCAAGCAGATGTTCACCTGCCGTGTGGCACACACTCCATCGTCCACAGACTGGGTCGACAACA AAACCTTCAGCGTCTGCTCCAGGGACTTCACCCCGCCCACCGTGAAGATCTTACAGTCGTCCTGCGACGGCGGCGGGCACT TCCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCCAGGGACTATCAACATCACCTGGCTGGAGGACGGGC AGGTCATGGACGTGGACTTGTCCACCGCCTCTACCACGCAGGAGGGTGAGCTGGCCTCCACACAAAGCGAGCTCACCCTC AGCCAGAAGCACTGGCTGTCAGACCGCACCTACACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGACAGCACCAA GAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCCAGCCCGTTCGACCTGTTCATCCGCAAGT CGCCCACGATCACCTGTCTGGTGGTGGACCTGGCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGG GAAGCCTGTGAACCACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCCACCCTGCCGGTG GGCACCCGAGACTGGATCGAGGGGGAGACCTACCAGTGCAGGGTGACCCACCCCCACCTGCCCAGGGCCCTCATGCGGT CCACGACCAAGACCAGCGGCCCGCGTGCTGCCCCGGAAGTCTATGCGTTTGCGACGCCGGAGTGGCCGGGGAGCCGGGA CAAGCGCACCCTCGCCTGCCTGATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTGCAGC TCCCGGACGCCCGGCACAGCACGACGCAGCCCCGCAAGACCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGCCTGGAG GTG ACCAGGGCCGAATGGGAGCAGAAAGATGAGTTCATCTGCCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGACCGTCC AGCGAGCGGTGTCTGTAAATCCCGGTAAA***TGA***
IgG_4 heavy chain sequence *Italic: human leader,*	*ATGGACTGGACCTGGAGGATCCTCTTCTTGGTGGCGGCCGCCACAGGCGCGCACTCC***CAGGTGTCCCTGAGAGAATCTGG CGGAGGACTGGTGCAGCCTGGCAGATCTCTGAGACTGAGCTGTACCGCCAGCGGCTTCACCTTTAGACACCACGGCATG ACCTGGGTCCGACAGGCTCCTGGAAAAGGCCTGGAATGGGTCGCCTCTCTGTCTGGCTCTGGCACCAAGACACACTTCG CCGACTCTGTGAAGGGCAGATTCACCATCAGCCGGGACAACAGCAACAACACCCTGTACCTGCAGATGGACAACGTGC**

bold: D1 V_H sequence, <u>underlined: hu γ_4 sequence,</u> ***bold italic: stop codon***	**GCGACGAGGACACCGCCATCTACTATTGTGCCAAGGCCAAGAGAGTGGGCGCCACCGGCTATTTCGATCTGTGGGGAA GAGGCACCCTGGTCACCGTTTCTTCT**<u>GCTAGCACCAAGGGCCCATCCGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCT CCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCC CTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCC TCCAGCAGCTTGGGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTG AGTCCAAATATGGTCCCCCATGCCCATCATGCCCAGCACCTGAGTTCCTGGGGGGGACCATCAGTCTTCCTGTTCCCCCCAAA ACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAG GTCCAGTTCAACTGGTACGTGGATGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCA CGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGGCCTCCCGTCCTCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAGCCACAGGTGTACACCCTGCC CCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCG TGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTC CTCTACAGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCT GCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAA***TGA***</u>

Table S2. PIPE PCR details and primers for amplification of the four fragments

Name	Template	Fwd primer	Rev primer	Fragment size (bp)	Extension time (s)
Frag. 1 – IgE	D1VH	GCCACAGGCGCGCACTCCCAGGTGTCC CTGAGAGAAT	ATGGGCTCTGTGTGCTAGCAGAAGA AACGGTGACCAGGGT	360	5
Frag. 2 – IgE	E	GCTAGCACACAGAGCCCATCCGTCTTC CCCTTGACCCGCTGCTGCA	ACCGCGGCTAGCTGGAACCCAGAGC AGCAGAAACCCAATGAGTTG	4076	56.6
Frag. 1 – IgG4	D1VH	GCCACAGGCGCGCACTCCCAGGTGTCC CTGAGAGAAT	AAGACGGATGGGCCCTTGGTGCTAG CAGAAGAAACGGTGACCAG	376	5
Frag. 2 – IgG4	G4	GCTAGCACCAAGGGCCCATCCGTCTTC CCCCTGGC	ACCGCGGCTAGCTGGAACCCAGAGC AGCAGAAACCCAATGAGTTG	3773	52.4
Frag. 3 – all	D1VL	TTCCAGCTAGCCGCGGTGACATCGTGA TGACACAGAGCCCTAGC	CGCCGCCACCGTACGCTTGATTTCCA CCTTGGTGCCCTGGCCAA	313	4.3
Frag. 4 – all	K for E/G4	CGTACGGTGGCGGCGCCATCTGTCTTC ATCTTCCCGCCATCTG	GGAGTGCGCGCCTGTGGCGGCCGC CACCAAGAAGAGGATC	4126	57.3

PCR products were digested with DpnI (#R0176S, New England Biolabs (NEB), Ipswich, MA, USA) and analysed on a 1 % agarose gel. Fragments were ligated by mixing 100 ng/µl of the four PCR products in a 1:1:1:1 ratio and incubated for 1 h at RT. Transformation into competent *E. coli* top 10 (#C3019H, NEB) cells was done according to the manufacturer's instructions and bacterial colonies were cultivated on agar plates supplemented with 200 µg/ml hygromycin B (#1287, Carl Roth, Karlsruhe, Germany) overnight at 37 °C. Colony PCR was conducted to check for heavy and light chain inserts. DNA was released from bacterial colonies via heat treatment (95 °C for 10 min) and mixed with 0.5 µl suitable forward and reverse primers, 5 µl 2x Phusion Flash High-Fidelity PCR master mix (#F548S, Thermo Fisher Scientific). Colony PCR program was started with an initial denaturation at 98 °C for 30s, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C or 15s, extension at 72 °C for 7 s and ended in a final extension step at 72 °C for 10 min and a cool down to 4 °C. Sequences were confirmed via cycle sequencing (Mix2seq overnight kit; Eurofins Genomics, Ebersberg, Germany). Finally, Expi293F cells (#A14527, Thermo Fisher Scientific, Waltham, MA, USA) were transfected with the plasmids and 50 µg/ml hygromycin B (#1287, Carl Roth) was added 72 h after transfection to maximise antibody yield. After seven days of culture, antibodies were purified by affinity chromatography using either a HiTrap KappaSelect column (#17545811, GE healthcare, Chicago, IL, USA) for IgE, or a HiTrap Protein A column (#17040201, GE healthcare) for IgG4 antibodies. Antibodies were eluted with either 0.1 M glycine (pH 2.5, IgE) or 0.1 M citric acid (pH 3, IgG4) and pH was immediately neutralised by adding 100 µl of Tris-HCl (pH 9.3). Antibody concentration was measured by UV absorption at 280 nm with a UV/VIS spectrophotometer (DeNovix DS-11 FX+, DeNovix Inc.), using the extinction coefficients from the protein sequence.

SDS-PAGE

2 µg of the PIPE-cloned D1 antibodies as well as isotype controls, human plasma IgE (#16-16-090705, Athens Research & Technology, Athens, GA, USA) and human myeloma IgG4 (#16-16-090707-4M, Athens Research & Technology) were diluted in PBS, mixed with 10 µl non-reducing or reducing Laemmli sample buffer, and heated for 5 min at 95 °C. Samples and markers (#26619, Page Ruler, Thermo Fischer Scientific) were loaded into Mini PROTEAN TGX gels (#4561084, Bio-Rad, Hercules, CA,

USA) and electrophoretically separated for 20 min at 250 V. Gels were stained with SimplyBlue™ SafeStain (#LC6060, Thermo Fisher Scientific) according to the manufacturer's instructions.

Specificity ELISA

For specificity determination, ELISA plates (Maxisorp 96-well plates, Thermo Fisher Scientific) were coated with 3 µg/ml of BLG (#L01030, Sigma-Aldrich, St. Louis, MO, USA) or 3 µg/ml of recombinant control allergen Bet v 1 (produced as previously described[S22,S23]) in coating buffer, pH 9.6 (#421701, Biolegend, San Diego, CA, USA), overnight. Plates were washed once with 0.05 % TBS-T and blocked with 1% BSA in 0.05% TBS-T for 2 h at RT. After two washing steps, D1 IgE or D1 IgG4 were added at concentrations from 1 to 0.03 µg/ml, and incubated for one hour, at RT. After washing, HRP-labelled anti-human IgE antibodies 1:6,000 (#A18793, Invitrogen, Carlsbad, CA, USA), or anti-human IgG4 1:12,000 (#9200-05, Southern Biotech, Birmingham, AL, USA), were added and incubated for one hour at RT. After washing the reaction was developed by TMB (#00-4201-56, Invitrogen) and stopped with 1 M H_2SO_4. The absorbance was measured at 450 nm with an Infinite 200M PRO plate reader (Tecan, Männedorf, Switzerland).

Sandwich ELISA for soluble BLG in milk

1 µg/ml of a commercial anti-BLG antibody (#Ab112893, Abcam, Cambridge, UK) was coated onto ELISA plates. After washing and blocking as described above, 2.5 - 0.05 µg/ml of BLG (#L01030, Sigma-Aldrich) as standard, raw cow milk (diluted 1:5,000 – 1:40,000), ultra-high temperature processed (UHT) cow milk, sheep milk and goat milk from a local store (1:500 – 1:1,000) were added and incubated for one hour, at RT. After washing, 1 µg/ml of D1 IgE or 0.25 µg/ml D1 IgG4 antibodies were added for one hour, RT and detected by HRP-labelled anti-human IgE and IgG4 as described above. For comparison, milk samples were tested with anti-BLG ELISA set (#E10-125, Bethyl Laboratories, Inc., Montgomery, Texas, US) according to the manufacturer´s instructions.

ImmunoCAP ISAC112

For testing D1 IgE specificity, ImmunoCAP ISAC112 microarray (Thermo Fisher Scientific, WO) was used, following the manufacturer's instructions by diluting 1 µg of the antibody in control serum obtained from a non-allergic person and devoid of specific antibodies to milk.

Patients´ characteristics

Blood samples of CM-allergic patients (n=3) and healthy controls (n=2) were collected in the Cruces University Hospital and provided by the Basque Biobank, Spain (http://www.biobancovasco.org). These patients presented IgE-mediated allergy symptoms, positive skin prick test responses to milk, and with specific IgE to cow's milk in ImmunoCAP (Table S3).

Paediatric patients diagnosed with CMA by the Alergologia Dept., Ospedale Pediatrico Bambino Gesù, Rome, Italy were all sensitized to cow´s milk (n=14), with one group (n=6) reacting positive to an oral milk challenge and the other sensitised but tolerant group not reacting to oral milk challenge (n=8) (see patients´ characterisation in Table 3). The serum were retrospectively collected, after the informed consent from the patients were signed, in accordance with the Helsinki Declaration of 1975. Experiments were performed under approval of the ethical committee of the Pediatric Hospital Bambino Gesù IRCCS, Rome and by the Ethical Committee of Clinical Research of the Basque Country (CEIC-E) (PI2015182 and PI2016156).

Table S3: Patients' characteristics

Patient	Status	Age (months)	Sex	IgE to milk (ImmunoCAP, kU/L)
1	healthy control	108	F	nA
2	healthy control	48	F	nA
3	allergic [a)]	72	F	46.3
4	allergic	16	F	> 100.0

5	allergic	84	M	62.5
6	allergic	123	M	2.6
7	allergic	22	F	4.8
8	allergic	40	M	1.1
9	allergic	84	M	53.8
10	allergic	197	F	51.6
11	allergic	41	F	100.0
12	tolerant [b)]	23	M	2.5
13	tolerant	52	M	0.3
14	tolerant	31	M	0.3
15	tolerant	103	M	0.1
16	tolerant	51	M	0.2
17	tolerant	30	M	0.0
18	tolerant	51	F	1.3
19	tolerant	70	F	0.0

a) Patients with clinical history of CMA, positive skin prick test responses, and *in vitro*–specific IgE to cow's milk and BLG (Patient 3 - 5) and positive reaction in a milk challenge (Patient 6 - 11).
b) Patients with a clinical history of CMA, but without reacting in a milk challenge.
*) means ± SD of 2 instead of 3 repetitions

Quantification of serum antibody levels by ELISA
ELISA plates were coated with 3 µg/ml of BLG (#L01030, Sigma-Aldrich) in coating buffer, pH 9.6 (#421701, Biolegend) overnight, washed with 0.05 % TBS-T once and blocked for 2 h at RT with 0.05 % TBS-T. After washing, sera (diluted 1:2 for IgE, up to 1:100 for IgG4) were added, for calibration, a dilution series of D1 IgE (250 – 3 ng/ml) or D1 IgG4 (4000 – 62.5 ng/ml) antibodies, and incubated for one hour at RT. Detection, development and readout were carried out as described in the ELISA experiments above.

Inhibition ELISA
ELISA plates were coated with 3 µg/ml of BLG (#L01030, Sigma-Aldrich) in coating buffer, pH 9.6 (#421701, Biolegend) overnight, washed with 0.05 % TBS-T once and blocked for 2 h at RT with 0.05 % TBS-T. D1 IgG4 was added in increasing concentrations (0.1 nM to 1000 nM) and incubated for one hour at RT. Next, D1 IgE was incubated in concentrations (0.015 nM to 1 nM), for one hour at RT. Bound IgE was detected using HRP-labelled anti-human-IgE antibody diluted 1:6,000 (#A18793, Invitrogen), as described above.

Basophil activation tests and BAT inhibition
We followed the protocol previously described.[S24] Briefly, ficoll-isolated (Ficoll-Paque PLUS, GE Healthcare) PBMCS of healthy donors were IgE-stripped by incubation in lactic acid buffer (13.4 mM lactic acid #W261114-1KG-K, Sigma-Aldrich), 4.09 g of NaCl, 0.186 g KCl in 500 ml of dH_2O and adjust pH to 3.9) for 4 min on ice. After two washing steps, cells were sensitized with 5 nM D1 IgE in the presence of 10 ng/ml IL-3 (Miltenyi Biotec, Bergisch Gladbach, Germany), for 2 hours at 37 °C. Sensitised cells were seeded into a 48-well plate at a concentration of $1x10^6$ cells per well. Cells were then stimulated with 1000 ng/ml BLG (#L7880, Sigma-Aldrich), control allergen casein 1000 ng/ml (#C7078, Sigma-Aldrich) or medium, in the presence of 2 ng/ml IL-3, for 40 min at 37 °C. In the BAT inhibition experiments, D1 IgG4 antibody (final concentration: 10-100 nM) was added with the BLG. Cells were transferred to FACS tubes and washed with PBS. After centrifugation (300 x g for 5 min),

cells were re-suspended in 1 ml PBS and stained with LIVE/DEAD™ fixable near-IR viability dye (#L10119, Invitrogen) for 25 min on ice in the dark. After another washing step, cells were stained with PerCP-Cy5.5-labelled anti-HLA-DR (#560652, BD Biosciences, San Jose, CA, USA), PE-Cy7-labelled anti-FcεRI (#25-5899-42, Invitrogen), BV510-labelled anti-CD123 (#563072, BD Biosciences), and FITC-labelled anti-CD63 (#557288, BD Biosciences) antibodies, and incubated in PBS-BSA for 25 min on ice in the dark. After the last washing step, cells were re-suspended in 175 µl of PBS and measured in MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec) and analysed with FlowJo software (version 10.0.7, Tree Star, Ashland, OR, USA).

Flow cytometry and intracellular cytokine determinations

Cells were IgE-stripped and sensitised with D1 IgE as described above and $1x10^6$ cells were seeded directly into FACS tubes. BLG, casein or medium, as well as 2 ng/ml IL-3 were added and incubated for 6 h at 37 °C with GolgiStop (0.66 µl/ml, #554724, BD Biosciences) and GolgiPlug (1 µl/ml, #555029, BD Biosciences). The tubes were then incubated at 4 °C overnight. Cells were stained with PE-Cy7-labelled anti-FcεRI (#25-5899-42, Invitrogen), BV510-labelled anti-CD123 (#563072, BD Biosciences) and FITC-labelled anti-CD63 antibodies (#557288, BD Biosciences), for 25 min on ice in the dark. After washing with PBS-BSA, cells were re-suspended in 250 µl fixation/permeabilisation solution (#554722, BD Biosciences), and incubated for 20 min on ice in the dark. After washing with permeability wash buffer (#51-2091KZ, BD), cells were stained with BV421-labelled anti-IL-4 (#500826, Biolegend), PerCP-Cy5.5-labelled anti-IL-13 antibodies (#501911, Biolegend) and 50 µl of Brilliant Buffer (#563794, BD Biosciences) for 25 min on ice in the dark. After the last washing step, cells were re-suspended and analysed as described above.

Statistics

All statistical analyses were performed using Graph Pad Prism 8 (GraphPad Software LLC). Blood serum data were analysed with Shapiro-Wilk tests to analyse data for normal distribution, Mann-Whitney-U test and spearman correlation. Repeated measures ANOVA with Greenhouse-Geisser correction and Sidak multiple comparison test were calculated for the basophil data. For the cytokine analyses of the basophils, a two-way ANOVA with Greenhouse-Geisser correction and Bonferroni multiple comparison test were conducted. Basophil inhibition experiments were analysed with Friedmann-Test and Dunn's multiple comparison test for three groups.

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Supplementary Figures and Legends

Figure S1: Species-specificity of the anti-BLG D1 IgE and D1 IgG4 antibodies. For the in-house ELISA 0.1 µg per well of commercial anti-BLG antibodies (#Ab112893, Abcam) were coated onto ELISA plates and incubated with the following samples: raw cow milk, ultra-high temperature processed (UHT) cow milk, sheep milk, or goat milk (x-axis). For detection of BLG, D1 IgE (1 µg/ml) or D1 IgG4 (0.25 µg/ml) antibodies were added and detected with HRP-labelled anti-human IgE and gG4. For quantification (y-axis), BLG was serially diluted (0.05 – 2.5 µg/ml) and values compared hereto. The commercial sandwich ELISA set for BLG was used according to the manufacturer´s instruction and quantified using provided standards. Columns represent the mean of duplicates from three experiments, error bars indicate +/- SD between experiments.

Figure S2: PIPE-cloned D1 IgE and D1 IgG4 for quantification of natural antibodies to BLG in patients' sera.

BLG was coated onto ELISA plates and incubated with sera (diluted 1:2 for IgE and between 1:5 and 1:100 for IgG4 depending on sera IgG4 content) of CM-sensitized patients (allergic, n=9, or tolerant in milk challenge, n=8). Natural bound IgE (A) and IgG4 (B) was quantified comparing to dilution series of D1 IgE or D1 IgG4, all detected by HRP-labelled anti-human IgE or anti-human IgG4. Data points represent the mean of three independent experiments. Differences were calculated by Mann-Whitney test: U = 11, p = 0.0089); *P < .05, **P < .01, and ***P < .001.

Figure S3: Basophil gating strategy in flow cytometry. Peripheral blood mononuclear cells (PBMCs) of one exemplary donor are shown, stained with anti-HLA-DR (PerCP-Cy5.5), anti-FcεRI (PE-Cy7), anti-CD123 (BV510), anti-CD63 antibodies (FITC). Basophils were gated as $CD123^{+}FcεRI^{+}HLA\text{-}DR^{-}$, after excluding doublets and dead cells.