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Memory B cells from older people express normal levels of cyclooxygenase-2 and produce higher levels of IL-6 and IL-10 upon *in vitro* activation

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Abstract

Worldwide the elderly population is increasing. The elderly show deficiencies in immune function. B lymphocytes are essential elements of the immune system responsible for antibody production. This laboratory previously showed that activated human B cells isolated from young adults express cyclooxygenase-2 (Cox-2) and that Cox-2 is essential for optimal antibody responses. Recent data suggests that Cox-2 expression decreases with age in mouse bone tissue. There is no information regarding Cox-2 expression in B cells from older human subjects. We investigated the expression and activity of Cox-2 in naïve and memory B cells from older people. We show that B cells from older subjects show similar Cox-2 protein expression and activity, antibody production and proliferation compared to younger people. However, we found that activated memory B cells from older people produce higher levels of IL-6 and IL-10 compared to young adults. Therefore, the dysregulated cytokine production could contribute to immune senescence in the elderly.

Keywords

human B cells; elderly; cyclooxygenase-2; antibody production; IL-6; IL-10

1. Introduction

B lymphocytes serve as key defenders against infection. Once activated, B cells undergo a series of transformations that culminate in antibody production. Cox-2 protein levels are dramatically elevated shortly after B lymphocyte activation and Cox-2 expression is a prerequisite for optimal antibody production [1]. While Cox-2 expression is increased in activated B lymphocytes, cyclooxygenase-1 (Cox-1) is ubiquitously expressed in most cell types and does not appear to be essential for optimal human antibody synthesis. Both Cox-1 and Cox-2 are involved in the conversion of arachidonic acid to prostaglandins (PGs). PGs are small lipid mediators which regulate a wide array of physiological and immune functions [2]. PGE₂, an abundant PG, is produced by many cell types and is a mediator of pain, fever and

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swelling, which are common features of inflammatory diseases [3]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and indomethacin target the activity of Cox-1/Cox-2 and reduce PGE₂ synthesis. Therefore, NSAIDs are recommended in cases of pain and fever and are prescribed for inflammatory-related diseases. About half of NSAID prescriptions are for people over 60 years old [4].

Aging has a profound impact on the immune system. Immunosenescence is characterized by quantitative (decrease in cell number) and qualitative (decline in cell activity) changes [5,6] which are accompanied by a pro-inflammatory environment [7,8]. These changes result in decreased immunity and, in general, poor responses to vaccinations. B cells consist of a heterogeneous population comprised of naïve (newly generated) and memory (antigen experienced) cells. The ratio between naïve and memory B cells varies in human subjects and is influenced by age [9,10].

Cox-2 inhibition, in the case of Cox-2 deficient mice or caused by Cox-2 selective drugs or by NSAIDs, dramatically hinders antibody production in human peripheral blood mononuclear cells and B cells *in vitro* and decreases antibody titers to vaccination in mice and humans [11,12]. Studies to date on Cox-2 expression in B cells were done using only young human subjects (18-35 years old) who show a predominant naïve B cell phenotype. There are no data regarding the expression of Cox-2 in B cells from older subjects. Recent research showed that Cox-2 expression is reduced in the femurs of old mice which results in impaired bone healing in case of femur fracture [13]. In B cells, a decrease in Cox-2 expression/activity would be expected to impair antibody synthesis which could, at least in part, explain why the elderly produce less antibody. Herein, we investigated Cox-2 protein expression and activity (by measuring PGE₂ production) and antibody production in naïve and memory B cells from older people (over 60 years old). Since immunosenescence can be characterized by changes in cytokine synthesis, IL-6 and IL-10 levels in activated B cells from older versus younger people were also determined. High levels of IL-6 are indicative of a pro-inflammatory environment whereas IL-10 promotes antibody synthesis and blunts inflammation.

2. Material and Methods

2.1. Reagents

F(ab')₂ goat anti-human IgG, F(ab')₂ (herein called anti-Ig) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), CpG oligodeoxynucleotide 2395 (5'-TCGTCGTTTTCGGCGCGCGCCG-3') from Coley Pharmaceutical Group (Wellesley, MA) and arachidonic acid from (Nu-Check-Prep, Elysian, MN). Human B cells were stained with the following antibodies: CD19 (APC conjugated), CD27 (Pe conjugated), CD69 (APC-Cy7 conjugated) (BD Biosciences, San Jose, CA) and Cox-2 (Fitc conjugated; Cayman Chemical, Ann Arbor, MI). Human IgM and IgG quantitation kit was purchased from Bethyl Laboratories (Montgomery, TX).

2.2. Human B lymphocyte isolation and culture conditions

One unit of blood was obtained from healthy donors as approved by the University of Rochester Institutional Review Board and Office for Human Subjects Protection. We considered young donor individuals to be between 18-35 years old (n=10; 6 males and 4 females) and older individuals to be those over 60 years old (60-72 years old; n= 9; 3 males and 6 females). Given the sample size (n= 4-10) Mann-Whitney test and Student's t test were chosen for statistical analysis. Donors who were taking NSAIDs (including low dose aspirin) or had an iron level lower than 12.3 were excluded from the study. Volunteers were healthy, were not obese or diabetic, were not taking steroids or diabetic medication and women volunteers were not pregnant. The racial category of the participating subjects was white or other (choose not to

answer). All blood donations were collected in the morning (8-9 AM). An aliquot of blood (1 ml) was used to prepare plasma which was used for ELISA. Peripheral blood mononuclear cells were obtained by density-gradient centrifugation of buffy coat using Ficoll-Paque Plus. CD19 positive cells were captured with a magnet, washed and detached using CD19 Detachabead (DynaL Inc, Brown Deer, WI). B cells were stained with CD19 and CD27 antibodies and then sorted on a FACS Aria Cell Sorter (BD Biosciences) into naïve (CD19⁺CD27⁻) and memory (CD19⁺CD27⁺) B cells (> 98% purity).

Naïve and memory B cells (1×10^6 cells/ml, unless otherwise specified) were cultured in RPMI1640 media (Invitrogen Life Technologies) supplemented with 10% FBS, 50 μ M β -mercaptoethanol (Eastman Kodak, Rochester, NY), 10 mM Hepes (U.S. Biochemical, Cleveland, OH), 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA), 50 μ g/ml gentamicin (Invitrogen Life Technologies, Carlsbad, CA) and 10 μ M arachidonic acid. B cells were stimulated with anti-Ig (2 μ g/ml) plus ODN CpG 2395 (1 μ g/ml) (concentrations determined by a titration experiment) for variable time points as described in the figure legends.

2.3. Flow cytometry

B lymphocytes were cultured for 2 days in the presence of mitogens (anti-Ig plus CpG 2395). Cells were surface stained for CD69 (BD Biosciences) for 20 minutes at 4°C, washed in staining buffer (PBS with 0.3% BSA) and then pelleted by centrifugation. For Cox-2 intracellular staining, cells were fixed and permeabilized (Caltag Laboratories) followed by staining with Cox-2 antibody (1 μ g/ 10^6 cells; Cayman Chemicals). An equal number of live cells (based on forward and side scatter) were acquired and were analyzed on a LSRII (BD Bioscience, San Jose, CA) flow cytometer using FlowJo software (Tree Star).

2.4. Measurement of PGE₂ synthesis

B cells were stimulated with anti-IgG plus CpG 2395 for 2 days after which supernatants were harvested. PGE₂ synthesis was determined using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI), as recommended by the manufacturer.

2.5. Proliferation assays

Activated B cells (1×10^5 cells/ ml) were cultured in triplicate in 96-well round bottom plates. Cell cultures were pulsed with [³H]-thymidine (1 μ Ci/well) for 24h and the incorporation was determined by scintillation spectroscopy.

2.6. IgM and IgG enzyme-linked immunosorbent assay (ELISA)

Activated B cells were cultured in triplicate in 96-well plates for 7 days. Supernatants were collected and used for IgM and IgG detection using the human-specific ELISA kit (Bethyl Laboratories) as recommended by the manufacturer.

2.7. IL-6 and IL-10 cytokine ELISA

Stimulated (anti-Ig plus CpG 2395) naïve and memory B cells were cultured for two days *in vitro* after which supernatants were harvested. IL-6 and IL-10 production was determined by ELISA as recommended by the manufacturer (BD Pharmingen, San Jose, CA).

2.8. Statistical analysis

Mann-Whitney test was used for statistical analysis and p values of *, p<0.05; **, p<0.001; ***, p<0.0001 are considered significant. A second test, Student's t test was also used and the results of the t test are shown in the figure legends.

3. Results

3.1. Cox-2 expression and activity show similar patterns in naïve and memory B cells from young and older people

Recent published data indicate that Cox-2 levels decrease in mouse bone tissue with age [13]. We were therefore interested in whether or not B cells from elderly humans would also have a deficit in Cox-2 expression. In order to investigate Cox-2 expression and activity in B cells from older people, we enrolled older volunteers (over 60 years old). Younger subjects (18-35 years old) were used as controls. The percentage of naïve and memory B cells in the peripheral blood is reported to change with age; there is an increase in the percentage of memory B cells in the older individuals [9,10]. Therefore, we first analyzed the percentage of naïve and memory B cells in the peripheral blood of young and older subjects enrolled in the study. To identify memory B cells we used the CD27 marker in combination with CD19. CD27 is absent on naïve B cells but is highly expressed on memory B cells [10,14]. PBMC were isolated from young and older subjects and were stained with anti-CD19 and anti-CD27 antibodies. Figure 1 (A and B) shows that younger volunteers have ~ 75% naïve B cells (CD19⁺CD27⁻) and ~ 25% memory B cells (CD19⁺CD27⁺) in the peripheral blood. Older subjects showed a scattered range in terms of the percentage of naïve and memory B cells; older volunteers had between 2-70% naïve B cells and between 30-98% memory B cells. We did not detect a significant change in the number of B cells isolated from older compared to young volunteers. There was no correlation between the B cell number and racial category, gender and actual age of young and older subjects.

Because of the significant difference in the percentage of naïve and memory B cells in young and older volunteers it was essential to isolate B cells based on their naïve and memory phenotype. Highly purified B cells were stained with CD19 and CD27 and were sorted into naïve (CD19⁺CD27⁻) and memory (CD19⁺CD27⁺) B cells. In order to determine Cox-2 expression, naïve and memory B cells were activated with broad polyclonal B cell activators, namely anti-Ig plus oligodeoxynucleotide (ODN) CpG 2395 for 48h after which cells were harvested and stained for Cox-2. Figure 2A shows that anti-Ig plus CpG 2395 activated naïve B cells from young and older people upregulated Cox-2 expression. Similarly, memory B cells from both young and old increased Cox-2 expression upon activation (Figure 2B). Naïve and memory B cell population upregulated Cox-2 to similar levels. Overall though there was no difference in Cox-2 expression levels between naïve or memory B cells from young or old.

In order to see if the level of Cox-2 protein expression paralleled Cox-2 activity, PGE₂ production was determined in both activated naïve and memory B cells from young and older subjects. Figure 3 (A and B) shows that PGE₂ levels were increased in activated naïve and memory B cells from young and older people. There was no difference in PGE₂ production (and hence Cox-2 activity) between naïve and memory B cells or between young and older people. There was no difference in Cox-2 expression and activity in naïve and memory B cells from young and older volunteers exposed to lower CpG 2395 concentrations (0.25 and 0.5 µg/ml) with or without anti-Ig (data not shown).

3.2. Naïve and memory B cells from older people show similar activation and proliferation patterns to young subjects

It was reported that immunosenescence causes a decrease in cell proliferation [15,16]. Therefore, we analyzed the expression of activation markers and the proliferation of naïve and memory B cells from young and older subjects during the culture period. The activation marker used was CD69 which is elevated on activated B cells. Figure 4 (A-D) shows that in both naïve and memory B cells CD69 expression is increased upon activation and there is no difference between naïve and memory B cells from young and older people.

Cell proliferation was assayed using the [3 H]-thymidine assay (Figure 4E and 4F). On day 2 naïve and memory B cells from young and older people had ~2,000 cpm after which it increased dramatically reaching a peak on day 3 (~15,000 cpm for young and ~12,000 cpm for older people). Memory B cells from young and older people showed a comparable proliferation profile. There was no significant difference in proliferation of B cells from older people compared to young subjects.

3.3. Naïve and memory B cells from young and older people produce similar antibody levels

Several reports have shown that the elderly produce less antibody compared to younger subjects [17,18]. Taken into consideration that Cox-2 expression is linked to optimal antibody synthesis [1] and Cox-2 expression and activity showed no differences in naïve and memory B cells in older subjects as compared to young volunteers in this study (Figure 2 and 3), we hypothesized that older people would produce similar levels of antibody *in vitro*. In order to test our hypothesis, naïve and memory B cells from young and older volunteers were activated with anti-Ig plus CpG 2395 for 7 days after which supernatants were harvested and used for antibody ELISA. Activated naïve B cells (5A and 5B) from older people produced ~12,000 ng/ml IgM and ~200 ng/ml IgG. These antibody levels were comparable to those synthesized by naïve B cells isolated from young subjects (~7,300 ng/ml IgM and ~240 ng/ml IgG). Memory B cells from older people synthesized more IgM (~70,000 ng/ml) compared to young subjects (~31,000 ng/ml) (Figure 5C). However, because of the scattered profile, the difference is not statistically significant. In contrast, IgG production in memory B cells from young and older people show very similar levels (~4,000 ng/ml in young and ~5,400 ng/ml in older people) (Figure 5D).

Because older people have a higher percentage of memory B cells in their peripheral blood and because memory B cells from older subjects produced slightly higher levels of antibody *in vitro*, the levels of antibody in the plasma of young and older subjects were also determined and there were no differences between IgM and IgG levels in the plasma of young versus older volunteers (data not shown).

3.4. Memory B cells from older people produce more IL-6 and IL-10 after *in vitro* activation

IL-6 participates in a plethora of functions including inflammation and immunity [19]. IL-6 is required for B cell differentiation as well as for antibody synthesis [19]. Due to the fact that IL-6 overproduction is associated with autoimmune diseases [20] and that older people are more likely to develop rheumatoid arthritis, we investigated the levels of IL-6 in activated B cells from older subjects. Naïve and memory B cells from young and older people were activated *in vitro* with anti-Ig plus CpG 2395 for 2 days after which supernatants were tested for IL-6 using ELISA. Figure 6A shows that naïve B cells from young and older subjects produce similar levels of IL-6 upon activation (~2,000 pg/ml). However, IL-6 production in activated memory B cells was significantly different between young and older subjects. Memory B cells from older people produced higher levels of IL-6 after activation (~3,000 pg/ml) compared to young subjects (~1,000 pg/ml) (Figure 6B).

IL-10 is considered an “anti-inflammatory” cytokine that promotes antibody production and is synthesized by B cells upon activation [21]. IL-10 is involved in the maintenance of optimal immune responses and is considered to act as a feed-back control cytokine [22]. Since memory B cells from older people showed a heightened production of IL-6, IL-10 levels in naïve and memory B cells from young and older subjects were also assayed. Activated naïve B cells from older people showed an increase in IL-10 synthesis (~1,500 pg/ml) compared to naïve B cells from young subjects (~700 pg/ml) (Figure 6C). However, memory B cells from older people showed an even more pronounced increase (~1,600 pg/ml) compared to young subjects (~250 pg/ml) (Figure 6D).

4. Discussion

Aging is associated with decreased immunity, poor response to vaccination and increased susceptibility to infections [17,18,23]. While immunosenescence is well documented in human T cells (partly due to the involution of the thymus) [24], most of the studies focusing on B cell activation, proliferation and antibody production associated with aging were done using mouse models [5,15,16].

We recently showed that activated human B lymphocytes from young human beings express Cox-2 and that Cox-2 expression is required for optimal antibody production and inhibition of Cox-2 by NSAIDs hinders human antibody production [11,12]. There are no data regarding Cox-2 expression in B cells in the human elderly population. So far, Cox-2 expression was analyzed only in the bone and macrophages of old mice. It was reported that Cox-2 expression was decreased in the femur of old mice which increases the risk of fractures [13]. Conversely, mouse macrophages upon activation showed increased Cox-2 activity, demonstrated by increased PGE₂ production [25]. PGE₂ is a proinflammatory and protumorigenic mediator and therefore NSAIDs, which inhibit Cox-2 activity and decrease PGE₂ levels, are recommended as chemo-preventive agents [26]. NSAIDs are also prescribed for inflammation and pain (e.g. rheumatoid arthritis) and the largest group of NSAIDs users are people over 60 years old [4,27]. These facts raise an interesting scenario: what if B cells from older people express less Cox-2 (which may lead to decreased antibody synthesis) should NSAIDs be recommended for the elderly? On the other hand, if B cells from the elderly produce elevated levels of PGE₂ (similar to mouse macrophages) should the elderly be encouraged to use more NSAIDs?

Our compelling data demonstrate that Cox-2 expression and activity (as shown by PGE₂ production) is increased upon activation at similar levels in naïve and memory B cells. Furthermore, Cox-2 expression and activity in B cells from older people is comparable to B cells from young subjects. Ibuprofen, the popular over-the-counter NSAID, equally inhibited Cox-2 activity in naïve and memory B cells from young and older people (data not shown). Consequently, NSAIDs are effective in inhibiting Cox-2 expression and activity in both young and older people, independent of the percentage of naïve and memory B cells in their peripheral blood.

A main feature of immunosenescence is decreased antibody production caused by quantitative (mainly due to reduced proliferation and isotype switching of B cells) or qualitative changes (reduced antigen-specific antibody titers) [9,28,29] leading to a poor response to vaccination and more infections in the elderly [18,30,31]. We found that activated B cells from older people produced similar levels of antibody *in vitro*. Memory B cells from older people also produced more IgM upon activation. However, we found that IgM and IgG levels in the plasma of young and older people were nearly identical. The fact that B cells from older people produced similar antibody levels as young people might be explained by the activators used in our experiments. In order to activate the B cells *in vitro*, anti-Ig in combination with ODN CpG 2395 was used [32]. Anti-Ig binds to the surface of B cells and ensures activation of both naïve and memory (isotype switched) B cells [32]. ODN CpG serves as a ligand for toll-like receptor 9 (TLR9), increases Cox-2 expression in B cells and is reported to act as an adjuvant in vaccination [33]. Using anti-Ig plus ODN CpG 2395 we show that B cells from older people respond to activation by increasing CD69 expression, proliferation and antibody production similar to young subjects highlighting the role of ODN CpG as a potential adjuvant.

“Inflammaging” refers to increased pro-inflammatory cytokine production in the elderly [7]. IL-6, one of the most studied cytokines, is increased in activated human mononuclear cells [8] and in human serum in inflammatory diseases [20]. It was reported that an elevated IL-6 environment increases IgG production in a mouse model *in vivo* [34]. Interestingly, we show

that only the memory B cells from older people produced high levels of IL-6. We speculate that activated memory B cells from older people produced high levels of IL-6 in order to promote antibody synthesis. On the other hand, IL-6 is required for experimentally-induced as well as for naturally occurring autoimmune diseases [35]. The fact that memory B cells from older people produce more IL-6 suggest that those elderly which have a higher percentage of memory B cells in their peripheral blood would synthesize more IL-6 upon activation and be more susceptible to developing autoimmune diseases.

IL-10 is considered an anti-inflammatory cytokine dampening the effects of pro-inflammatory mediators [21] and it was showed that B cell production of IL-10 can prevent arthritis in mice [36]. We found that activated memory B cells from older people, the same cell population which synthesized high levels of IL-6, also produced increased levels of IL-10. Although TNF α , another pro-inflammatory cytokine, is associated with aging, we could not detect TNF- α in activated B cells *in vitro* (data not shown). PGE₂ induces type-2 (IL-10 production) and inhibits type-1 responses (TNF- α) in macrophages [37]. Therefore, it is likely that increased levels of PGE₂ produced by activated B cells *in vitro* would induce IL-10 production and inhibit TNF- α synthesis.

Our study compared the functions of naïve and memory B cells isolated from the peripheral blood of young and older people. In order to discriminate between naïve versus memory B cells, the CD27 marker was used. However, there are several reports showing that the B cell compartment is much more complex, comprising small populations of isotype switched and non-switched naïve and memory B cells [38,39]. While these studies analyzed the surface markers in total B cell populations, it is challenging to conduct complex *in vitro* experiments using a low and limited number of cells. Nevertheless, B cells heterogeneity [14,38] and dysregulated B cell development in bone marrow and spleen of the elderly [5,24] appear to be contributing factors to the multifaceted process of immune aging. Furthermore, defects in T cell development and function [5;23] and abnormalities in T cell-B cell interactions [40] also have to be considered as part of immunosenescence.

Our study highlights the finding that although B cells from older people exhibited a similar pattern of activation, proliferation and antibody production as compared to young subjects, memory B cells from older people showed an increased production of IL-6 and IL-10. Therefore, dysregulation of cytokine production is another participating factor in immunosenescence.

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Figure 1. Older people show a lower percentage of naïve B cells but a higher percentage of memory B cells in their peripheral blood

Peripheral blood was collected from young (18-35 years old; n= 8) and older (over 60 years old; n= 9) people and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation, stained with CD19 and CD27 antibodies and analyzed on a Becton Dickinson LSRII. **A.** Young volunteers have ~75% naïve B cells (CD19⁺CD27⁻) whereas older volunteers have a significantly lower percentage of naïve B cells (~40%). **B.** Older volunteers have an increased percentage (~60%) of memory B cells (CD19⁺CD27⁺) whereas young volunteers have ~ 25% memory B cells in the peripheral blood. Graphs show the mean and SEM. *, p< 0.05 was calculated using the Mann-Whitney test. Student's t test gave similar results (**, p= 0.0087 for Figure 1A and **, p=0.0059 for Figure 1B).

Figure 2. Naïve and memory B cells from older people increase Cox-2 expression upon activation
Sorted naïve and memory B cells from young (n= 8) and older (n= 8) people were stimulated with anti-Ig plus CpG 2395 for 2 days. Cells were stained with an anti-Cox-2 antibody and analyzed on a Becton Dickinson LSRII. Graphs show the mean fluorescence intensity (MFI) of Cox-2 staining. **A** and **B**. Naïve and memory B cells from both young and older people increased Cox-2 expression upon activation. Untreated cells show a lower MFI (~500) compared to activated cells (~ 800 for young and ~1,100 for memory from older people). Graphs show the mean and SEM. *, $p < 0.05$ and **, $p < 0.005$ was calculated using the Mann-Whitney test. Analysis using Student's t test resulted in: Figure 2A: ***, $p = 0.0002$ (when comparing young (no treatment) to young (aIg+ CpG 2395) and *, $p = 0.0472$ (when comparing older (no treatment) to older (aIg+CpG 2395) samples; Figure 2B: **, $p = 0.0037$ for young (no treatment) versus young (aIg+CpG) and $p = 0.064$ (not significant) when comparing older (no treatment) to older (aIg+CpG 2395). There is no significant statistical difference between young (aIg+ CpG 2395) and older (aIg+ CpG 2395) samples.

Figure 3. Naïve and memory B cells from older people increase PGE₂ synthesis upon activation
Sorted naïve and memory B cells from young (n= 9) and older (n= 8) people were stimulated with anti-Ig plus CpG 2395 for 2 days after which supernatants were harvested and used for PGE₂ detection. Unactivated naïve and memory B cells produce ~ 75 pg/ml PGE₂ whereas activated naïve and memory B cells (from both young and older subjects) produce ~150 pg/ml PGE₂. Graphs show the mean and SEM. *, p< 0.05, **, p< 0.005 and ***, p<0.0001 was calculated using the Mann-Whitney test. According to Student's t test the p values are: Figure 3A: *, p=0.013 for young (no treatment) versus young (aIg+ CpG 2395) and **, p= 0.004 for older (no treatment) versus older (aIg+ CpG 2395); Figure 3B: ***, p=0.003 for young (no treatment) versus young (aIg+ CpG 2395) and ***, p< 0.0001 for older (no treatment) versus older (aIg+ CpG 2395). There is no significant statistical difference between young (aIg+ CpG 2395) and older (aIg+ CpG 2395) samples.

Figure 4. Naïve and memory B cells from older people increase expression of CD69 and proliferate after *in vitro* activation

Sorted naïve and memory B cells from young and older people were activated *in vitro* with anti-Ig plus CpG 2395. On day 2 cells were harvested and stained for CD69 after which cells were analyzed on a Beckton Dickinson LSR II. **A** and **B** show the histograms of CD69 expression in anti-Ig plus CpG 2395 activated (black line) compared to no treatment (gray contour) naïve and memory B cells. The histograms are representative for naïve and memory B cells in young and older people. **C** and **D** show the fold induction in CD69 MFI of activated cells versus no treatment cells. There is an increase in CD69 MFI in both naïve and memory B cells from young (n=7) and older (n=7) subjects. [^3H]-thymidine was added to the activated naïve and memory B cells from young and older people and proliferation was assayed (**E** and **F**). Graphs show the mean and SEM of counts per minute (cpm) from young (n= 4) and older (n= 5) people. Naïve and memory B cells showed similar proliferation patterns and there are no significant differences between the two age groups.

Figure 5. B cells from older people produce similar levels of antibodies compared to younger subjects

Sorted naïve and memory B cells from young and older people were activated with anti-Ig plus CpG 2395 for 7 days after which supernatants were harvested and used to determine IgM and IgG production. **A** and **B**. Activated naïve B cells from young and older people synthesize similar levels of IgM (~8,000 ng/ml) and IgG (~200 ng/ml). **C** and **D**. Activated memory B cells from older people produce more IgM (~70,000 ng/ml) but similar levels of IgG (~4,000 ng/ml) compared to young subjects (~25,000 ng/ml and ~4,000 ng/ml, respectively). Graphs show the mean and SEM.

Figure 6. Activated memory B cells from older people increased IL-6 and IL-10 levels

Sorted naïve and memory B cells from young and older people were stimulated with anti-Ig plus CpG 2395 for 2 days after which supernatants were harvested and used for IL-6 and IL-10 detection. **A** Naïve B cells from young and older subjects produce similar levels of IL-6 (~2,000 pg/ml). **B**. Memory B cells from older people produce significantly more IL-6 (~3,000 pg/ml) as compared to young subjects (~1,000 pg/ml). **C**. Naïve B cells from older people produce slightly more IL-10 (~1,500 pg/ml) compared to naïve B cells from young people (~700 pg/ml). **D**. Memory B cells from older people produce significantly more IL-10 (~1,600 pg/ml) compared to young subjects (~250 pg/ml). **, p < 0.005 was calculated using the Mann-Whitney test. The p values according to t test were: Figure 6B, ***, p = 0.0009 and Figure 6D, *, p = 0.035.