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Gene expression analysis revealed downregulation of complement receptor 1 in clonal B cells in cold agglutinin disease

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Abstract

Cold agglutinin disease (CAD) is a rare B-cell lymphoproliferative disorder of the bone marrow, manifested by autoimmune hemolytic anemia caused by binding of monoclonal IgM autoantibodies to the I antigen. Underlying genetic changes have previously been reported, but their impact on gene expression profile has been unknown. Here, we define differentially expressed genes in CAD B cells. To unravel downstream alteration in cellular pathways, gene expression by RNA sequencing was undertaken. Clonal B-cell samples from 12 CAD patients and IgM-expressing memory B cells from 4 healthy individuals were analyzed. Differential expression analysis and filtering resulted in 93 genes with significant differential expression. Top upregulated genes included *SLC4A1*, *SPTA1*, *YBX3*, *TESC*, *HBD*, *AHSP*, *TRAF1*, *HBA2*, *RHAG*, *CA1*, *SPTB*, *IL10*, *UBASH3B*, *ALAS2*, *HBA1*, *CRYM*, *RGCC*, *KANK2*, and *IGHV4-34*. They were upregulated at least 8-fold, while complement receptor 1 (*CR1/CD35*) was downregulated 11-fold in clonal CAD B cells compared to control B cells. Flow cytometry analyses further confirmed reduced CR1 (CD35) protein expression by clonal CAD IgM+ B cells compared to IgM+ memory B cells in controls. CR1 (CD35) is an important negative regulator of B-cell activation and differentiation. Therefore, reduced CR1 (CD35) expression may increase activation, proliferation, and antibody production in CAD-associated clonal B cells.

Keywords: cold agglutinin disease, gene expression, complement receptor 1

Introduction

Cold agglutinin disease (CAD) is a rare B-cell lymphoproliferative disorder of the bone marrow characterized by autoimmune hemolytic anemia (AIHA) mediated by binding of monoclonal IgM autoantibodies to the I antigen [1]. The pathology and genetic features of CAD have been studied in detail during the last decade, leading to the recognition of CAD as a distinct lymphoproliferative disease by the 2022 World Health Organization classification of hematolymphoid tumors and the International Consensus Classification of Mature Lymphoid Neoplasms [2–6]. *KMT2D* and *CARD11* genes are recurrently mutated in CAD B cells. *KMT2D* has loss-of-function mutations, and *CARD11* has gain-of-function mutations [3]. More recently, a more complete mutational landscape of CAD has been described. Of interest, *CARD11* and *CXCR4* mutations were correlated with hemoglobin levels, suggesting pathogenic involvement [7]. Further, trisomy 3 and 12 or 18 are common in CAD, with the presence of trisomy 12 and 18 correlating with response to treatment [2]. Since CAD is a clonal B-cell disease, B-cell-directed therapy is now the treatment of choice for most patients [8]. Recently, inhibition of complement protein C1s by sutimlimab has been documented as an alternative, highly efficacious approach with selective upstream inhibition of the classic complement pathway and halted hemolysis [9].

Here, we present novel RNA-sequencing (RNA-seq) data from 12 CAD patients. Differential expression analysis revealed that the complement receptor 1 (*CR1/CD35*) gene was the most significantly downregulated gene in CAD B cells compared with controls. Flow cytometry analysis further confirmed reduced CR1 (CD35) protein expression on clonal CAD B cells. CR1 (CD35) is a membrane-bound complement receptor and an important negative regulator of B-cell differentiation towards immunoglobulin-producing plasma cells.

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Moreover, it is shown to be downregulated on B cells in some autoimmune diseases, i.e. rheumatoid arthritis and systemic lupus erythematosus [10–16]. Its reduction may, therefore, contribute to CAD pathogenesis.

Materials and methods

Materials and RNA-seq analysis

Peripheral blood and bone marrow samples were obtained from CAD patients enrolled in a prospective trial [8]. Additionally, peripheral blood was obtained from healthy controls. The diagnostic criteria for CAD have been reported previously [8]. Clonal B cells from bone marrow of 12 CAD patients were enriched by fluorescence-activated cell sorting as published before [17] (Supplementary Fig. S1). Immunophenotype of the clonal B cells for each patient is shown in Supplementary Table S1. Normal memory B cells (CD19+/CD27+/IgM+) from four healthy controls were stained for surface antigens with antibodies purchased from BioLegend [anti-CD27 (clone O323) and anti-IgM (clone MHM-88)], Beckman Coulter [anti-CD19 (clone J3-119)], and Becton Dickinson [anti-CD3 (clone SK7) and anti-CD45 (clone 2D1)]. Both CAD and healthy control samples were sorted using a FACS Aria Ilu high-speed sorter (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with 408, 488, and 633 nm lasers. DNA and RNA were extracted simultaneously from the same cells using Qiagen AllPrep DNA/RNA Micro Kit. DNA was analyzed previously and the results have been published [2, 3, 7]. RNA library preparation was performed using Kappa Total RNA prep combined with Twist capturing system Human Core Exome (according to manufacturer recommendations), followed by library QC and sequencing using NextSeq500 HighOutput flow cell with 2 × 75 bp paired-end sequencing that was performed at the Genomics Core Facility at Oslo University Hospital/University of Oslo.

Analysis to assess overall similarity between samples from CAD patients and controls

Two methods were used to check overall similarities of total gene expression between CAD B cells and normal memory B cells. First, sample distances were calculated using the Poisson Distance implemented in the 'PoiClaClu' R package (Supplementary Fig. S2A). Then the R function 'dist' was used to calculate the Euclidean distance between samples (Supplementary Fig. S2B). Both methods resulted in clustering of controls within patients' samples, a result expected from appropriate control's samples. Control samples were very homogeneous, while CAD samples were heterogeneous.

Differential expression analyses

Reads were aligned to hg38 with the use of STAR v2.7.0f. Analyses were performed on Services for Sensitive Data (TSD) server at the University of Oslo. Reads were counted with both STAR and FeatureCounts, and differential expression was analyzed using both DESeq2 and EdgeR. Results from FeatureCounts followed by DESeq2 were used for the main analysis (order of presented genes). Results from STAR followed by EdgeR were then used for validation. Genes were chosen based on results from FeatureCounts followed by DESeq2 with P-adj < 0.001 (P-value adjusted for multiple testing; top 530 genes) and from STAR followed by EdgeR with FDR < 0.05 (false discovery rate; top 550 genes). Only genes that were detected as statistically significant by both methods were used for further analyses. Additionally, immunoglobulin lambda light chain (IGL) genes were removed, since CAD samples were sorted for immunoglobulin kappa light chain (IGK) expressing B cells. T-cell receptor genes were also removed. The final list contained 306 statistically significant genes. In search of biologically relevant changes in gene expression, the list was further scrutinized by keeping only genes with fold change <–8 or >8 and median number of reads, in at least one group, at minimum of 50 reads. The final list contains 93 genes. This stringent cutoff for differential expression analysis was applied to rule out any spurious results.

Gene expression of the final 93 genes was visualized using hierarchical clustering and heatmap generated by Bioconductor package. Normalized number of reads for each sample is presented, to account for differences in library size. In order to present the data, a log2 transformation was used. All raw data presented in Tables and Figures are normalized with the use of DESeq2 to account for differences in library sizes. In addition, Volcano plot (https://huygens.science.uva.nl/VolcaNoseR2/) was used for visualizing top 306 statistically significant genes.

Fusion protein testing

Testing for fusion genes, large indels, and other structural variants was performed with the use of STAR, STAR-fusion, and Manta programs.

Flow cytometry validation of CR1 (CD35) expression

Flow cytometry analysis was performed as described before with the addition of antibody for CR1 (CD35) from BioLegend [PE anti-human CD35 Antibody (clone E11)] [17]. In short, viable cells were gated using the forward scatter versus side scatter dot plot, then CD45 bright, low side scatter events (i.e. lymphocytes) were selected. CD5+ and CD19– events (i.e. T cells) were gated out, leaving only B cells. Finally, monoclonal B cells were separated from the polyclonal B cells using the immunoglobulin light chain gate, taking advantage of the fact that B-cell clones show either kappa or lambda light chain restriction [3, 17]. CR1 (CD35) expression was assessed separately in IGK and IGL expressing B cells [17]. Since our cohort did not contain patients with IGL expressing cold agglutinins, IGL+ B cells were used as in-sample controls. CR1 (CD35) expression in IGK and IGL expressing B cells was analyzed both for CAD patients and for controls.

Results

Gene expression by RNA-seq analysis

The 93 most differentially expressed genes in the samples of CAD patients are listed in Supplementary Table S2. The top 20 differentially expressed genes were: *SLC4A1*, *SPTA1*, *YBX3*, *Tescalcin* (*TESC*), *HBD*, *AHSP*, *TRAF1*, *HBA2*, *RHAG*, *CR1* (*CD35*), *CA1*, *SPTB*, *IL10*, *UBASH3B*, *ALAS2*, *HBA1*, *CRYM*, *RGCC*, *KANK2*, and *IGHV4-34* (Supplementary Table S5; Fig. 1A, B and F). Nineteen of these 20 genes were upregulated at least 8-fold compared to controls. Only one, namely *CR1* (*CD35*), was downregulated about 11-fold. P-value and P-value adjusted for multiple testing were equal or lower than 3.0×10^{-12} and 2.7×10^{-9}, respectively, therefore considered highly significant.

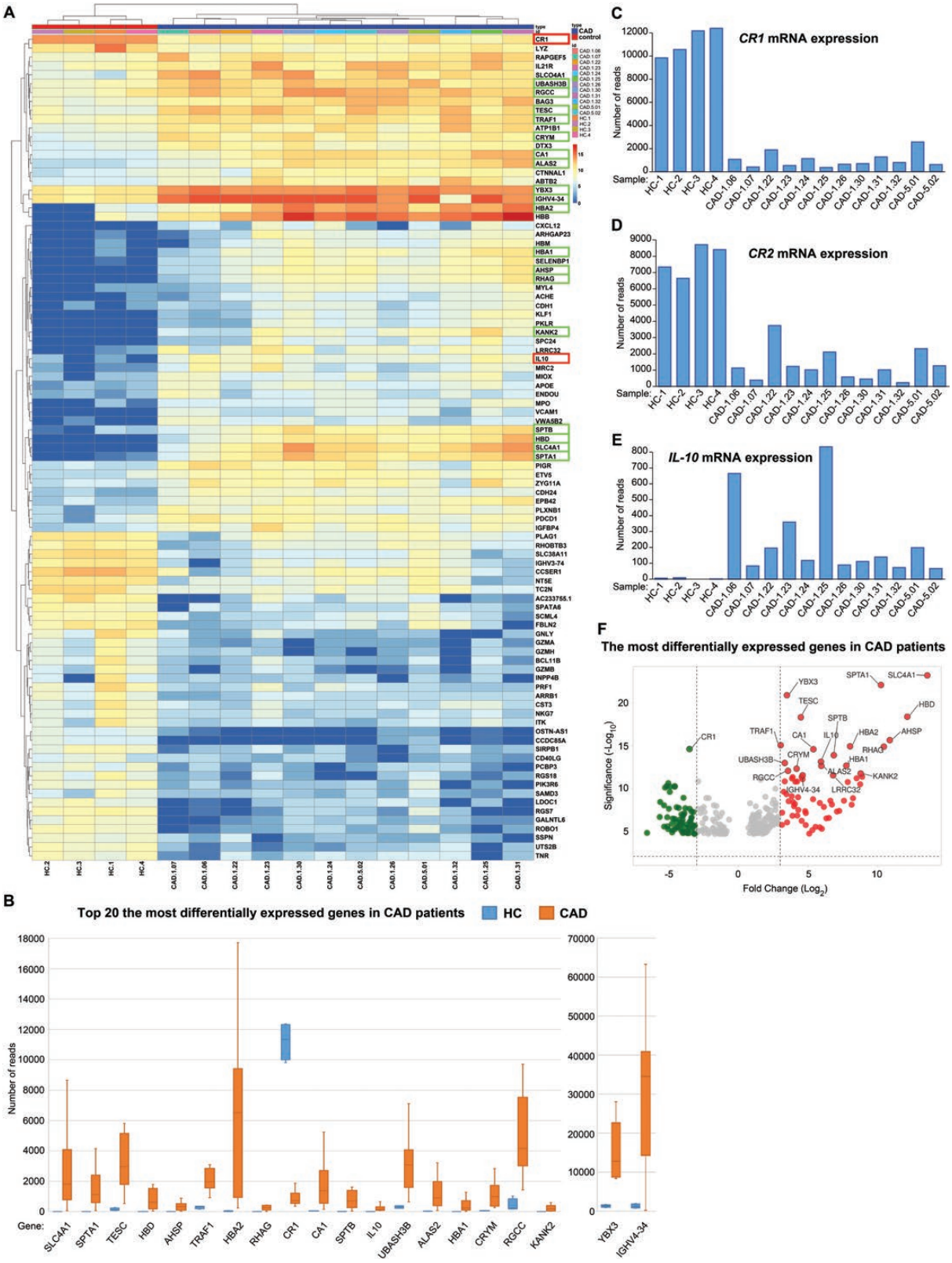

Figure 1. Analysis of the most differentially expressed genes in CAD patients with a focus on biologically relevant changes. (A) Heatmap showing 93 of the most differentially expressed genes. Colors correspond to expression levels. Top 20 genes are marked with green boxes, with exception of *CR1* (*CD35*) and *IL-10* that are marked with red boxes. (B) Box plots visualizing gene expression levels for top 20 differentially expressed genes. All genes are highly upregulated in comparison to controls, with the exception of *CR1* (*CD35*) gene that is highly downregulated in CAD. Controls are shown in blue and CAD patients in orange. Minimum and maximum values together with median, first and third quartile are shown. There is separate scale for *YBX3* and *IGHV4-34* due to significant difference in expression. (C–E) Visualization of *CR1* (*CD35*), *CR2* (*CD21*), and *IL-10* expression based on RNA-seq data for CAD patients and controls. For *CR1* (*CD35*), *CR2* (*CD21*), and *IL-10* the difference between CAD and controls is about 11, 6, and 60 times, respectively. (F) The most differentially expressed genes in CAD. Volcano plot is visualizing top 306 statistically significant genes detected by two

CR1 (*CD35*) was downregulated, and its expression level was very similar within each group, but the difference between patients and controls was about 11 times (Fig. 1C). When using STAR followed by EdgeR, *CR1* (*CD35*) was assigned at the top of the most differentially expressed genes. Since *CR1* (*CD35*) was strongly downregulated, we investigated complement receptor 2 (*CR2/CD21*) in our RNA-seq data and found *CR2* (*CD21*) also to be downregulated in CAD, but not as pronounced as *CR1* (*CD35*). The *P*-value is 1.3×10^{-6}, while *P*-value adjusted for multiple testing is 1.1×10^{-4}, but with a 6-fold change (Fig. 1D). Therefore, *CR2* (*CD21*) was not included in the list of most differentially expressed genes.

Interleukin 10 (*IL-10*) was highly upregulated (Fig. 1E). Controls had practically no expression of *IL-10*, while CAD B cells had substantial expression of this cytokine (*P*-value 7.6×10^{-14} and *P*-value adjusted for multiple testing 1.2×10^{-10}).

The most significantly upregulated pathways, according to Reactome (https://reactome.org/), were connected to O_2/CO_2 exchange in erythrocytes with 6 genes: *HBA1*, *HBB*, *HBA2*, *RHAG*, *SLC4A1*, and *CA1* in the top 25 most differentially expressed genes (Fig. 1A; Supplementary Tables S2 and S3). In addition, genes associated with erythrocyte membrane design, *SLC4A1*, *SPTA1*, *SPTB*, and *EPB42* were upregulated and were among the top 30 differentially expressed genes (Fig. 1A; Supplementary Table S2).

Validation of CR1 (CD35) expression by flow cytometry

Reduced *CR1* (*CD35*) gene expression was validated by flow cytometry (Fig. 2; Table 1). CR1 (CD35) protein expression was assessed in blood or bone marrow samples obtained from 15 CAD patients (11 frozen and 4 fresh samples; Fig. 2A). Additionally, five non-clonal B-cell samples from CAD patients in complete remission (Fig. 2B) and blood samples from five controls (Fig. 2C) were analyzed. For each sample, CR1 (CD35) expression was compared between IgM+ CD27+ CD19+ memory B cells expressing IGK+ and the CAD clone (if possible to separate), as well as IgM+ memory B cells expressing IGL+, respectively. The latter served as non-clonal patient-specific normal B-cell controls. It was considered important to have patient-specific controls as CR1 (CD35) expression may vary between individuals. In CAD patients, CR1 (CD35) expression by clonal IGK+ B cells was significantly lower than by IGL+ control B cells (Table 1; Fig. 2A and D) as indicated by a median fluorescence intensity that was 11 times lower in clonal B cells than in normal IGL+ B cells. In addition, there were five CAD samples without detectable clonal CAD B cells at the time of testing, and three of those were dominated by immature B cells likely related to regeneration after recent B-cell-directed therapy. In these cases, IGK+ and IGL+ B cells had almost the same level of CR1 (CD35) expression (Fig. 2B and D). B cells from controls showed similar expression of CR1 (CD35) by both IGK+ and IGL+ B cells (Fig. 2C and D). Samples from clonal CAD patients usually showed a bimodal CR1 (CD35) expression by IGK+ B cells, but not by IGL+ B cells (Fig. 2A).

Fusion gene testing

Extensive testing for fusion genes, large indels, and other structural variants with the use of three programs (STAR, STAR-fusion, and Manta) was performed. No fusion genes were detected.

Discussion

We report dysregulation of gene expression in B cells derived from CAD patients compared to normal memory B cells. Using RNA-seq analysis, we found 93 genes that were highly differentially expressed in CAD B cells, including *CR1* (*CD35*) that was downregulated, and *SLC4A1*, *SPTA1*, *YBX3*, *TESC*, *HBD*, *AHSP*, *TRAF1*, *HBA2*, *RHAG*, *CA1*, *SPTB*, *IL10*, *UBASH3B*, *ALAS2*, *HBA1*, *CRYM*, *RGCC*, *KANK2*, and *IGHV4-34* that were upregulated. Importantly, using flow cytometry analysis, we confirmed reduced membrane expression of CR1 (CD35) in CAD B cells compared to normal memory B cells.

CR1 (CD35) is the receptor for C3b and C4b complement peptides [18]. Its main functions are inhibition of the complement cascade [19] and regulation of B-cell proliferation [20–22]. CR1 (CD35) blocks proliferation induced by B-cell receptor (BCR) activation, and in addition, inhibits differentiation of B cells into plasmablasts and plasma cells, as well as their immunoglobulin production [10]. CR1 (CD35) ligation decreases phosphorylation of key molecules of the BCR-induced signaling cascade [11]. Among other upstream targets in the classical complement pathway, CR1 (CD35) inhibits opsonization with C3b, which is a major hemolytic mechanism in CAD [16]. There are several studies showing an association of CR1 (CD35) with autoimmune diseases, but not much is known about CR1 (CD35) association with other diseases. However, there are some studies on CR1 (CD35) in malaria, HIV, SARS, carcinoma of the gallbladder, and other diseases [20]. CR1 (CD35) downregulation on lymphocytes, erythrocytes, and other types of cells is characteristic of several diseases [20]. Reduced expression of CR1 (CD35) has been observed on B cells in autoimmune conditions, e.g. in rheumatoid arthritis [10, 23] and systemic lupus erythematosus [11].

We found that CR1 (CD35) is highly downregulated in clonal B cells from CAD patients. This finding is of interest because complement, in addition to its critical role for hemolysis in CAD [1, 16], has been shown to control B- and T-cell responses, and thus is a regulator of adaptive immunity [24]. Although we have focused on *CR1* (*CD35*), we also found *CR2* (*CD21*) expression to be downregulated in CAD. CR1 (CD35) has different functions depending on the cell type by which it is expressed. In B cells, the main function of CR1 (CD35), together with CR2 (CD21), is regulation of proliferation [21, 22]. CR1 (CD35) binding to C3b and CR2 (CD21) binding to C3d inhibits BCR-mediated activation,

methods as explained in materials and methods. Log2 fold change is shown on *X*-axis and significance (calculated as −Log10 of *P*-value) on Y-axis. Log2 fold change threshold is set so that values <−3 and >3 are considered significant (it corresponds to fold change <−8 and >8). Top 20 genes are marked with names. Genes marked as green are downregulated while genes marked as red are upregulated. Note: panel A shows Log2 transformed data (transformation is necessary for visualization purposes), other panels however are showing raw data after normalization only, necessary to account for differences in library size. HC: controls, *X*-axis: patient or control, *Y*-axis: number of reads.

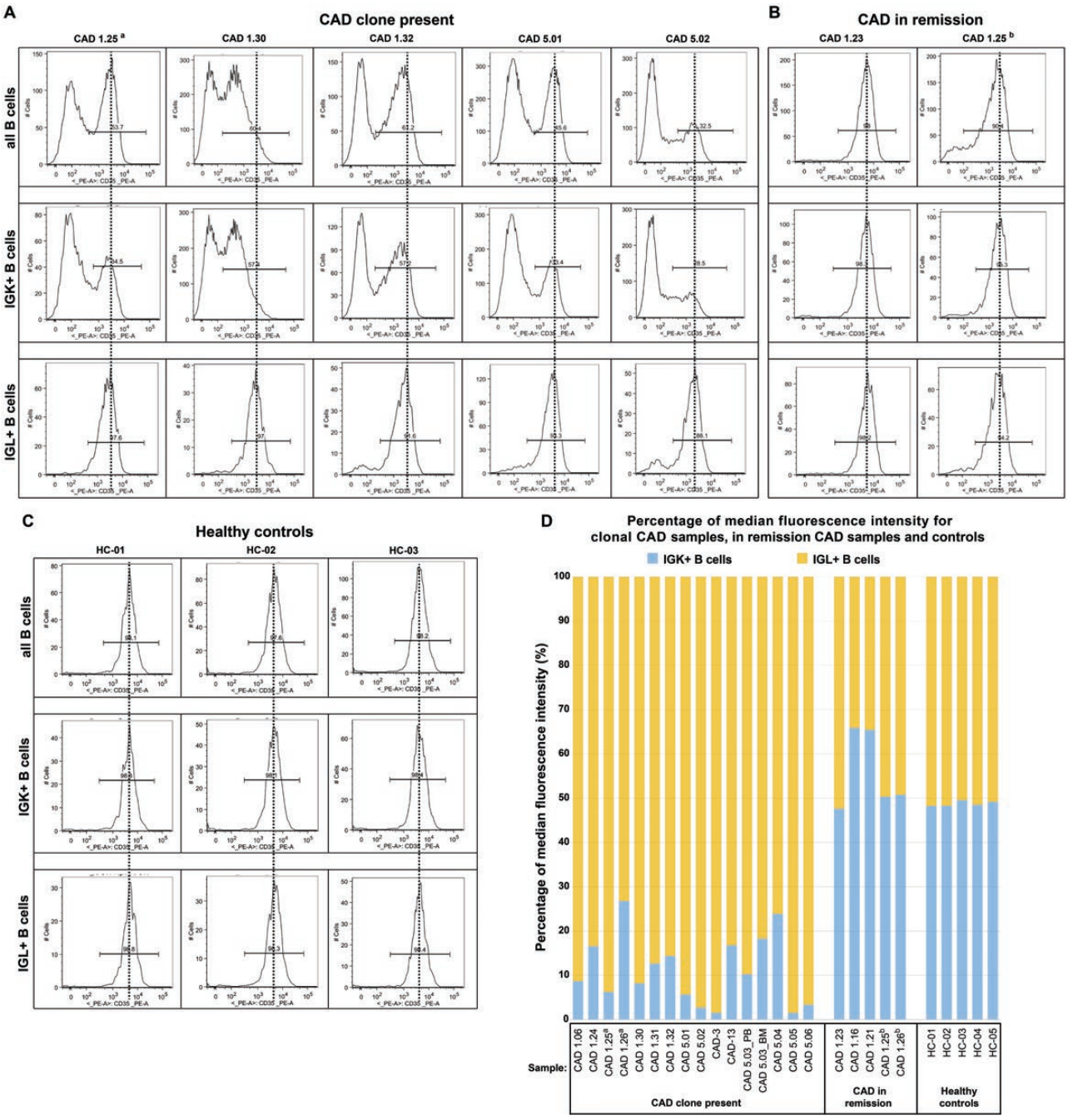

Figure 2. Validation of CR1 (CD35) expression by flow cytometry. (A) Samples from CAD patients with demonstrated CAD clone. (B) Samples from CAD patients in remission. (C) Controls. (D) Median fluorescence intensity (100% stacked column chart; values are shown in columns that are stacked to represent 100%) for IGK+ B cells (containing clonal CAD B cells) and normal IGL+ B cells from the same patient. IGK+ and IGL+ B cells from CAD patients in remission and control samples are also presented. Samples with clonal B-cell population show a significant reduction in CR1 (CD35) expression in clonal CAD B cells compared to normal IGL+ B cells. In contrast, patients in remission at the time of testing and controls (HC) show similar CR1 (CD35) expression between IGK+ and IGL+ B cells. The median fluorescence intensity is used for comparisons, since it is the most informative measure of central tendency for skewed distributions or distributions with outliers. Note: IGK+ B cells: IGM+ memory B cells expressing IGK, IGL+ B cells: IGM+ memory B cells expressing IGL. [a]Sample from a CAD patient with demonstrated CAD clone at the time of testing, [b]sample from a CAD patient in remission at the time of testing.

proliferation, and antibody production [21]. Therefore, downregulated expression of CR1 (CD35) and CR2 (CD21) may be part of a mechanism that allows autoreactive CAD B cells to proliferate. Downregulation of CR1 (CD35) and CR2 (CD21) expression may thus result in increased antibody production. CR1-mediated downregulation of B-cell proliferation [10, 11, 15] through autoantigen/suboptimal antigen binding to BCR has previously been demonstrated in autoimmune disease. Both CR1 (CD35) and CR2 (CD21) were reported to be downregulated in several autoimmune diseases, e.g. systemic lupus erythematosus [25]. There is contradictory data on the role of CR2 (CD21) in B-cell proliferation. However,

Table 1. CR1 (CD35) expression measured by flow cytometry, mean and median fluorescence intensity for different B-cell populations

Samples	CAD clone	Material	Total B cells CD19+ CD20+		IGK+ B cells		CAD clone B cells		Normal/IGL+ B cells	
			Mean FI	Median FI	Mean FI	Median FI	Mean FI	Median FI	Mean FI	Median FI
CAD 1.06	CD5+ K+	BM	1004	261	986	255	973	245	3413	2571
CAD 1.24	CD5– K+	BM	847	491	715	415	572	347	2112	1745
CAD 1.25	CD5– K+	PB	1703	885	1222	264	806	177	3138	2656
CAD 1.26	CD5– K+	PB	3937	3001	3486	2527	2613	1665	5429	4548
CAD 1.30	CD5– K+	BM	720	251	585	224	585	224	3148	2520
CAD 1.31	CD5+ K+	PB	736	605	735	605	726	596	4787	4114
CAD 1.32	CD5– K+	BM	1473	731	1181	408	1181	408	2862	2428
CAD 5.01	CD5– K+	BM	1644	344	1212	170	1212	170	3291	2841
CAD 5.02	CD5+ K+	BM	711	88	490	62	208	48	2017	1728
CAD-3	CD5+ K+	BM	2354	1429	1886	773	289	49	4034	3155
CAD-13	CD5– K+	PB	981	486	1002	524	1002	524	3306	2597
CAD 5.03[a]	CD5+ K+	PB(F)	3771	2855	3198	2000	902	497	5437	4347
CAD 5.03[a]	CD5+ K+	BM(F)	504	221	474	215	432	210	1776	938
CAD 5.04	CD5+ K+	BM(F)	2086	1321	2036	1295	1841	1099	4505	3504
CAD 5.05	CD5+ K+	BM(F)	1450	110	1065	75	449	55	4162	3611
CAD 5.06	CD5– K+	BM(F)	814	103	625	94	424	88	3617	2520
CAD 1.23	No clone	BM(F)	5973	5261	5733	5054	–	–	6275	5560
CAD 1.16	No clone[b]	BM	567[c]	295[c]	1070	731	–	–	612	379
CAD 1.21	No clone[b]	BM	587[c]	366[c]	916	677	–	–	562	358
CAD 1.25	No clone	BM	2246	1669	2898	2422	–	–	2922	2392
CAD 1.26	No clone[b]	BM	587[c]	377[c]	814	363	–	–	533	352
HC-01	No clone	PB(F)	5378	4664	5228	4537	–	–	5592	4867
HC-02	No clone	PB(F)	5421	4594	5278	4458	–	–	5611	4770
HC-03	No clone	PB(F)	5135	4293	5126	4250	–	–	5124	4325
HC-04	No clone	PB(F)	8652	7242	8531	7170	–	–	8824	7616
HC-05	No clone	PB(F)	7355	5966	7204	5906	–	–	7618	6103

[a]The same patient.
[b]Total B cells CD19+ (mostly immature B cells in this sample, CD20 weak/neg, IGK/IGL neg).
[c]CD20 neg B cells included.
Abbreviations: IGK+ B cells, IGM+ memory B cells expressing IGK; IGL+ B cells, IGM+ memory B cells expressing IGL; FI, fluorescence intensity; BM, bone marrow; PB, peripheral blood; (F), fresh material; HC: controls.

a recent report indicates that co-clustering of CR2 (CD21) with BCR inhibits proliferation and antibody production by B cells, especially at suboptimal levels of BCR stimulation [22]. This would suggest that pronounced downregulation of CR1 (CD35) and CR2 (CD21) enables autoreactive B cells to proliferate and survive.

CR1 (CD35) on erythrocytes functions as an inhibitor of the complement cascade [19]. In this study, gene expression analyses as well as flow cytometry analyses were performed on B cells retrieved from a biobank established for samples from CAD patients enrolled in previous clinical studies. Erythrocytes were not available for flow cytometry analyses and could not be investigated. Downregulation of CR1 (CD35) expression on erythrocytes has previously been reported in other diseases, including autoimmune disease [26–29]. Reduced expression of CR1 (CD35) on erythrocytes in CAD has previously been shown in case series, but systematic studies are lacking [26, 30, 31].

IL-10 gene expression was substantially upregulated in CAD B cells with almost no expression in controls. Primed B cells can secrete IL-10 after CD40 and BCR ligation [32], and activated IL-10 secretors include $CD24^{hi}CD27+$ and $CD27^{hi}CD38^{hi}$ plasmablast B-cell compartments [33]. IL-10 may have anti-inflammatory feedback and regulatory effects, but its precise role in human B-cell biology is still unclear. High expression in CAD B cells could suggest recent BCR ligation and activation of the signaling cascade together with CD40-pathway activation, such as provided help from T helper (Th) cells. Several studies have shown increased expression of IL-10 and a role of IL-10 in AIHA [34–37], and IL-10 expression in B cells may be positively correlated with disease severity [38]. It has also been suggested that genetic factors influencing IL-10 production may increase the risk of developing AIHA [39]. Neutralization of IL-10 has been considered for treatment also in CAD [36].

In addition, several genes connected to cancer were overexpressed in CAD B cells. These include the cold shock protein YBX3, which regulates mRNA transcription, splicing, and translation, and has a role in modulating stress responses, inflammation, and cancer development [40, 41]. TESC plays a role in hematopoietic stem cell differentiation and growth, and its high expression contributes to invasive and metastatic activity in colorectal cancer [42]. Tumor necrosis factor

receptor-associated factor 1 (TRAF1) is involved in the classical NF-kB activation and is overexpressed in many B-cell malignancies, including chronic lymphocytic leukemia, non-Hodgkin lymphoma, and Burkitt lymphoma/leukemia [43–45]. The protein tyrosine phosphatase UBASH3B is upregulated in prostate cancer [46], and its overexpression in breast cancer promotes invasion and metastasis [47].

Other overexpressed genes, such as *HBA1*, *HBB*, *HBA2*, *RHAG*, *SLC4A1*, and *CA1*, code for proteins involved in the uptake and release of oxygen and carbon dioxide in erythrocytes. Moreover, genes connected to hereditary spherocytosis, *SLC4A1*, *SPTA1*, *SPTB*, and *EPB42*, which is the most common red blood cell membrane disorder [48], were overexpressed in the samples of CAD patients. These genes, which code for erythrocyte membrane proteins, were essentially not expressed in controls, whereas their expression in CAD B cells was substantial. It should be noted that expression of hemoglobin and other erythrocyte-related genes is not limited to erythroid cells only, but is also found in a variety of other cells [49–51]. Also, genes of the *SLC4* family are overexpressed in many cancer types, which may have diagnostic value and therapeutic potential in cancer treatment [52–54].

The above results of our RNA-seq analysis suggest that CAD B cells contain substantial amounts of mRNA for genes that are typically expressed in erythrocytes, which is puzzling. Our stringent flow-cytometry sorting criteria rule out the possibility that sorted CAD B cells were simply contaminated with erythroid precursors or mature erythrocytes. However, one possible scenario is the transfer of mRNA from these cells to clonal B cells after phagocytosis. Indeed, erythrocyte phagocytosis by human lymphocytes was shown over 50 years ago [55, 56], and more recent studies confirmed that B cells have phagocytic capabilities [57–60]. Multiple studies have shown that plasma cells can phagocyte erythrocytes, especially in disease states such as multiple myeloma [61–64] and monoclonal gammopathy of undetermined significance [65]. In addition, it was shown that B cells are able to phagocyte 3-μm latex beads coated with anti-IgM antibodies, and that B-cell phagocytosis is required for potent humoral response [66].

Recent experiments may further explain the mechanism of RNA transfer into CAD clonal B cells. First, immune response to sheep erythrocytes is driven by cytosolic recognition of sheep erythrocyte RNA through the RIG-I-like receptor–mitochondrial anti-viral signaling adaptor pathway in phagocytes [67], suggesting that erythrocyte RNA can be transported into the phagocyte cytoplasm. Second, some malignant IgM+ B cells, such as CLL cells, can phagocytose large (3 μm) particulate antigens and present Ag to T cells [68]. Third, very recent alloimmunization experiments demonstrated that B cells can activate T cells by presenting cytosolic erythrocyte antigens by an unknown mechanism, possibly by trogocytosis [69].

Since BCR on clonal CAD B cells is autoreactive against I antigen present on erythrocytes [1], it is likely that CAD B cells can bind erythrocytes by interacting with I antigen on their surface. Moreover, in light of published literature, it is plausible that after such binding some erythrocytes will be phagocytosed by CAD B cells, and in this way, erythrocyte RNA can be transferred to B cells' cytoplasm. Although erythrocytes cannot synthetize new RNA molecules, they still contain a large number of RNA transcripts [70, 71]. Alternatively, instead of internalizing erythrocytes, CAD B cells could, in fact, have internalized erythrocyte extracellular vesicles (RBCEV) that are increasingly generated after complement activation and are increased in patients with AIHA and other diseases with complement-mediated lysis [72]. Importantly, RBCEV have been demonstrated to very efficiently transfer mRNA into target cells [73]. Elevated levels of RBCEV have been also found in CAD patients; moreover, it correlated with the severity of anemia and hemolytic features [74].

In addition to BCR, Fc and complement receptors might be taking part in erythrocyte uptake and phagocytosis. Phagocytosis via Fc and complement receptors was shown many years ago [75]. CR1 (CD35) and Fc receptors were shown to cooperate in uptake of particles covered by complement proteins and immunoglobulins [20]. In summary, it is plausible, but unproven that the partial erythrocyte-like gene expression profile detected in CAD B cells was not derived from CAD B cells, but rather from erythrocytes, either whole erythrocytes or RBCEV. This RNA transfer could be an imprint of the B-cell specificity in this disease. Further studies are required to investigate the potential effect of erythrocyte antigens and erythroid RNA on CAD B cells.

One of the genes that was found to be highly upregulated in all CAD samples, except for one, was *IGHV4-34*. This was expected and underscores that our analysis was performed on CAD B cells since it is well established that the *IGHV-4-34* gene is used by the clonal B cells in almost all CAD patients [17].

Conclusion

In conclusion, we demonstrate that CR1 (CD35) expression is significantly downregulated in clonal B cells in CAD, both at the mRNA and the protein level. Since CR1 (CD35) blocks proliferation induced by the BCR, downregulation of CR1 (CD35) in CAD might be responsible for increased proliferation and survival of autoreactive B cells. Deregulation, secondary to CR1 (CD35) downregulation, could lead to enhanced activation, IgM production, and secretion of autoantibodies in CAD B cells. We also found that *IL-10* and some other genes were substantially upregulated in CAD B cells, but their role in CAD requires further investigations.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Conflict of Interests

Authors declare no financial conflict of interest with regard to publication of this manuscript.

Author contributions

AM, IØ, GT, JD, AT, SB and GET designed the study. AM and IØ performed the analyses. AM, IØ, GT, JM, JD, LAM and GET discussed the results. GT, JD, AT, SB and GET supervised the study. JD, AT, SB and GET reviewed the diagnostic patient samples and collected the clinical data. AM, JD, JM, LAM and GET prepared the manuscript. All authors have critically read the manuscript.

Ethical approval

The patients included in this study were enrolled in a clinical trial. The study was approved by the Regional Committee for Medical and Health Research Ethics of South-East Norway (2012/131/REK).

Patient consent statement

Written informed consent was procured by using consent forms approved by the Regional Committee for Medical and Health Research Ethics of South-East Norway.

Data availability

In accordance with Norwegian legislation and the ethic approval of the study, all sensitive data are stored in protected databases at Oslo University Hospital. On request, the data will be made available for other institutions. Additional approval might be required before sharing. However, non-sensitive data will be shared upon request. For the original data, please contact the corresponding author.

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