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Naïve and memory B cells in the rhesus macaque can be differentiated by surface expression of CD27 and have differential responses to CD40 ligation

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Abstract

The rhesus macaque (RM) model has the potential to be an invaluable tool for studying B cell populations during pathogenic infections, however, to date, there has been no definitive delineation of naïve and memory B cell populations in the RM. This has precluded a rigorous analysis of the generation, persistence and resolution of a pathogen-specific memory B cell response. The present study utilized multiple analyses to demonstrate that CD27 expression on B cells is consistent with a memory phenotype. Compared to CD20+CD27– B cells, CD20+CD27+ B cells were larger in size, and preferentially accumulated at effector sites. Direct sequence analysis revealed that CD20+ CD27+ B cells had an increased frequency of point mutations that were consistent with somatic hypermutation and at a functional level, CD40 ligation improved CD20+CD27– but not CD20+ CD27+ B cell survival *in vitro*. These data provide definitive evidence that the naïve and memory B cell populations of the RM can be differentiated using surface expression of CD27.

Keywords

B cell; Memory; Rhesus macaque

1. Introduction

The RM model is an essential tool utilized in the study of human immunodeficiency virus (HIV) infection and vaccine development. Notably, the early depletion of resting memory T cells from the gut in simian immunodeficiency virus (SIV) infected macaques (Veazey et al., 1998; Li et al., 2005) led to an improved understanding of the kinetics of the loss of T cells from the gut in human HIV infection (Brenchley et al., 2004; Brenchley and Douek, 2008). Analysis of B cell subsets has improved our understanding of antibody producing cells in humans (Agematsu et al., 1995, 1997; De Mito et al., 2001), but very few studies have focused on B cell subsets in the RM. A mechanistic understanding of the immunological

characteristics of specific B cell populations in the macaque model is needed to facilitate translation of findings from studies in rhesus to application in humans. Effective utilization of RMs for modeling human disease requires the precise resolution and sensitive detection of relevant immune populations. Indeed, ineffective validation of the veracity of cell markers has led to confusion in non-human primate models that have negatively impacted scientific progress. For example, CD56 was utilized as an NK cell marker in RMs based on its staining profile in humans (Tseng et al., 1993) until it was demonstrated that CD56 marks monocytes and not NK cells in RMs (Carter et al., 1999; Webster and Johnson, 2005).

Memory and naïve B cells have distinct phenotypic and functional characteristics. In humans, memory B cells are physically larger than naïve B cells (Agematsu et al., 1997) and have increased surface expression of activation markers including CD95, CD80 and CD86 (Shi et al., 2003; Good et al., 2009). Memory B cells in humans have also been shown to express CD27, a TNF-related type II transmembrane protein that is expressed on the surface of multiple lymphocyte cell populations (Agematsu et al., 2000; Nolte et al., 2009). Previous human studies have demonstrated that CD20+CD27+ B cells have more mutations within the immunoglobulin gene that are consistent with somatic hypermutation compared to CD20+ CD27- B cells (Agematsu et al., 1997; Klein et al., 1998). In addition, functional differences have also been described between CD20+CD27+ and CD20+CD27- B cells. CD20+ CD27+ B cells differentiate into antibody secreting cells within 4 days of CD40 ligation while CD20+CD27- B cell populations proliferate within 4 days of CD40 ligation and produce antibodies with different kinetics from the CD20+CD27+ B cell population (Fecteau and Neron, 2003). Work from our lab and others demonstrates differential disruption in CD20+ CD27+ versus CD20+CD27- subsets in the RM model of SIV infection (Kuhrt et al., 2010; Peruchon et al., 2009), and obtaining a mechanistic picture of how these cells are altered during infection requires definitive resolution of memory B cells.

Although it is generally assumed that B cell subsets in non-human primates are similar to those in humans, fundamental differences are known to exist. For example, anti-human antibodies to both CD19 and CD20 can be used to stain B cells in humans, but only anti-human antibodies to CD20 and not CD19 consistently identify a distinct cell population in the RM (Sopper et al., 1997). Additional differences between human and RM B cells can be found in the structures of the antibodies produced. Although humans and RMs both have 4 IgG isotypes, the intron lengths are variable between species (Scinicariello et al., 2004). Lastly, differences in immunoglobulin A (IgA) subtypes also exist. Two subtypes have been identified in humans (IgA1 and IgA2) while RMs have only 1 IgA subtype which exhibits high levels of heterogeneity (Scinicariello and Attanasio, 2001). These physical differences in antibodies between species may be indicative of physical differences in B cells between humans and RMs. The considerable value of the RM as a surrogate model for human disease and the potential for discrepancies between models demonstrates the essential need to functionally validate the major markers used to discriminate hematopoietic populations.

The goal of the present study was to establish CD27 as a bona fide memory B cell marker in the RM, and to define the role of surface CD27 expression in an animal model increasingly exploited for studies of human pathogens. Specifically, we wanted to determine whether CD27 could be used to accurately differentiate naïve and memory B cells, as a step toward elucidating the roles of naïve and memory B cells during chronic viral infection. Our results demonstrated that CD20+CD27+ B cells displayed characteristic size and activation marker expression associated with memory populations, accumulated at effector sites, bore somatic hypermutations, and failed to be protected from apoptosis following CD40 binding. In contrast, CD20+CD27- B cells were smaller in size and had lower activation marker expression, demonstrated a lack of somatic hypermutation and were protected from spontaneous apoptosis through binding of the CD40 receptor. Together, these studies

empirically validate CD27 as a phenotypic marker of memory B cells in the RM, provide insight into the role of CD40 ligation in survival and activation of B cell subsets, and allow for the separate analysis of naïve and memory B cell populations during pathogenic infections.

2. Materials and methods

2.1. Animals

Peripheral blood, tissue and bronchoalveolar lavage samples were obtained from SIV negative colony-bred RMs (*Macaca mulatta*) of Indian origin at the Oregon National Primate Research Center. Additional blood samples were obtained from normal RMs awaiting other studies at the University of Pittsburgh. Cord blood from clinical cesarean sections was obtained from the California National Primate Research Center. All animals were maintained and used in accordance with the guidelines of the Animal Care and Use Committees at their respective institutions.

2.2. Flow cytometry analysis of whole blood

Flow cytometric analysis was performed on fresh whole blood and umbilical cord blood samples. Two hundred microliters of citrate treated whole blood was obtained in a blood collection tube (BD Diagnostics, Franklin Lakes, NJ) and incubated for 15 min with ammonium chloride solution to promote red blood cell lysis. Remaining cells were centrifuged and washed 2 times with Dulbecco's Phosphate buffered saline (dPBS). Titrated biotinylated or directly fluorochrome conjugated antibodies to extracellular targets were incubated with cells at room temperature for 15 min. Following incubation, cells were washed once with 4 ml cold (4 °C) dPBS with 0.1% of fetal bovine serum (FBS) and 5 micromolar sodium azide (wash buffer). Cells were then incubated with LIVE/DEAD fixable near IR cell stain (Invitrogen, Carlsbad, CA) at room temperature for 10 min. Cells were then washed twice with wash buffer and fixed with 1% paraformaldehyde in phosphate buffered saline (PBS). Lymphocytes were differentiated using forward and side scatter characteristics on an LSRII (BD Biosciences, San Jose, CA). B cell populations were analyzed using anti-human CD20 Pacific Blue (2H7, eBioscience, San Diego, CA), CD27 PE (M-T271, BD Bioscience, San Jose, CA), CD40 FITC (5C3, BD Bioscience), CD86 PE-Cy5 (IT2.2, eBioscience) and CD95 APC (DX2, BD Bioscience). List-mode multiparameter data files were analyzed using the FlowJo software program (Version 8.8.6; Tree Star Inc., Ashland, OR).

2.3. Flow cytometry analysis of lymphoid and effector organ sites

Mononuclear cells from peripheral blood, lymph nodes and bronchoalveolar lavage were isolated through centrifugation over Lymphocyte Separation Media (Mediatech, Manassas, VA) gradients and either stained fresh or cryopre-served prior to staining. All samples were stained for CD20, CD27 and viability and analyzed on an LSRII.

2.4. Blood processing and cell separation based on expression of CD20 and CD27

Purified PBMC were isolated prior to fluorescence activated cell sorting (FACS) based cell separations. Thirty milliliters of citrated whole blood were utilized for purification. PBMC were isolated using Lymphocyte Separation Media. Purified PBMC were stained with CD20, CD27 and CD4 in order to identify B cell and T cell populations. The sort was performed in single cell mode (yield mask=0, purity mask=32 and phase mask=16) verified at greater than 95% purity in post-sort analysis. Cells were sorted on a BD FACSaria into tubes containing FBS. Following isolation, cells were immediately pelleted and processed for RNA isolation.

2.5. Sequence analysis of immunoglobulin gene for somatic hypermutation

Total RNA was extracted from sorted cells using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA) following the manufacturers protocol and the AMV Reverse Transcription System (Promega, Madison, WI) was used to prepare cDNA. Amplification of cDNA was carried out using nested PCR catalyzed by high fidelity Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA). Each round of PCR was performed in a volume of 25 μ l using oligonucleotide primers with specificity for IgM IGHV1/7 chain family genes for 35 cycles (outer nested reaction) and 30 cycles (inner nested reaction) as previously described (Margolin et al., 2006). PCR products were size selected from electrophoresis in 1.25% agarose and purified using the PureLink Quick Gel Extraction Kit (Invitrogen). IgM H chain libraries were constructed using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) per manufacturer's instructions. Purified plasmids were obtained from *E. coli* (One Shot, Top Ten Chemically Competent cells (Invitrogen) using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The presence of a gene insert was verified by gel electrophoresis following a 1 hour digestion with EcoR1 enzyme. Insert containing clones were analyzed for DNA sequence using the M13 forward primer and an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) at the University of Pittsburgh Genomics and Proteomics Core laboratory (Pittsburgh, PA).

2.6. Sequence alignment

IGHV1/7 nucleic acid sequences for RMs (GenBank/EMBL AY161053–71 and AY161078–79 (Bible et al., 2003)) were used to create a genetic database. Distance based analysis was performed with Geneious software version 4.7.4 (Auckland, New Zealand) using Tamura-Nei distance estimates and a neighbor joining algorithm to create a phylogenetic tree for the IGHV1/7 genes. Experimental sequences obtained from CD20+CD27+ and CD20+CD27– B cells from RMs were instilled into the database individually to determine germline sequence identity. Functional IGHV1/7 sequences were next aligned using Geneious Alignment (Geneious software version 4.7.4) to database sequences within an identity cluster. Data are reported as percent homology to germline sequence.

2.7. Isolation of PBMC and activation through ligation of CD40

Cells were isolated over Lymphocyte Separation Media (Mediatech), plated at 1×10^6 cells/ml in a 24 well tissue culture plate (2 ml/well) and incubated in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, penicillin and streptomycin. Cells were either treated with anti-CD40 antibody (MAB89, Abcam, Cambridge, MA) at 200 ng/ml or were left untreated for 24 h. After 24 h, cells were stained for CD20, CD27, CD95 and Annexin V (Caltag, Carlsbad, CA) and treated with a viability dye. Cells were fixed in 1% cold paraformaldehyde and analyzed on an LSRII.

2.8. Statistical analysis

Paired Student's T test, T test with unequal variance and 1 way ANOVA with repeated measures and Bonferroni's multiple comparison test were used to compare differences between groups. All Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Phenotypic characterization of CD27 in peripheral blood in RMs

Surface molecule expression and size discrimination were utilized for initial phenotypic parsing of B cell subsets. For these studies, peripheral blood cells were stained with

antibodies specific for CD20, CD27, CD40, CD86 and CD95, and analyzed by flow cytometry. The CD20+ B cell population was subsequently resolved based on CD27 surface expression, which has been used to define memory cells in humans (Yang et al., 1996; Agematsu et al., 1997; Klein et al., 1998; Avery et al., 2005). For the 6 animals analyzed in this study, an average of 6.9% of peripheral blood lymphocytes were CD20+; 44.9% of CD20+ cells expressed CD27 (Fig. 1). More CD20+CD27+ cells expressed CD95 ($54.9\% \pm 10\%$) and CD86 ($79\% \pm 13\%$) compared to CD20+CD27- cells, of which $8.3\% \pm 3.1\%$ and $71.6\% \pm 17.9\%$ expressed CD95 and CD86 respectively. Additionally, more CD20+CD27- B cells expressed surface CD40 ($55.4\% \pm 11.1\%$) compared to CD20+ CD27+ cells ($21.9\% \pm 7.3\%$). CD20+CD27+ cells were also significantly larger (average geometric mean of FSC = 734.6 ± 199.7) than CD20+CD27- cells (average geometric mean of FSC = 633.6 ± 185.6) (Fig. 2). These data demonstrated that the pattern of activation marker expression and size of CD20+CD27+ B cells is consistent with a memory phenotype.

3.2. Increased surface expression of CD27 on B cells in effector sites

To further validate CD27 as a memory B cell marker in RMs, the frequency of CD20+CD27+ B cell populations in effector sites was compared to B cells from peripheral blood and lymph nodes. Previous studies in T cells have demonstrated that extralymphoid effector sites have an increased abundance of effector memory T cells (Pitcher et al., 2002). Given that interactions between immune cells and pathogens occur at these effector sites, it was anticipated that these locations would include higher frequencies of memory B cells (Brandtzaeg et al., 1999). To test this hypothesis, the distribution of B cells expressing CD27 was analyzed in lymphoid and effector sites. Following detailed analysis of tissue from three animals, lymphoid sites (lymph nodes) had fewer CD27 expressing CD20+ B cells (25%) compared with 45% found in peripheral blood. This was in contrast to effector sites (lungs and gut), which had 70% and 75% of CD20+ B cells expressing CD27, respectively (Fig. 3). These data demonstrated that higher percentages of CD20+CD27+ B cells were present in effector sites.

3.3. B cells from neonatal RMs do not express CD27

To compare the distribution of CD27 on antigen-naïve versus antigen-experienced B cells, expression of surface CD27 was evaluated in embryonic cord blood samples. By extension from human literature (Agematsu et al., 1997), we predicted that very few CD20+CD27+ B cells would be present in cord blood. Indeed, minimal levels of CD27 were observed in CD20+ B cells from RM umbilical cord blood ($2.4\% \pm 2.5\%$, average of 3 animals) compared to expression observed in peripheral blood B cells in adult RMs ($44.9\% \pm 17.1\%$) (Fig. 4). These data confirmed that B cells that have not encountered antigen do not express CD27.

3.4. Surface expression of CD27 on B cells in the RM corresponds with somatic hypermutation of the immunoglobulin gene

A defining molecular feature of memory B cells is the presence of somatic hypermutation of the immunoglobulin gene variable region (McHeyzer-Williams and McHeyzer-Williams, 2005). Therefore, we examined the somatic hypermutation status of the CD20+CD27+ B cells from RMs. For these analyses, RM peripheral blood cells were sorted by FACS into CD20+CD27+ and CD20+CD27- populations (Fig. 5a). Following mRNA extraction from these cells, cDNA amplification of the immunoglobulin M heavy chain variable region (IGHV) 1/7 was performed using nested PCR (Margolin et al., 2006). Sequences from one representative subgroup (IGHV1p) are shown in Fig. 5b, while Table 1 summarizes all 40 IGHV1/7 family sequences obtained. Sequence analyses of these amplified regions from 5 independent animals revealed that CD20+ CD27+ cells in the RM demonstrated significantly less sequence homology to germline sequences (95.5%) compared to the CD20+CD27- cells (99.4%) (Fig. 5c and Tables 1 and 2). Somatic hypermutation was

confirmed by analysis of mutation sites; many of the observed mutations occurred in and around the complementarity determining regions. These data provided a clear indication at the molecular genetic level that CD27 expression denotes memory B cells.

3.5. CD40 ligation induces naïve but not memory B cell survival

To build on differences brought to light in the phenotypic and molecular genetic analysis, we sought to establish whether CD20+CD27⁻ and CD20+CD27⁺ B cell populations are functionally distinct. CD20+CD27⁻ and CD20+CD27⁺ B cells were predicted to respond to activating stimuli such as CD40 ligation with unequal kinetics. CD40 ligation is essential for upregulation of activation markers, class switching and B cell maturation (Splawski et al., 1993; Arpin et al., 1995; van Kooten and Banchereau, 2000). Activation of CD40 with CD40L (CD154) and the addition of interleukin (IL)-2, IL-4 and IL-10 for 4 days *in vitro* has previously been shown to result in the expansion of human naïve B cells and the maturation of memory B cells (Fecteau and Neron, 2003; Fecteau et al., 2006). In this study, treatment of B cells with anti-CD40 antibody for 24 h altered the relative ratios of CD20+CD27⁻ and CD20+CD27⁺ B cells, but did not result in expansion of either population. Following a 24 hour incubation, there was a significant difference ($P<0.05$) in the percentage of CD20+CD27⁻ B cells between the group treated with anti-CD40 antibody ($64.2\% \pm 16.7\%$) and the untreated group ($49.3\% \pm 18.8\%$) (Fig. 6a). In the CD20+CD27⁺ B cell population, the percentage of cells decreased following treatment with anti-CD40 antibody compared to untreated cells. An average of $35.9\% \pm 16.8\%$ were CD20+CD27⁺ after 24 h of treatment compared to $50.7\% \pm 18.8\%$ after 24 h with no treatment ($p<0.05$) (Fig. 6b). CD40 treatment increased the ratio of naïve cells and decreased the ratio of memory cells.

A protective effect of CD40 treatment was observed in the naïve B cell population. A significant difference ($p<0.05$) in the absolute number of CD20+CD27⁻ cells was also observed between anti-CD40 antibody treated ($42,874 \pm 41,820$ cells) and untreated ($26,604 \pm 16,707$ cells) groups (Fig. 6c). In the CD20+CD27⁺ population, in contrast to the higher number of cells observed in the anti-CD40 antibody treated CD20+CD27⁻ population, slightly fewer cells were present in the treated group ($19,430 \pm 18,173$) than were in the untreated group ($27,956 \pm 20,756$ cells) (Fig. 6d). These data demonstrated that CD40 binding aids in the maintenance of naïve but not memory B cell numbers.

3.6. CD40 binding results in a reduction of the percentage of apoptotic CD20+CD27⁻ B cells

To further understand the effects of CD40 mediated activation in the rhesus model, we measured the effect of CD40 ligation on activation and apoptosis. First, we assessed whether anti-CD40 antibody treatment of naïve and memory B cells increased the expression of the activation marker CD95. Treatment with anti-CD40 antibody resulted in significant ($P<0.05$) three-fold increase in percentage of CD95⁺ CD20+CD27⁻ cells from $13\% \pm 5.5\%$ in the untreated group to $30\% \pm 3.8\%$ (Fig. 7a). Although the CD20+CD27⁺ B cell population had higher basal levels of CD95 expression, no differences were observed between untreated and CD40 antibody treated groups (Fig. 7b).

A prior *in vitro* study by Hu et al. demonstrated that CD40 ligation can improve the survival of human tonsillar memory B cells (Hu et al., 1997). In order to test whether CD40 ligation had differential protective effects on CD20+CD27⁻ and CD20+CD27⁺ B cell populations in the RM, we measured Annexin V staining on anti-CD40 antibody treated and untreated cells. Moderately fewer CD20+CD27⁻ B cells appeared apoptotic following 24 hour treatment with anti-CD40 antibody compared to untreated CD20+CD27⁻ cells, representative plots of each population are shown in Fig. 7c and d. In the untreated group,

28.8%±9.4% of CD20+CD27⁻ cells stained with Annexin V and in the anti-CD40 antibody treated group, this was modestly reduced to an average of 22.4%±9.3% (Fig. 7e). No effect of treatment was observed in the CD20+CD27⁺ B cells (Fig. 7f). Together, these results indicated that CD40 binding increased the activation and slightly improved the survival of the CD20+CD27⁻ B cell population.

4. Discussion

The RM model is a valuable tool for the production of improved vaccines and therapeutic treatments for numerous human diseases but fundamentally important immunological parameters have not been definitively characterized. Phenotypic and molecular analysis demonstrated that surface expression of CD27 distinguishes naïve and memory B cell populations, as determined at the phenotypic and genetic levels. Further analysis demonstrated that functional differences in survival, activation and apoptosis following CD40 ligation were present between CD20+CD27⁻ and CD20+ CD27⁺ B cells. Together, these data will aid in the understanding of the B cell response to pathogenic infections in the RM model.

This study established that surface expression of CD27 marks memory B cells in the RM model. We showed here that sorted CD20+CD27⁺ B cells had somatic mutations in the IGHV1/7 region, many of which occurred in and around complementarity determining regions. Additional data, including the demonstration that CD20+CD27⁺ B cells have increased expression of activation markers, the increased abundance of CD20+CD27⁺ B cells in effector sites compared to lymphoid sites, and the increased size of CD20+ CD27⁺ B cells compared to CD20+CD27⁻ B cells further supports this finding. Finally, antigen-naïve B cells obtained from RM umbilical cord blood had very little CD27 expression. The further characterization of B cells from these findings in the RM model provides a basis for additional functional studies of B cell populations.

Functionally, CD20+CD27⁻ B cells were more sensitive to CD40 receptor binding than the CD20+CD27⁺ B cell population. CD40 binding resulted in CD20+CD27⁻ B cell survival and in contrast, exacerbated spontaneous cell reductions in CD20+CD27⁺ B cells *in vitro* either by hastening the apoptosis of these cells or by driving them through the maturation process. CD40 ligation also resulted in an increased percentage of CD20+CD27⁻ B cells expressing CD95 and a significant reduction in the percentage of apoptotic cells compared to untreated controls.

CD40 ligation may have multiple mechanistic outcomes depending on the specific B cell population in which it is activated. Previous studies in humans have demonstrated that CD40 ligation plays a central role in the maturation and differentiation of naïve and memory B cells. Specifically, CD40 ligation results in increased expression of surface CD95 and is essential for the development of class switched antibody producing cells (Schattner et al., 1995; Lagresle et al., 1996; Conley et al., 2009). CD40 ligation also results in B cell activation through a signaling cascade that includes the activation of the I κ B kinase (Kosaka et al., 1999). Interestingly, in the current study, CD40 ligation resulted in an upregulation of CD95 expression in naïve B cells but not in the memory B cell compartment indicating that analyzing the populations separately provides a clearer picture of which cell populations become activated. In addition, our results indicated that CD40 mediated changes could be observed within 24 h which may be more physiologically relevant than prior studies which measured changes after 4 or more days. Further, the present study provides more detailed information about the effect of CD40 ligation on the number of cells present and the percentage of those cells which were apoptotic. Interestingly, in this study, it was demonstrated that CD40 mediated effects on the naïve B cell population were not mirrored

in the memory B cell population, indicating that the role of CD40 ligation is different between the two populations.

The utility of the RM is clear in studies of simian immuno-deficiency virus (SIV) infection, a system used to model HIV infection in humans. While HIV cannot infect RMs, HIV and SIV are genetically and structurally similar (Chakrabarti et al., 1987). Thus, understanding the similarities (and differences) between HIV and SIV has led to a greater understanding of human HIV infections, with the RM providing an invaluable preclinical animal model for the evaluation of therapeutic and vaccine strategies to prevent infection and/or disease. Prior SIV studies in RMs have demonstrated reductions in CD20+ B cell numbers in RMs during acute infection (Mattapallil et al., 2004), and that there are differential responses to infection between CD20+CD27– and CD20+CD27+ cell populations (Kuhrt et al., 2010). These data indicate that the regulation of CD20+CD27– and CD20+CD27+ B cells is different during infection and different SIV induced alterations may be occurring in each population and needs to be studied further.

The experiments reported here establish CD27 as a marker of memory B cells in RMs and demonstrate that CD20+CD27+ and CD20+CD27– B cells have functionally distinct responses to CD40 binding. These critical analyses of lymphoid populations improve our collective understanding of B cells in the RM and will enhance our ability to translate findings from the RM model to humans.

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Abbreviations

RM	rhesus macaque
HIV	human immunodeficiency virus
SIV	simian immunodeficiency virus
IGHV	immunoglobulin M heavy chain variable region

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Fig. 1.

Surface expression of CD27 and activation markers on RM B cells. (a.) Representative flow cytometry analysis of CD20+ peripheral blood B cells based on CD27 expression. The gate for positive CD27 levels was set against background staining of myeloid cells which are not known to express this marker. The CD27+ and CD27- subsets were subsequently examined for expression of CD95, CD86 and CD40. Averaged results for percent of cells expressing CD95 (b.), CD86 (c.) and CD40 (d.). n=6 independent animals. Means were compared using paired Student's T test.

Fig. 2. CD20+ CD27+ cells are larger than CD20+ CD27- peripheral blood cells. (a.) Representative flow cytometry analysis of relative sizes of CD20+ CD27- (black line) and CD20+ CD27+ subsets (gray line). (b.) Averaged geometric mean values of FSC for 6 independent animals. Means were compared using a paired Student's T test.

Fig. 3. Surface expression of CD27 and activation markers on B cells from tissue compartments. (a.) Representative flow cytometry analysis of CD27 expression on CD20⁺ cells in peripheral blood, axillary lymph node, mediastinal lymph node, small intestine and bronchial alveolar lavage samples. (b.) Plotted values for each replicate, the line represents the average value for all samples (n=3 independent animals).

Fig. 4.

CD20+ B cells from umbilical cord blood do not express CD27. (a.) Analysis of CD27 expression on CD20+ B cells from 3 umbilical cord blood samples. (b.) Averaged CD27+ B cell expression from adult peripheral blood (n=6 independent animals) and cord blood samples (n=3 independent animals). Means were compared using a T test with unequal variance.

Fig. 5.

CD20+CD27+ cells but not CD20+CD27- cells demonstrate somatic hypermutations at Ig variable regions. (a) Representative plots for cell sorting parameters and post-sort purity analysis of sorted populations. (b) Representative sequence samples from IGHV1p family genes, shaded areas denote complementarity determining regions. (c) Percent homology to germline sequences for IGHV1 family genes isolated from CD20+CD27- and CD20+CD27+ B cells. Results are representative of 40 unique sequences obtained from 5 individual animals. Means were compared using a T test with unequal variance.

Fig. 6.

Binding of CD40 on CD20+CD27- but not CD20+CD27+ B cells results in protection from spontaneous cell losses in a 24 hour culture system. PBMC were cultured in the presence or absence of 200 ng/ml of anti-CD40 antibody for 24 h. Cultures were harvested and then analyzed for CD20 and CD27 expression in order to differentiate the percentages and numbers of B cell populations. (a) Percentage of CD20+CD27- B cells and (b) CD20+CD27+ B cells. (c) Absolute number of CD20+CD27- B cells and (d) CD20+CD27+ B cells. Results are representative of 6 independent experiments run in duplicate. Error bars represent the standard error of the mean. Mean values for both culture conditions and freshly isolated cells were compared using 1 way ANOVA with repeated measures and Bonferroni's multiple comparison test.

Fig. 7.

The percentage of CD20+CD27- B cells expressing CD95 increases and the percentage of CD20+CD27- B cells that bind Annexin V decreases after anti-CD40 antibody treatment. PBMC were cultured in the presence or absence of 200 ng/ml of anti-CD40 in triplicate for 24 h. Cultures were harvested and then analyzed for CD95 expression within (a) CD20+CD27- or (b) CD20+CD27+ B cells. Representative flow cytometry plots of the CD20+CD27- B cell population following 24 hour culture (c) without anti-CD40 antibody treatment and (d) with anti-CD40 antibody treatment. The average percentage of Annexin V staining for all 6 animals within the (e) CD20+CD27- or (f) CD20+CD27+ B cell populations. Mean values for both culture conditions and freshly isolated cells were

compared using 1 way ANOVA with repeated measures and Bonferroni's multiple comparison test.

Table 1

Sequence homology of IGHV1/7 sequences isolated from sorted CD20+CD27- B cells in RMs.

Parental sequence ^a	Animal ID ^b	Framework mutations ^c	CDR mutations ^d	Total mutations ^e	Percent identity ^f
IGHV7a	RO245	0	0	0	100
IGHV7a	RO245	1	1	2	99.3
IGHV7a	RO245	1	1	2	99.3
IGHV7a	RO245	1	1	2	99.3
IGHV7a	RO245	1	0	1	99.7
IGHV7a	RO245	1	0	1	99.7
IGHV7a	RO310	4	1	5	98.3
IGHV7a	RO316	3	0	3	99.0
IGHV7a	RO316	1	0	1	99.7
IGHV7a	RO316	1	1	2	99.3
IGHV7a	RO316	1	1	2	99.3
IGHV7a	RO442	4	1	5	98.3
IGHV7a	RO442	1	1	2	99.3
IGHV7a	RO442	1	1	2	99.3
IGHV1c	RO310	1	2	3	99.0
IGHV1l	RO310	0	0	0	100
IGHV1l	RO316	2	2	4	98.6
IGHV1l	RO316	0	0	0	100
IGHV1l	RO316	0	0	0	100
IGHV1l	RO316	0	0	0	100
IGHV1p	RO245	1	0	0	99.7
IGHV1p	RO310	0	0	0	100
IGHV1p	RO316	1	0	0	99.7
Average		1.1	0.6	1.6	99.4
Standard Deviation		1.1	0.7	1.6	0.5

^aIGHV1/7 germline parental sequence as determined by using Tamura-Nei distance estimates and a neighbor joining algorithm.

^bIdentification for RMs.

^cThe number of mutations in the framework regions of the Ig V region gene.

d The number of mutations in the complementarity determining regions of the Ig V region gene.

e Total number of nucleotide changes from the parental sequence in the immunoglobulin variable region of the sequenced cells.

f Relative identity of the experimentally obtained sequence to the germline sequence.

Table 2

Sequence homology of IGHV1/7 sequences isolated from sorted CD20+CD27+ B cells in RMs.

Parental sequence ^a	Animal ID ^b	Framework mutations ^c	CDR mutations ^d	Total mutations ^e	Percent identity ^f
IGHV7a	RO245	0	0	0	100
IGHV7a	RO245	0	0	0	100
IGHV7a	RO316	5	4	9	96.9
IGHV7a	RO316	4	4	8	97.2
IGHV7a	RO632	15	8	23	92.0
IGHV7a	RO632	14	8	22	92.4
IGHV7b	RO316	4	4	8	97.2
IGHV7b	RO442	19	5	24	91.7
IGHV1k	RO310	10	1	11	96.2
IGHV1l	RO310	37	16	53	81.6
IGHV1l	RO316	0	0	0	100
IGHV1l	RO442	16	1	17	94.1
IGHV1p	RO245	9	2	11	96.2
IGHV1p	RO245	7	2	9	96.9
IGHV1p	RO310	7	1	8	97.2
IGHV1p	RO310	6	2	8	97.2
IGHV1p	RO316	2	1	3	99.0
IGHV1p	RO442	14	4	18	93.8
Average		9.4	3.5	12.9	95.5
Standard Deviation		9.1	4.0	12.6	4.4

^aIGHV1/7 germline parental sequence as determined by using Tamura-Nei distance estimates and a neighbor joining algorithm.

^bIdentification for RMs in study.

^cThe number of mutations in the framework regions of the Ig V region gene.

^dThe number of mutations in the complementarity determining regions of the Ig V region gene.

^eTotal number of nucleotide changes from the parental sequence in the immunoglobulin variable region of the sequenced cells.

^fRelative identity of the experimentally obtained sequence to the germline sequence.