

MR. LARS HEEDE BLOM (Orcid ID : 0000-0003-2027-727X)

MR. LAU FABRICIUS LARSEN (Orcid ID : 0000-0001-6433-3961)

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The immunoglobulin superfamily member CD200R identifies cells involved in type 2 immune responses

Lars H. Blom¹, Britta C. Martel^{1,2}, Lau F. Larsen¹, Camilla V. Hansen¹, Malene P. Christensen¹, Nanna Juel-Berg¹, Thomas Litman², and Lars K. Poulsen¹

1) Department of Dermatology and Allergy, Allergy Clinic, Copenhagen University Hospital Herlev and Gentofte, Denmark

2) LEO Pharma A/S, Ballerup, Denmark

Corresponding authors

Lars Heede Blom; Allergy Clinic, Department of Dermatology and Allergy, Copenhagen

University Hospital Herlev and Gentofte, Kildegårdsvej 28, Dept. 22, 1st floor, Denmark; Tel

+45 38 67 23 37 lars.blom@regionh.dk

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Keywords

T lymphocytes; Th2; peanut-specific Th cells, CD154, CD200R, AD;

Abbreviations

CD200R (CD200R1)

CD200R2 (CD200RLa)

CD4⁺ T helper (Th)

Chemoattractant receptor–homologous molecule expressed on Th2 cells (CRTh2)

c-Jun N-terminal protein kinase (JNK)

Extracellular signal-regulated kinases (ERK)

GATA-binding protein 3 (GATA3)

Immunoreceptor tyrosine based inhibitory motifs (ITIM)

Median fluorescence intensity (MFI)

Mitogen-activated protein kinases (MAPK)

Non-allergic (NA)

Peripheral blood mononuclear cell (PBMC)

Peanut-allergic (PA)

Systemic lupus erythematosus (SLE)

T regulatory (Treg)

Th type 1 (Th1)

Cytotoxic Type 2 (Tc2)

Innate lymphoid type 2 (ILC2)

Abstract

Background: The pathology of allergic diseases involves type 2 immune cells, such as Th2, ILC2, and basophils exerting their effect by production of IL-4, IL-5, and IL-13. However, surface receptors that are specifically expressed on type 2 immune cells are less well documented. The aim of this investigation was to identify surface markers associated with type 2 inflammation.

Methods: Naïve human CD4⁺ T-cells were short-term activated in the presence or absence of IL-4, and analysed for expression of >300 cell-surface proteins. *Ex vivo* isolated PBMCs from peanut and non-allergic allergic subjects, were stimulated (14-16h) with peanut extract to detect peanut-specific CD4⁺CD154⁺T-cells. Biopsies were obtained for transcriptomic analysis from healthy controls and patients with extrinsic or intrinsic atopic dermatitis and psoriasis.

Results: Expression analysis of >300 surface proteins enabled identification of IL-4 up-regulated surface proteins, such as CD90, CD108, CD109, and CD200R (CD200R1).

Additional analysis of *in vitro* differentiated Th0, Th1, and Th2 cultures, identified CD200R as up-regulated on Th2 cells. From *ex vivo* isolated PBMCs, we found high expression of CD200R on Th2 and ILC2 cells and basophils. In peanut-allergic subjects the peanut-specific Th2 (CD154⁺CRTh2⁺) cells expressed more CD200R than the non-allergen specific Th2 (CD154⁻CRTh2⁺) cells. Moreover, co-staining of CD161 and CD200R identified peanut-specific highly differentiated IL-4⁺IL-5⁺ Th2 cells. Finally, transcriptomic analysis revealed

up-regulation of CD200R in lesional skin from subjects with an extrinsic atopic dermatitis phenotype compared to healthy skin.

Conclusion: These results indicate that CD200R expression strongly correlates with Th2 pathology; though, the mechanism is as yet elusive.

Introduction

Memory CD4⁺ T helper (Th) cells are central in orchestrating the adaptive immune system to direct immune responses, leading to the elimination of various pathogens. The importance of distinct Th cell subsets, such as Th type 1 (Th1), Th2, Th17 and T regulatory (Treg) cells for host-defense immune responses, has long been recognized. Th2 cells are involved in the immune responses against extracellular parasites and are essential in the initial and established responses of allergic inflammatory diseases. Th2 cell differentiation is initiated by endogenous or basophil released IL-4 (1,2). By binding to IL-4 receptor, IL-4 upregulates the expression of GATA-binding protein 3 (GATA3), the master transcription factor for Th2 cell differentiation (3). Th2 cells function through their production of the hallmark Th2 cytokines IL-4, IL-5, and IL-13 (4). IL-4 plays a crucial role not only in Th2 differentiation but also in B cell IgE isotype switching, whereas IL-5 promotes eosinophil maturation, tissue recruitment, activation, and survival. IL-13 is critical for mucus production by goblet cells, airway hyper-responsiveness, and stimulates keratinocyte production of pro-inflammatory cyto- and chemokines in the skin (5). At least two Th2 cell subpopulations have been described; an early subtype characterized by production of IL-4 and IL-13, and a highly differentiated “inflammatory” subtype characterized by additional production of IL-5 (6-9) and we speculate potentially other cytokines, such as i.e. IL-31. In addition to the Th2 cells, the recently identified group 2 innate lymphoid cells (ILC2s) contribute to type 2 inflammation by release of IL-5 and IL-13 (10).

Differentiated Th2 cells have been associated with expression of a few surface receptors, such as the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2), C-C motif chemokine receptor 8 (CCR8), and ST2 (11-13). Additionally, IL-5 producing “inflammatory” Th2 cells were recently shown to co-express CRTh2, CD161, and hematopoietic prostaglandin D synthase whereas IL-5 negative Th2 cells were CRTh2 positive but did not express CD161 and hematopoietic prostaglandin D synthase (7).

Identification of stably expressed surface molecules on cells involved in type 2 inflammation, such as Th2 cells, could provide new therapeutic targets for the treatment of allergic or autoimmune diseases. Therefore, it is essential to identify new surface markers associated with cells involved in type 2 inflammation. In this study, we used a combination of *in vitro* Th2 cell differentiation and flow cytometric expression analysis of >300 surface molecules to detect novel IL-4 up-regulated surface proteins on newly activated naïve CD4⁺ T cells.

Thereby, we were able to identify high expression of the inhibitory transmembrane protein belonging to the immunoglobulin superfamily, CD200R (CD200R1) on Th2 cells.

Furthermore, high expression of CD200R was detected both on peanut-specific and non-peanut specific human Th2 cells as well as other cells involved in type 2 inflammation such as ILC2, Tc2, and basophils.

Materials and Methods

Subjects

Buffy coats used in the *in vitro* parts of the study originate from blood portions from donors whose identity has been kept anonymous by the Blood Bank (National University Hospital Copenhagen, Denmark). Nine peanut and eight non-allergic subjects matched in regards to age and sex were recruited from the Allergy Clinic (Copenhagen University Hospital Herlev

and Gentofte) and had signed written informed consent. The protocol was approved by the local ethics committee (file number: 46312). Peanut allergic subjects, with a positive oral challenge or convincing medical history of an immediate allergic reaction to peanut, a positive ImmunoCAP score for peanut (>3.5 kUA/L; Thermo Fisher Scientific, Uppsala, Sweden), and a positive basophil activation test (unpublished data) were included in this study. The non-allergic subjects had negative peanut-specific IgE (< 0.35 kUA/L) levels and no clinical or family history of peanut allergy.

For the skin mRNA analysis, a dataset of 31 subjects was available from a cohort consisting of 36 individuals who have previously been characterized with respect to various inflammatory parameters (14-16). Description of skin tissue sampling and patient characteristics are published elsewhere (16). In short, fourteen patients with atopic dermatitis (AD), nine patients with psoriasis and eight healthy controls were included in this study. Five of the patients with AD have been characterized as having extrinsic AD (AD_{ex}, allergic AD, with high total serum IgE), and nine patients had intrinsic AD (AD_{in}, non-allergic AD, normal total serum IgE) (16).

In vitro and *ex vivo* cultures

PBMCs and naïve CD4⁺ T cells were purified as previously described (17). The naïve CD4⁺ T cells were polyclonally activated and cultured under Th0, Th1 or Th2 culture conditions for 12 h, 36 h, 60 h and 7 days, respectively. CD4⁺ T cells were polyclonally activated for 18 h. A complete description of cell culture conditions and production of peanut extract can be found in the Methods section in this article's Supplementary information.

Ex vivo analysis of peanut specific CD4⁺ T cell

For detection of CD154⁺ peanut-specific CD4⁺ T cells, 45×10^6 PBMCs ($5-7.5 \times 10^6$ cells/mL) were stimulated with 100 mg/mL peanut extract for 14-16 h. For more information, see the Methods section in this article's Supplementary information.

Flow cytometry

The cells were phenotypically characterized by combinations of live/dead amino reactive dyes, and antibodies against surface and intracellular markers. Data acquisition was performed with an LSR II flow cytometer (BD Biosciences, San Diego, USA), and data were analyzed with FlowJo software (TreeStar, Ashland, USA). Analysis and presentation of distributions was performed using SPICE version 5.1 (18). For further information, see the Methods section in this article's Supplementary information.

Quantitative real time PCR and microarray analysis

A complete description of quantitative real time PCR and microarray analysis can be found in the Methods section in this article's Supplementary information.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.0 (GraphPad Software, La Jolla, CA, USA). Two-tailed Student's *t* test or 2-way ANOVA followed by Tukey correction were used for determining statistical significance; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. Bar graphs are represented as means $\pm$ SD. The horizontal bar represents group means.

Results

Surfaceomics of IL-4 primed naïve CD4⁺ T cells

A systematic expression analysis of >300 surface proteins was performed by flow cytometry using a commercial kit and fluorescent cell barcoding. Surface proteins upregulated by IL-4, were identified by comparing the median fluorescence intensity (MFI) of Th cells cultured with and without IL-4, respectively (Supplementary data 1 and gating strategy in figure 1A). Within the first 12-36 h of culture, IL-4 induced expression of primary activation markers, such as CD25, CD69, CD154, and CD226 (Supplementary data 1). After 60 h of stimulation, the cell adhesion and transmigration molecules CD31, CD58, the complement decay-accelerating factor CD55, as well as the immunoregulatory molecules CD108, CD279 (PD-1), and CD210 (IL-10R) were induced by IL-4 (Supplementary data 1). Furthermore, CD90 was found to be up-regulated on a subpopulation of blasted cells (Figure 1A) after 60 h of stimulation (Supplementary data 2). At all time-points (Figure 1B and Supplementary data 1), up-regulation of the IL-4 receptor (CD124), the chemokine receptor CD184 (CXCR4), the inhibitory receptor CD200R, as well as CD109 was observed (Figure 1A). Of the IL-4 induced surface molecules CD90, CD108, CD109, and CD200R were chosen for additional *in vitro* and *ex vivo* analysis (Supplementary data 2).

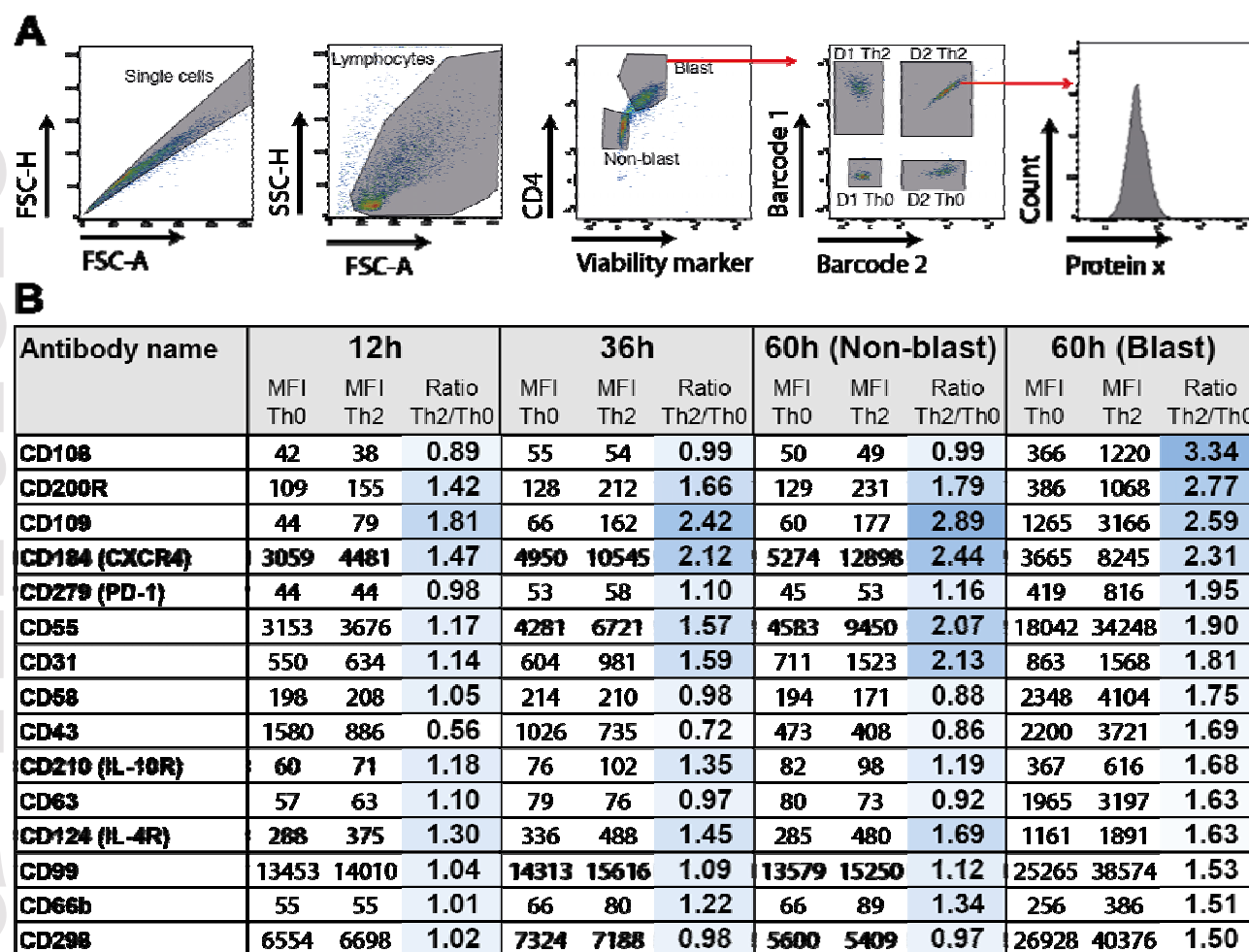


Figure 1. Surfaceomics of IL-4 primed naïve CD4⁺ T cells. Naïve CD4⁺ T cells were primed with and without IL-4 for 12, 36, and 60 h. **A**) Gating strategy for identification of, from left, singlets, lymphocytes, blasted or non-blasted cells, barcoding of the two different donors (D1 and D2) and culture conditions (Th0 and Th2) and protein expression of surface molecule x. **B**) 50% and more up-regulated targets from (n = 2) healthy individuals, sorted after the MFI of the cultures with (Th2) and without IL-4 (Th0) at time point “60 h blast”.

IL-4 induces specific CD90 and CD200R expression

To test if CD90, CD108, CD109 or CD200R were selectively up-regulated by Th2 cells, we analyzed for expression of the identified surface markers on *in vitro* primed Th0, Th1, and Th2 cells (Figure 2). Even though CD90 and CD109 was up-regulated under Th2 culture conditions at day 7, the selectively Th2 cell association could not be confirmed on established

Th2 cells (Figure 2A, 2B, data not shown). Unexpectedly, CD108 expression was significantly up-regulated under Th1 culture conditions (Figure 2A, 2B). CD200R was significantly up-regulated on IL-4 primed cells at day 3 and day 7 when compared with the Th0 cultures (Figure 2B). However, a significant difference in CD200R expression between Th1 and Th2 cultures was only found on day 3 and not on day 7 (Figure 2B). This was supported by a significantly increased expression of *CD200R* mRNA in cells cultured under Th2 conditions after 24 h and 48 h of culture (Figure 2C).

We also wanted to investigate if Th2 cultured cells (Figure 2D) – i.e. cells producing the Th2 subtype cytokines IL-4, IL-5, IL-13, and IL-31 differed in their expression of CD200R. After 7 days of Th2 culture, an additional significant increase in CD200R expression was found within cells producing cytokines associated with a more terminally differentiated Th2 phenotype namely IL-5 or IL-31 (Figure 2E).

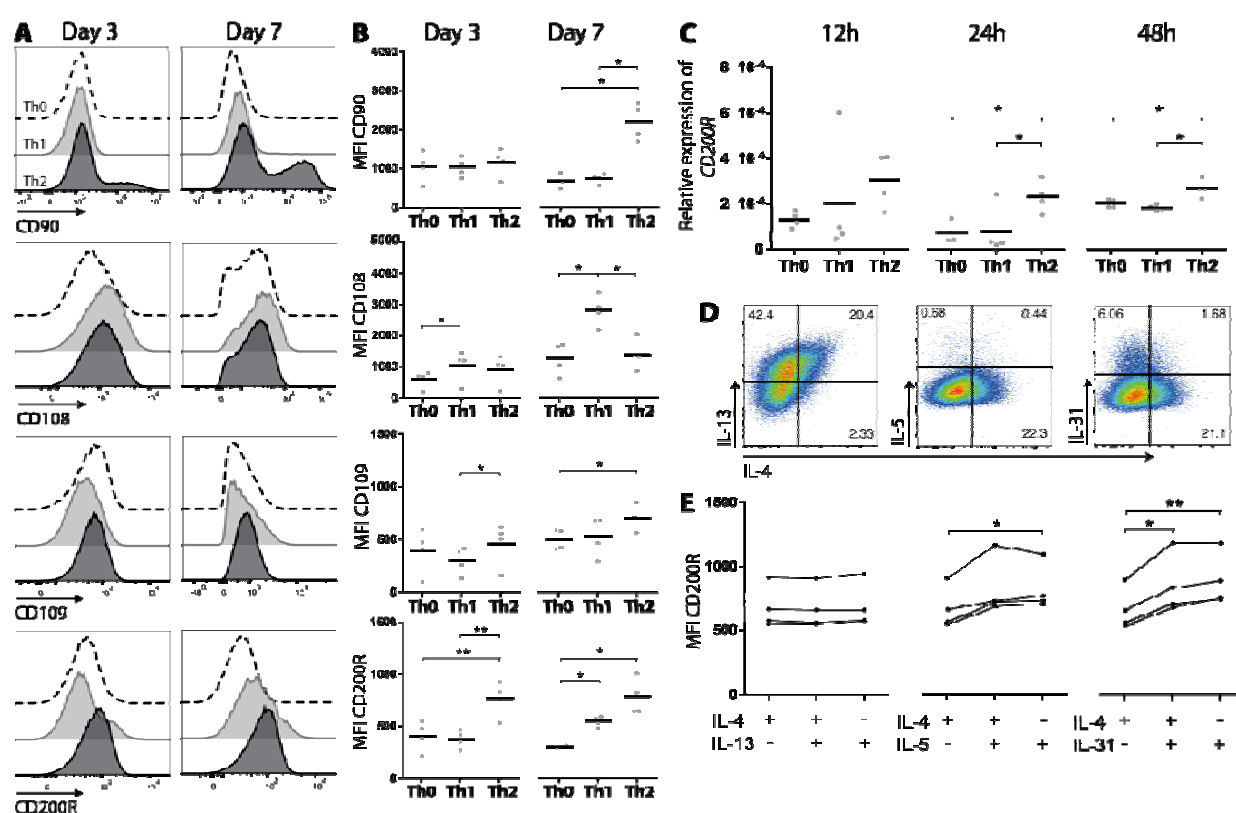


Figure 2. IL-4 induces specific CD90 and CD200R expression. Naive CD4⁺ T cells were stimulated under Th0 (dotted line), Th1 (light gray fill) or Th2 (black fill) culture conditions for 12 h, 24 h, 48 h, 72 h, and seven days. **A)** Representative histograms of CD90, CD108, CD109, and CD200R at day 3 and 7. **B)** MFI for CD90, CD108, CD109, and CD200R at day 3 and 7 of (n = 4) individuals. **C)** Relative mRNA expression of *CD200R* at the time points 12 h, 48 h, and 72 h of (n = 4) individuals. **D)** Representative percentage of cells positive for the Th2 cytokines IL-4, IL-5, IL-13, and IL-13 at day 7 in Th2 primed cultures. **E)** MFI of CD200R for Th2 primed cells producing combinations of IL-4, IL-5, IL-13, and IL-31 at day 7 of (n = 4) individuals. (*) p<0.05, (**) p<0.01. The horizontal bar represents group means.

CD200R is expressed by Th2, ILC2, and basophils

Next, we studied if CD200R expression on PBMCs was general or especially associated with Th2 cells. First, expression of CD200R on circulating unstimulated PBMCs from healthy donors was investigated. Basophils and the type 2 cytokine producing cell subsets; Th2, type 2 cytotoxic T cells (Tc2), and ILC2 had the highest expression of CD200R (Figure 3A). Of interest, the Th2 cell type associated marker CRTh2 was co-expressed by cells with high levels of CD200R (Figure 3B). Expression of the CD200R ligand CD200 was also investigated on PBMCs (Supplementary data 3), and found to be most highly expressed by IL-3 receptor (CD123) positive B cells, B cells, eosinophils, and plasmacytoid dendritic cells (pDC) (Supplementary data 3).

Within polyclonally re-activated peripheral CD4⁺ T cell subsets, highly differentiated Th2 cells (IL-4⁺IL-5⁺) showed a tendency towards higher expression of CD200R compared with less differentiated Th2 cells (IL-4⁺IL-5⁻) (Figure 3C). Interestingly, high expression of CD200R was also detected on IL-9 producing Th9 cells, whereas IL-31⁺ Th2 and IL-22⁺ Th22 cells, were characterized by an intermediate expression of CD200R (Figure 3C). In contrast, only low expression of CD200R was detected on other CD4⁺ T cell subsets, such as

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Tr1, Th1, Th17, and Tregs (Figure 3C). Expression of the CD200R ligand CD200 was low on all investigated CD4⁺ T cell subsets (data not shown). Dividing the expression of CD200R in High, Dim and Low regions (Figure 3D) the highly differentiated Th2 cells (IL-4⁺IL-5⁺) and Th9 cells showed the largest fraction cells within the CD200R^{High} region (Figure 3E). In addition, the majority of the non-Th2 subsets were categorized within the CD200R^{Low} region (Figure 3E).

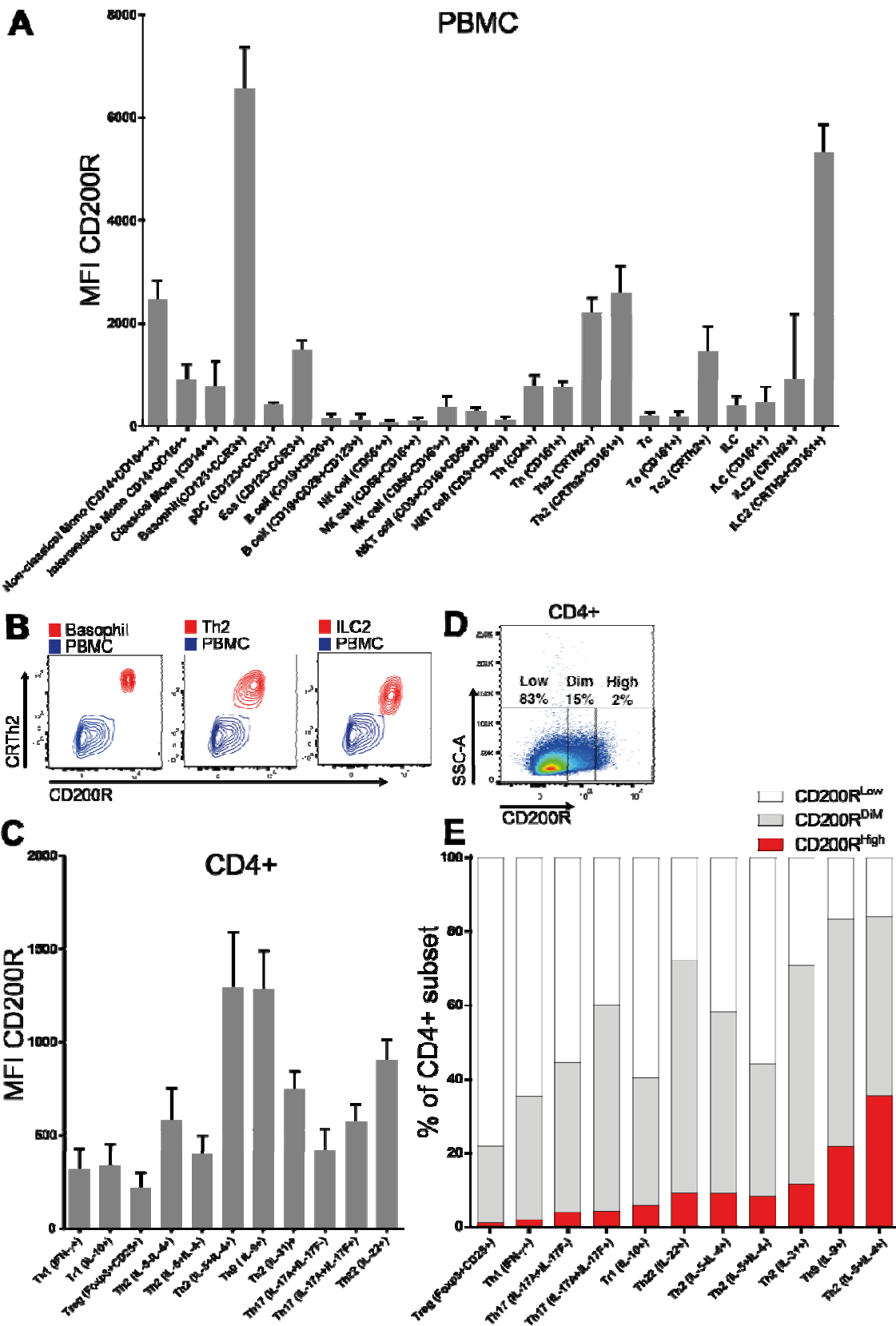


Figure 3. CD200R is expressed by type 2 T and ILC cells as well as basophils. *Ex vivo* PBMCs and CD4⁺ T cells were isolated (n = 8). **A)** Expression of CD200R on unstimulated circulating cell types.

B) Representative dot plots of CRTh2 and CD200R in Basophils, Th2 cells and ILC2 cells (all red) vs. PBMCs (blue). **C)** Expression of CD200R on various CD4⁺ T cell subsets after 18 h of *ex vivo* polyclonal re-activation. **D-E)** Representative dot plot (**D**) and expression (**E**) of CD200R divided in CD200R^{Low}, CD200R^{Dim}, CD200R^{High} gates of various CD4⁺ T cell subsets after 18 h of *ex vivo* polyclonal re-activation. Bar graphs are represented as means $\pm$ SD.

Peanut-specific IL-5⁺ Th2 cells are characterized by high CD200R expression

Peanut-specific CD4⁺ T cells from non-allergic (NA) and peanut allergic (PA) subjects were identified by expression of the activation marker CD154 after short time (14-16h) *ex vivo* culture (Supplementary data 4A). In contrast to the general population of peripheral Th2 cells, peanut-specific CD4⁺ T cells of PA subjects were characterized by co-expression of CRTh2 and CD161 (Figure 4A), indicative of a highly differentiated Th2 cell profile. In PA subjects, expression of CD200R, was generally 7-fold higher on Th2 cells (CRTh2⁺CD154⁺) compared with non-Th2 cells (CD4⁺CRTh2⁻, $p < 0.0001$, MFI; 1198 vs 183, Figure 4B). No change in CD200R expression was detected in the peripheral CD4⁺ T cell population when comparing less differentiated Th2 cells (CRTh2⁺CD161⁻) with more differentiated Th2 cells (CRTh2⁺CD161⁺) in the PA subjects (Figure 4B). In contrast in the PA subjects, highly differentiated peanut-specific Th2 cells (CD154⁺CRTh2⁺CD161⁺) showed higher expression of CD200R than less differentiated peanut-specific Th2 cells (CD154⁺CRTh2⁺CD161⁻) ($p < 0.0006$, MFI; 2455 vs 1681, Figure 4B). Moreover in the PA subjects, highly differentiated peanut-specific Th2 cells (CD154⁺CRTh2⁺CD161⁺) expressed even more CD200R than the highly differentiated non-peanut specific Th2 cells (CD154⁻CRTh2⁺CD161⁺) ($p < 0.0002$, MFI; 2455 vs 1362, Figure 4B). Furthermore, expression of CD200R on basophils and ILC2 cells was investigated in the PA subjects (Figure 4C and alternative gating strategy Supplementary data 4B and 4C). Even though the CD200R expression was high on both

basophils and ILC2 cells, there was no difference in the CD200R expression comparing control and peanut extract stimulated cultures (Supplementary data 4D). In addition, expression of CD200R on basophils and ILC2 cells from PBMC cultures stimulated with peanut extract did not differ between NA and PA subjects (data not shown).

The CD154⁺ peanut-specific CD4⁺ T cells in the PA subjects showed a primary Th2 profile with production of both IL-4 and IL-5 (Figure 4D), which is characteristic for highly differentiated Th2 cells. These highly differentiated peanut-specific CD154⁺ Th2 cells had a significantly higher CD200R expression than Th2 cells with a less differentiated profile, characterized by production of IL-4 only ($p < 0.0018$, MFI; 2218 vs 1227, Figure 4E).

Moreover, peanut-specific CD154⁺IFN- γ ⁺ CD4⁺ T cells in the PA subjects had 6 fold lower CD200R expression than IL-4 single positive peanut-specific Th2 cells ($p < 0.0004$, MFI; 1227 vs 196, Figure 4E). Interestingly, in the peanut-specific CD4⁺ T cells of both NA and PA subjects the fraction of Th2 cells increased positively with higher CD200R expression (Figure 4F, 4G). Compared with the NA subjects, the peanut-responsive CD4⁺ T cells of PA subjects had the largest fraction of cells within the CD200R^{High} region (1% vs 44%, Figure 4A). In addition, the highly differentiated (IL-4⁺IL-5⁺) Th2 population were primarily found in the CD200R^{High} region of the peanut-responsive CD4⁺ T cells of the PA subjects (Figure 4B). In the peanut-specific CD4⁺ T cells of the PA subjects co-staining of CD161 and CD200R identified comparable fractions cells as seen by co-staining with CD161 and CRTh2 (35% vs 31%, Figure 4H, 4I). Analysing the cytokine phenotype of the CD161 either CD200R or CRTh2 co-expressing peanut-specific CD4⁺ T cells of the PA subjects showed comparable Th2 cytokine percentages and identification of primarily highly differentiated (IL-4⁺IL-5⁺) Th2 cells (Figure 4J, 4K). This indicates that CD200R in combination with CD161 can be used as an alternative to CRTH2 for the identification of highly differentiated (IL-4⁺IL-5⁺) Th2 cells.

We next analyzed transcriptomic data from lesional skin from five patients with ADex, nine patients with ADin and nine patients with psoriasis, and healthy skin from eight control subjects. When comparing lesional to healthy skin we found a significantly up-regulated expression of CD200R in ADex ($\text{Log}_2\text{-fold change (FC)} = 1.4$) and psoriasis ($\text{Log}_2\text{-FC} = 1.1$) (Figure 4L). Gene expression of the other CD200R family member CD200R2 (CD200RLa) was also investigated; however, its expression was not detected in our array data (Supplementary data 5). Interestingly, the CD200R ligand CD200 was significantly up-regulated only in skin from subjects with ADex, albeit with a low $\text{Log}_2\text{-FC} = 0.54$ (Supplementary data 5).

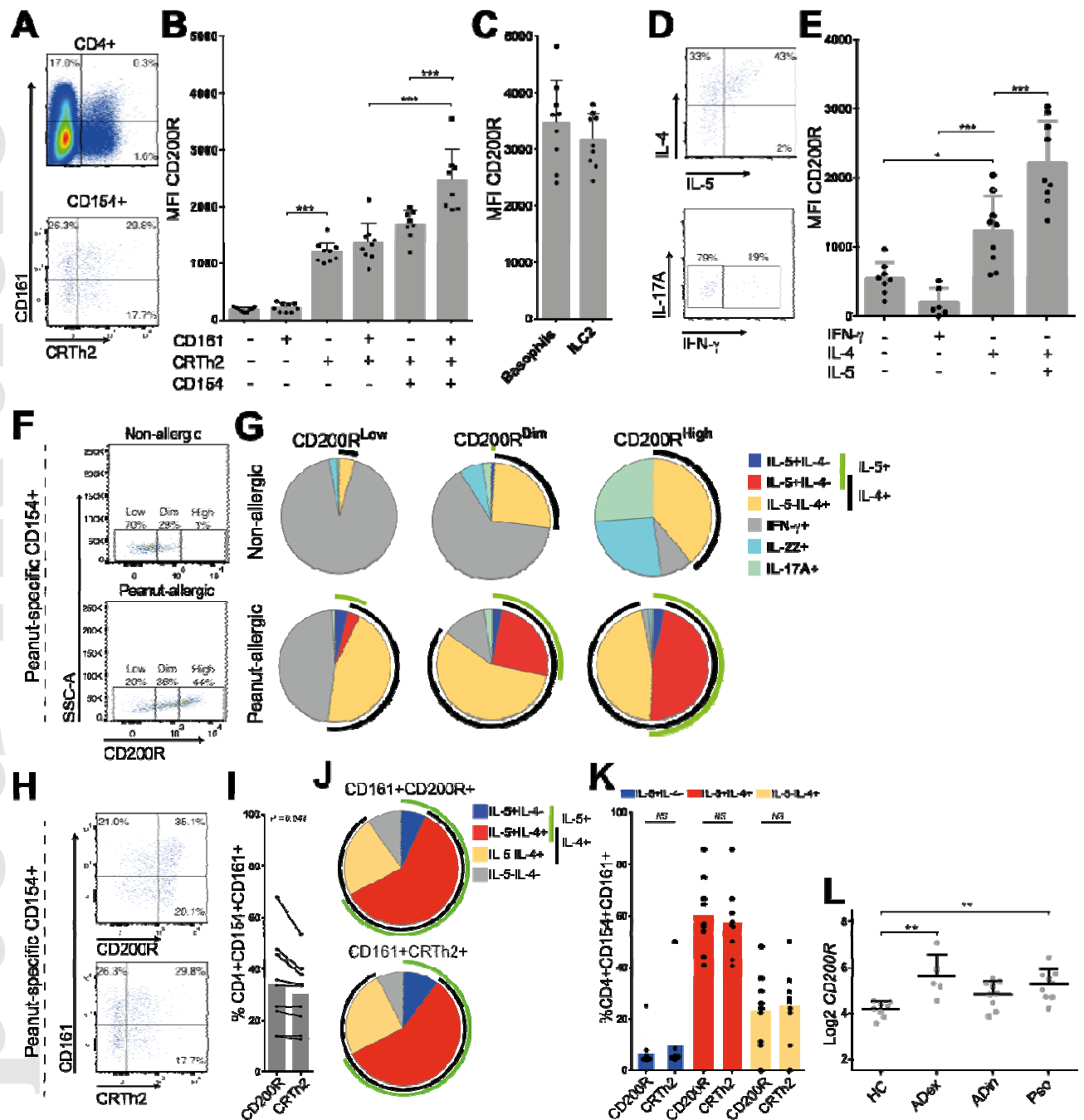


Figure 4. IL-5⁺ peanut-specific Th2 cells are characterized by high CD200R expression. Peanut-reactive CD4⁺ T cells were identified, from PA (n = 9) and NA (n = 8) subjects, by expression of CD154 after 14-16 h of *ex-vivo* stimulation with and without peanut extract. **A**) Representative phenotypic expression of CRTh2 and CD161 in CD4⁺ T cells in general and peanut-reactive CD154⁺ T cells of PA subjects. **B**) CD200R expression on CD4⁺ T cells positive for combinations of the phenotypic markers CD154, CD161, and CRTh2 in PA subjects. **C**) CD200R expression on basophils and ILC2 cells in PA subjects. **D**) Characteristic cytokines profile of peanut-reactive CD154⁺ T cells

in PA subjects. **E)** CD200R expression in peanut-specific CD154⁺ T cells producing combinations of IL-4, IL-5, and IFN- γ in PA subjects. **F)** Representative expression of CD200R and division of CD200R in Low, Dim, and High regions in the peanut-responsive CD4⁺ T cells of NA and PA subjects. **G)** Pie charts demonstrate the relative percentage of various CD4⁺ T cell subsets in the peanut-specific CD4⁺ T cells of NA and PA subjects within the CD200R^{Low}, CD200R^{Dim}, and CD200R^{High} regions. **H)** Representative phenotypic expression of CD161 and CD200R or CRTh2 in peanut-specific CD154⁺ T cells in PA subjects. **I)** Expression of CD200R and CRTh2 in peanut-specific CD154⁺CD161⁺ T cells in PA subjects. **J)** Pie charts demonstrate the relative percentage of combinations of IL-4 and IL-5 in the peanut-specific CD4⁺CD161⁺ T cells in PA subjects. **K)** Percentage of Th2 cell subpopulations in the peanut-specific CD4⁺CD161⁺ T cells in PA subjects. **L)** Transcriptome analysis of CD200R gene expression for healthy control skin (HC) (n = 8), ADex (n = 5), ADin (n = 9), and psoriasis (Pso) (n = 9) subjects. (*) p<0.05, (**) p<0.01, (***) p<0.001. The horizontal bar represents group means. Bar graphs are represented as means $\pm$ SD.

Discussion

Cells involved in type 2 inflammation are central in allergic sensitization and the pathogenesis of allergic diseases. By combining screening of >300 surface markers and fluorescent cell barcoding of *in vitro* differentiated CD4⁺ T cells we obtained an extensive, uniformly stained dataset enabling detection of novel IL-4 associated surface proteins. From this initial screening, we were able to identify CD90, CD108, CD109, and CD200R as associated with development of a Th2 profile, and consequently, these proteins were selected as candidates for further characterization. By using a series of *in vitro* and *ex vivo* approaches, we demonstrated for the first time that CD200R was in general expressed by cells involved in type 2 inflammation, such as Th2, Tc2, ILC2, and basophils. In addition, based on transcriptomic analysis of lesional skin, CD200R gene expression was found up-

regulated in the extrinsic (allergic) atopic dermatitis phenotype, underlining the connection of CD200R to an allergic phenotype. Further examination of peanut-specific CD4⁺ T cells from PA subjects, revealed that CD200R is not only expressed by Th2 cells but is especially associated with a highly differentiated Th2 phenotype characterized by IL-5 production. We confirmed on an allergen-specific level, that “inflammatory” IL-5⁺ Th2 cells were phenotypically characterized by CRTh2 and CD161 expression (7), and we are the first to document that CRTh2⁺CD161⁺ “inflammatory” Th2 cells co-express high levels of CD200R. Furthermore, gating on CD200R⁺CD161⁺ T cells identified identical Th2 subpopulations as seen when using CRTh2⁺CD161⁺ in the peanut responsive CD4⁺ T cells of PA subjects, making CD200R an alternative surface marker for identifying at least highly differentiated (IL-4⁺IL-5⁺) Th2 cells. Islam et al. observed that peripheral CD4⁺CCR8⁺ T cells were enriched for IL-5 (13). We speculate that this could be the same cell population that we have identified making co-staining of CCR8⁺ and CD200R relevant for future investigations of IL-5 producing cells.

Previous studies have shown an association of CD200R expression and development of type 2 immune responses. In agreement with our findings, *CD200R1* mRNA was recently found to be one of the top 10 up-regulated genes in peanut-specific CD4⁺ T cells of peanut allergic subjects (19). Moreover, Snelgrove et al. reported a high expression of CD200R by all granulocytes and DCs and a moderate expression on monocytes and peripheral blood T cells, especially Th2 cells (20). Interestingly, Stefano et al. showed that CD4⁺ T cells up-regulated expression of CD200R on murine effector T cells in response to pathogens that drive predominant Th1 or Th2 responses, thereby suggesting increased expression of CD200R as a marker of chronic infection (21). Of importance, they also reported a correlation between IL-4 production and CD200R expression on T cells from humans infected with the helminth *Schistosoma haematobium* (21). In line with this, we show that CD200R gene expression is

up-regulated in skin lesions from subjects with psoriasis, a non-type 2 immune response dominated disease. Further studies are needed to clarify if CD200R is a general marker of chronic infections and terminally differentiated T cells or if high expression of CD200R is restricted to cells involved in type 2 inflammation.

CD200R has a structure similar to its ligand, CD200. However, in contrast to most inhibitory receptors, CD200R does not contain immunoreceptor tyrosine based inhibitory motifs (ITIM); instead, the CD200R cytoplasmic domain contains a phosphotyrosine binding domain, NPxY (22). Upon ligand binding, CD200R signaling results in inhibition of the extracellular signal-regulated kinases (ERK), c-Jun N-terminal protein kinase (JNK) and Mitogen-activated protein kinases (MAPK) p38 pathways (22,23). Interestingly, strong T cell receptor activation of murine naïve CD4⁺ T cells has been shown to mediate activation of ERK resulting in a failure to produce endogenous IL-4 and subsequent inhibition of Th2 differentiation (24). We speculate that CD200R activation - and thereby inhibited ERK pathway activation - could be involved in the early priming phase of Th2 differentiation.

Knowledge of the immunological function of CD200R is patchy. Some studies report induction of type 2 immune responses by stimulation with the CD200R ligand CD200; in a mouse model system, using mixed lymphocyte reaction, incubation with CD200Fc decreased IFN- γ and IL-2 release and on the other hand increased IL-4 and IL-10 production (25). Furthermore, wild type DCs (CD200^{+/+} mice) induced increased IL-5 and decreased TNF- α and IFN- γ release from T cells compared to DCs from CD200^{-/-} mice (26). Moreover, D-vitamin was shown to up-regulate CD200 on peripheral human CD4⁺ T cells *in vitro*, and *in vivo* there was a trend towards up-regulation of CD200 in healthy, but not asthmatic individuals (27). We found a weak up-regulation of CD200 gene expression in subjects having an extrinsic AD phenotype, indicating that CD200 might promote the establishment of

type 2 inflammation. In addition, we found that B cells are the primary cell type that expresses CD200 in circulation. Additional phenotypic characterization of B cells could reveal the role of CD200 expressing B cells in type 2 inflammation. In a mouse model system, data indicated that interaction between CD200 and CD200R is implicated in the development of tolerogenic DCs that preferentially induce populations of Treg cells (28,29). Ligation of CD200R on human CD4⁺ T cells with CD200Fc *in vitro* reduced the differentiation of Th17 cells and reversed the defective induction of Treg cells by transforming growth factor beta in patients with systemic lupus erythematosus (SLE) (30). Our preliminary results, using CD200R ligand (CD200Fc) stimulation of *ex vivo* PBMCs from peanut allergic subjects, showed a tendency towards increased IL-5 but not IL-4 production in allergen-specific (Carboxyfluorescein succinimidyl ester^{low}) CD4⁺ T cells (data not shown). However, these are very preliminary results, and additional investigations are needed to clarify the function of CD200R on Th2 cells. We also found that circulating ILC2 and Tc2 cells were characterized by high expression of CD200R, which could indicate that CD200R is involved in a general regulation of type 2 cytokine production.

CD200R2 (CD200R1L, CD200RLa) has been described as an additional activating member within the human CD200R family (31,32). Gene expression of CD200R2 was investigated in this study, but we were not able to detect CD200R2. In contrast, the locus of CD200R1L was reported to be associated with risk of developing psoriasis and atopic dermatitis (33).

Interestingly, Fumagalli et al. were able to identify *CD200R1L* as a candidate for modulation of helminth infection (34).

Because of their dual role, both as biomarkers and as potential therapeutic targets, it is highly important to establish knowledge of phenotypic markers associated with differentiation of type 2 immune responses. Taken together, we demonstrated for the first time that CD200R is highly expressed on cells involved in type 2 immune responses, namely on Th2, Tc2, ILC2,

and basophils. All these cell types play a role in type 2 inflammation, and hopefully, future studies will add to the knowledge of the function of CD200R on these cell types.

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Author contributions

L.H.B. performed the experiments, analyzed the data, designed the study and wrote the manuscript; B.C.M. performed the experiments, analyzed the data, and critically reviewed the manuscript; L.F.L, N.J.B. organized selection of the patients and collection of the blood samples and critically reviewed the manuscript; C.V.H., M.P.C. performed the experiments and critically reviewed the manuscript; T.L. analyzed the data and critically reviewed the manuscript; L.K.P analyzed the data, contributed with design of the study, critically reviewed the manuscript, and supervised the project. All authors have commented and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interests.

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