Abstract
The maintenance of B cell tolerance is dependent on cell surface molecules that serve to modulate signaling through the B cell receptor (BCR), such as Fc gamma receptor IIb (FcγRIIb; CD32b) and complement receptor (CR) 1 (CD35) and 2 (CD21). Consequently, deficiency in these receptors might lead to autoimmunity. We have studied the basal expression of FcγRIIb, CR1 and CR2 on B cells in healthy male and female blood donors in comparison to rheumatoid arthritis (RA) patients to reveal any possible differences. Firstly, we found a gender associated difference in FcγRIIb expression, as healthy women had lower basal expression of FcγRIIb on B cells than men and the expression significantly decreased with age. Secondly, RA patients displayed lower numbers of FcγRIIb, CR1 and CR2 positive B cells and decreased FcγRIIb, CR1 and CR2 receptor expression compared to healthy controls. Furthermore, the proliferation capacity of RA B cells following low dose IgG anti-µ stimulus was investigated. Even though RA B cells had lower FcγRIIb expression, the receptor was still able to inhibit BCR-mediated proliferation, however, not as significantly as in B cells from healthy subjects. Notably, the RA B cells proliferated more frequently from a BCR-independent IL-2 stimulation than B cells from healthy controls, suggesting that IL-2 signals generated from any activated T cell may initiate an unspecific B cell proliferation in RA patients. In conclusion, the uncontrolled B cell response seen in RA patients may be associated with lower levels of inhibitory FcγRIIb, CR1 and CR2. The lower basal expression of FcγRIIb in females might contribute to the increased incidence of RA in women.
Introduction
Rheumatoid arthritis (RA) is an autoimmune disease characterized by autoantibodies and chronic joint inflammation [1]. Like many other autoimmune diseases has RA a clear gender bias as women are more often affected than men and female sex hormones such as estrogen modulate the disease (reviewed in [2]). Thus, increased estrogen levels as seen during pregnancy, are associated with amelioration of RA [3], while reduced levels, as seen post partum or at menopause, are associated with disease flare or onset of RA [4; 5]. Furthermore, studies in collagen-induced arthritis (CIA), an animal model of RA, have shown that the disease can be suppressed by estrogen treatment and that estrogen influences the B cell response [6; 7].

The involvement of B cells in RA pathology has been recognized. Thus, B cells can be found in infiltrates of the inflamed joints and removal of B cells by anti-CD20 depleting therapy results in improvement of the disease [8; 9]. In addition, specific autoantibodies such as rheumatoid factor (RF) or anti-cyclic citrullinated peptide antibodies can be found years before disease onset, pointing towards an early break in B cell tolerance [10; 11].

For the generation and control of an appropriate B cell immune response, balanced signaling through regulatory receptors is important. The inhibitory Fc gamma receptor IIb (FcγRIIb; CD32b) is a low affinity receptor that binds antigen-bound IgG. When FcγRIIb is cross-linked with the B cell receptor (BCR) it reduces B cell activation and antibody production [12; 13; 14; 15]. The importance of FcγRIIb in peripheral B cell tolerance has been indicated in FcγRIIb-deficient mice, which on a specific genetic background develop spontaneous lupus like symptoms [16] or enhanced susceptibility to CIA [17; 18]. In humans altered FcγRIIb expression on leukocytes has been linked to systemic lupus erythematosus (SLE) and a FcγRIIb allele variant is associated with the degree of joint damage in RA [19; 20; 21]. Other important co-receptors modulating B cell activation are complement receptor (CR) 1 (CD35) and 2 (CD21). CR1 binds C3b, iC3b and C4b and functions as a powerful inhibitor of both the classical and the alternative pathway C3- and C5-convertases, due to its decay-accelerating capacity and co-factor activity for C3b and C4b cleavage. CR1 has also been demonstrated to have an inhibitory effect on the proliferation of human B cells stimulated with a suboptimal dose of anti-IgM [22] or with pokeweed mitogen [23]. Simultaneous ligation of BCR and CR1 is also associated with reduced auto-antibody levels in SLE [24; 25]. CR2 is part of the CR2/CD19/CD81 complex on B cells and binds iC3d and C3dg fragments in an immune complex. Simultaneous cross-linking of BCR and CR2 lowers the threshold for B cell activation to produce a normal Ab response [26; 27; 28]. However, a dual effect of CR2 on B cell activation has also been observed as co-ligation of BCR combined with excess CR2-ligand down-modulated BCR reactivity [29]. CR1 and CR2 have also proven to be important in the maintenance of self-tolerance as anergic autoreactive B cells in mice can become responsive if deficient in CR1/2, and CR1/2-deficiency in the autoimmune prone lpr/lpr mouse strain results in increased autoimmunity [30]. In addition,
we have recently shown that CR1/2-deficiency render female, but not male, mice more susceptible to CIA when immunized with a low dose of CII [31]. Ovariectomy of wild type mice reduces CR1 expression on B cells and enhances the susceptibility to low dose CIA. The data implies a suppressive effect of CR1 on arthritis development in female mice that is retained by estrogen. Moreover, studies by Haas et al. [32; 33] show that murine CR1/2-deficient B cells display higher cell surface expression of CD19 and exhibit enhanced BCR-induced calcium responses following BCR crosslinking, suggesting that CR1/2 has a complement-independent negative regulatory effect on CD19 that dampens BCR-mediated signaling.

The role of the inhibitory FcγRIIb, CR1 and CR2 in B cell tolerance and in autoimmunity is still not fully known, but alterations in their expression and/or function may affect susceptibility to autoimmune disease in humans. Here we have investigated the expression of FcγRIIb, CR1 and CR2 in relation to the proliferation capacity of peripheral blood B cells from healthy individuals and RA patients.

**Material and Methods**

**Subjects**

Peripheral blood samples were obtained from 38 healthy individuals, 18 women and 20 men, at the blood donor centre at the Uppsala University hospital. The mean age of the healthy subjects was 52 years (range 36-71 years). Blood samples from 38 RA patients, 27 women and 11 men, were obtained from the rheumatology unit at the Uppsala University hospital, Sweden. All RA patients had an IgM rheumatoid factor (RF) seropositive disease and were diagnosed according to the American College of Rheumatology [34]. The current treatment regimens included TNF-inhibitors, prednisolon, non-steroid anti-inflammatory drugs and conventional disease-modifying anti-rheumatic drugs. The mean age of the patients was 59 years (range 33-76 years), the mean age at disease onset was 45 years (range 16-71 years) and the mean disease duration was 14 years (range 1-52 years). The RA patients were also grouped according to disease activity. Patients were defined as having an active disease if they showed clinical signs of acute inflammation in a minimum of one joint while visiting the rheumatology clinic, or having a non-active disease if they did not show any clinical signs of acute inflammation during the visit. The study was approved by the local ethics committee in Uppsala and informed consent was obtained from all participating subjects.

**Peripheral blood mononuclear cell (PBMC) isolation**

EDTA-treated venous blood samples were centrifuged for 15 min at 1800 rpm and the plasma was removed. The cells were resuspended in PBS-EDTA (0.2 mM) and then transferred on to a Ficoll-Paque (GE Healthcare, Uppsala, Sweden) layer. The samples were centrifuged for 30 min without brake at room temperature. The interphase, containing PBMC, was collected and washed in PBS-EDTA three times by centrifugation at 1200 rpm for 10 min. The cell concentration was thereafter determined in a Bürker chamber using trypan blue and a light microscope.
Antibody staining and FACS
Two hundred and fifty thousand PBMC were suspended in 100 µl of 1% BSA in PBS and were stained with FITC-conjugated mouse anti-human CD19 (clone HD37, Dako, Glostrup, Denmark) in combination with mouse anti-human CR1 conjugated to PE (clone E11, BD Pharmingen), mouse anti-human CR2 conjugated to PE (clone LT21, BioLegend) or anti-human FcγRIIb conjugated with biotin (clone GB3, kindly provided by Dr. U. Jacob, SuppreMol, Martinsried, Germany). All Ab were of mouse IgG1 isotype, and control samples were thus stained with PE-, FITC- or biotin-conjugated mouse IgG1 isotype controls (all from BD Pharmingen). The samples were stained for 25 min at 4°C in the dark and thereafter washed twice with 1% BSA in PBS for 5 min at 1200 rpm. A second staining with PE-conjugated streptavidin for 25 min at 4°C in the dark was performed on samples stained with biotin-conjugated antibodies. The lymphocytes were defined in a forward and side scatter diagram and the B cells were further defined by CD19 positive staining. The percentage of cells expressing FcγRIIb, CR1 or CR2 was calculated from the CD19+ B cells. Cells with a higher mean fluorescent intensity (MFI) value than the corresponding isotype control was considered positive.

B cell isolation using MACS
The PBMC were incubated with anti-CD2-biotin for 30 min at 4°C in the dark. The cells were subsequently washed and stained with magnetic beads conjugated to streptavidin and anti-CD14 labeled magnetic beads according to the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were thereafter put on a LS column in a magnetic field and the negative fraction, containing B cells, was collected. The B cell yield was 89 ± 8%.

B cell proliferation assay
Peripheral blood B cells, purified from 13 RA patients and 15 healthy controls, were suspended in RPMI medium, supplemented with 10% FCS, 10 mM Hepes, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamin (all from Sigma) and 100 000 cells were plated per well in a 96-well microtiter plate (Nunc, Roskilde Denmark). The B cells were stimulated with IL-2 (10 ng/ml), CpG type B (3 µg/ml) (ODN-2006, HyCult biotechnology, Uden, The Netherlands), F(ab)² anti-μ (3 µg/ml) (Fc5µ, Jackson Immuno Research, West Grove, USA) or IgG anti-μ, (3 µg/ml) (Biodesign International, Saco, USA) in different combinations. All stimulations were at least done in duplicates. The cells were cultured for 6 days at 37°C with 5% CO₂, and 1µCi of [³H]-thymidine (Amersham Pharmacia) was added per well during the last 22 h of culture. The plate was harvested using a beta plate 96-well harvester (Wallac/PerkinElmer, Waltham, USA) and [³H]-thymidine incorporation, detected as counts per minute (cpm), was measured using a liquid scintillation counter (β-plate Scint Wallac/PerkinElmer, Waltham, USA).
Figure 1. The expression of FcγRIIb, CR1 and CR2 on B cells in healthy individuals. A-C. The percentage of CD19 positive B cells expressing FcγRIIb, CR1 and CR2 in healthy women (n = 17-18) and men (n = 17-20). D-F. The mean fluorescent intensity (MFI) values for FcγRIIb, CR1, and CR2 on CD19 positive B cells (Student t-test; *p < 0.05). Correlation between age and FcγRIIb expression on CD19 positive B cells in women (G) and in men (H)(Pearson’s test).
Statistics
Statistical analysis was performed on receptor expression (percentage and MFI) and cell proliferation using Student $t$-test. A paired $t$-test was used comparing proliferation stimulated with IL-2 + CpG + IgG anti-µ vs. IL-2 + CpG + F(ab)$_2$ anti-µ. Correlation of FcγRIIb and age was analyzed using Pearson’s test.

Results
Women have lower FcγRIIb receptor expression on B cells than men
Studies regarding FcγRIIb in humans have long been hindered by FcγRIIb homology to FcγRIIa [35] and previous available antibodies have shown cross-reactivity with both receptors. Here we used a FcγRIIb specific antibody [36] to determine the basal FcγRIIb expression on CD19$^+$ peripheral blood B cells in healthy women and men. In addition, the cells were analyzed for CR1 and CR2 expression. The results demonstrate similar numbers of FcγRIIb, CR1 and CR2 positive B cells in women and in men (figure 1a-c). However, the expression value (MFI) of FcγRIIb was significant lower in women than in men ($p < 0.05$) (figure 1d), while no gender difference in CR1 and CR2 expression observed (figure 1e-f).

We have previously reported that the expression of CR1, but not CR2, on B cells is decreased with increasing age in women, an effect not seen in men [31]. This also seems to be true for FcγRIIb, as lower receptor expression correlated with increasing age in women, but not in men ($r^2 = 0.27$, $p = 0.03$ and $r^2 = 0.01$, $p =0.66$ respectively) (Figure 1g-h). As estrogen levels drop in middle aged women these data may imply that estrogen retains inhibitory receptor expression on B cells in women.

Reduced FcγRIIb, CR1 and CR2 on B cells in RA
We analyzed the expression of FcγRIIb, CR1 and CR2 on CD19$^+$ B cells from RA patients in comparison with healthy controls. We found that RA patients had significant lower numbers of FcγRIIb expressing B cells compared to healthy controls ($p < 0.05$) (figure 2a). Furthermore, the numbers of B cells expressing CR1 and CR2 were also reduced in RA patients compared to healthy individuals (CR1; $p < 0.001$ CR2; $p < 0.01$) (figure 2b-c). When analyzing the receptor expression on the B cells, we observed a decrease in FcγRIIb in the RA patients compared to the healthy controls ($p < 0.05$) (figure 2d). An even more pronounced reduction of receptor expression was observed for CR1 and CR2 in RA B cells compared to healthy controls (CR1, CR2; $p < 0.001$) (figure 2e-f). These data clearly demonstrate that RA B cells have poor Fc and CR expression.

Disease activity does not correlate with low receptor expression on B cells
To investigate if the reduced FcγRIIb, CR1 and CR2 expression on RA CD19$^+$ B cells was associated with disease activity we grouped the RA patients according to disease status as described in the material and methods. However, low receptor expression was not associated with disease activity, as both active and non-active RA patients displayed similar receptor expression (figure 3a-c).
Increased BCR-independent B cell proliferation in RA

We explored whether the lower receptor expression on RA CD19+ B cells affected the proliferation capacity of the cells. Thus, B cells from RA patients and healthy controls were stimulated with IL-2 alone, or with IL-2 in combination with CpG, IgG anti-µ or with F(ab)² anti-µ. A comparable spontaneous B cell proliferation (without any stimuli added) was observed in RA patients and healthy controls (Figure 4a). However, when the B cells were stimulated with IL-2, the B cells from the RA patients proliferated 4.5 times more than those from the healthy controls ($p < 0.05$) (Figure 4b). In order to rule out that this was due to contamination by T cells, the correlation between B cell impurity and IL-2 proliferation was calculated. No significant correlation was seen (data not shown), implying that T cell contamination do not account for the increased proliferation of IL-2 in the RA patients. Furthermore, when the cells were stimulated with IL-2 + CpG, IL-2 + CpG + IgG anti-µ or IL-2 + CpG + F(ab)² anti-µ no differences were observed between patients and healthy individuals (Figure 4c-e). However, we observed that the B cells from healthy individuals proliferated more frequently with IL-2 + CpG than when stimulated with IL-2 alone (7269 and 360 mean cpm, respectively; 20-fold increase) ($p < 0.01$), whereas the addition of CpG to IL-2 stimuli did not significantly increase the proliferation of RA B cells.
Figure 3. The expression of FcγRIIb, CR1 and CR2 on B cells in active versus non-active RA. The RA patients were grouped according to disease activity (see material and methods) at the time of blood sampling. The expression of FcγRIIb (A), CR1 (B) or CR2 (C) was analyzed on CD19 positive B cells in patients with active disease (n = 14-15) and in non active disease (n = 18-23).

(7269 and 1610 mean cpm, respectively; 5-fold increase) ($p > 0.05$) (figure 4b-c). This might indicate an impaired response to CpG in B cells from RA patients, since the RA B cells still had the capacity to proliferate more, as seen when stimulated with IL-2 + CpG + F(ab)$^2$ anti-µ (20380 mean cpm)(Figure 4e).

When using the F(ab)$^2$ anti-µ protein as stimulus we expected a higher proliferation than when the B cells were stimulated with IgG anti-µ as the IgG Fc-part would be able to interact with FcγRIIb and inhibit B cell proliferation. In agreement, healthy controls had significantly higher proliferation when the B cells were stimulated with F(ab)$^2$ anti-µ than with IgG anti-µ ($p < 0.001$) (Figure 4f). Notably, this effect was less striking in RA patients ($p < 0.05$) (Figure 4g), most likely as a result of lower FcγRIIb expression on B cells.

Discussion

FcγRIIb together with CR1 and CR2 are believed to play important roles in regulating B cell responses and in the maintenance of B cell tolerance [30; 37]. In this study we demonstrate that healthy women on average have 40% less FcγRIIb expression on their B cells compared with healthy men. In addition, the FcγRIIb levels on B cells decrease with age in women, but not in men. Interestingly, an age-dependant decline of CR1 on B cells has also been demonstrated in women [31]. This may suggest that the age-related drop of B cell inhibitory receptors is associated with the decline of estrogen in middle aged women. In fact, we have previously demonstrated that ovariectomy in mice reduces CR1 expression on B cells and Grimaldi et al reported that estrogen upregulates CD22 on B cells, a receptor known to downregulate B cell activation [31; 38]. The low levels of FcγRIIb on B cells in women, and particularly in older women, might contribute to the increased risk for autoimmune disease in females. Indeed, FcγRIIb is required for maintenance of self-tolerance in mouse models of RA [17; 39; 40], and a 43% increase of FcγRIIb expression on B cells is enough to re-establish tolerance in autoimmune mouse strains, proving that changes in expression levels are critical for sustaining self-tolerance [41]. Further, mice that develop spontaneous autoimmune diabetes (NOD) or lupus (BXSB and MRL/lpr) have been shown...
Figure 4. B cells proliferation in RA. The proliferation of B cells was measured in RA patients (n = 12-16) and healthy controls (n = 14-15) after incubation for 6 days without stimuli (A), or with IL-2 (B), IL-2 + CpG (C), IL-2 + CpG + IgG anti-µ (D) or IL-2 + CpG + F(ab)² anti-µ (Student t-test *p < 0.05) (E). Paired analysis of proliferation in B cells stimulated with IL-2 + CpG + IgG anti-µ or IL-2 + CpG + F(ab)² anti-µ in healthy controls (n = 15) (F) and in RA patients (n = 13) (G) (Paired t-test *p < 0.05, ***p < 0.001).

Indeed, RA patients had significantly reduced FcγRIIb expressing B cells compared with healthy controls. Further, B cells from RA patients expressed markedly lower levels of CR1 and CR2. One could argue that decreased CR2 expression is due to increased shedding of the receptor, however, RA patients shed the same amounts of CR2 as healthy individuals and they even display reduced levels of soluble CR2 [43]. Interestingly, synovial B cells express even less CR2 compared to peripheral blood B cells in RA patients [44]. The CR2-, as well as FcγRIIb- and CR1-deficiency on RA B cells might contribute to a dysregulated B cell, which have lost self-tolerance and is easily activated. Low expression levels to express lower levels of FcγRIIb on germinal-center B cells due to a polymorphism in the promoter region of the FcγRIIb gene. This suggests that the absolute level of FcγRIIb is critical for protection from autoimmunity [42].
of FcγRIIb [19; 21; 45], CR1 [21; 46] and CR2 [46] on B cells have also been observed in SLE. The impairment of FcγRIIb was most profound in SLE patients with an active disease [19]. In contrast, we could not associate the FcγRIIb nor the CR1 and CR2 expression on RA B cells with the inflammation status of the patients. FcγRIIb may affect the antibody production in the patients, but no correlation of FcγRIIb expression and autoantibody titers (IgG anti-cyclic citrullinated peptide antibodies) in the patients was found (data not shown). These findings points to a role of FcγRIIb, CR1 and CR2 in the initiation of the disease rather than in the disease progression.

Moreover, the impact of the altered Fc and CR expression on B cell proliferation was assessed in RA patients, in comparison with healthy blood donors. Many inhibitory surface molecules serve to counteract signaling through the antigen receptors of lymphocytes, and simultaneous cross-linking of FcγRIIb and BCR can negatively regulate B cell activation [13]. In concordance, when B cells from healthy controls were stimulated with F(ab)² anti-µ (together with IL-2 and CpG) the cells proliferated more than when stimulated with IgG anti-µ (together with IL-2 and CpG), as the Fc-part of the IgG anti-µ may interact with FcγRIIb and inhibit B cell activation. Since the RA patients showed lower levels of FcγRIIb on B cells one would expect to see less inhibition of proliferation when IgG anti-µ was used as stimuli. The proliferation of the RA B cells was inhibited when stimulated with IgG anti-µ (together with IL-2 + CpG) compared to F(ab)² anti-µ (together with IL-2 + CpG), however, not to the extent as seen in healthy B cells. It is possible that when using a suboptimal IgG anti-µ concentration as used here, the RA B cells still express enough FcγRIIb to inhibit BCR responses. However, constant stimulation or abundantly expressed IgG immune complexes as seen in a chronic autoimmune disease might overcome this inhibition and the B cells would become inappropriately activated.

Interestingly, the B cells from RA patients responded better to IL-2 than healthy controls and started to proliferate without any further engagement of the BCR in vitro. Since all RA patients tested for proliferation were RF positive, auto-reactive peripheral B cells have probably already been activated with antigen in vivo, likely resulting in the up-regulation of the IL-2 receptor (CD25), increasing the affinity for IL-2. In accordance, it has been reported that RA patients display highly activated CD25⁺ B cells [47]. Thus, antigen-bound B cells stimulated with IL-2 become activated and proliferate, while naïve B cells do not, supporting the idea that B cells from RA patients have already bound antigen in vivo [48]. Further, the proliferation mediated by IL-2 (without any further BCR engagement) suggests that RA B cells can become activated by any nearby activated T cell accompanied by IL-2 production, a process known as bystander activation [49].

TLR-9 has been found to contribute to the activation of RF positive B cells [50; 51], however, we found that the RA B cells had decreased proliferation to the TLR-9 ligand CpG compared to B cells from healthy subjects. This finding might be due to differences in intracellular signaling, or insufficient ligand uptake to the endosomal-located TLR-9. Recent studies by Chaturvedi et al. demonstrated that CpG uptake is
associated with BCR ligation, and CR2 have been shown to influence BCR internalization [52; 53; 54]. It is therefore possible that the hyporesponse to the TLR9-ligand seen in RA patients might be associated with the lower expression of CR2, resulting in less efficient CpG internalization via BCR to the TLR.

In conclusion, our findings demonstrate that B cells from RA patients have significantly lower levels of the inhibitory receptors FcγRIIb and CR1 together with the activating CR2. This modified expression is likely contributing to a break of tolerance leading to dysregulated B cells that generate auto-antibodies and autoimmunity. The low FcγRIIb expression in healthy women that even decreases with age, is likely a contributing factor to the increased susceptibility to RA in women.

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