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Class-switched marginal zone B cells in spleen have relatively low numbers of somatic mutations

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ABSTRACT

The vast majority of rodent splenic marginal zone (MZ)-B cells are naive IgM$^+$ cells. A small fraction of these MZ-B cells carry mutated V-genes, and represent IgM$^+$ memory MZ-B cells. Here we reveal further heterogeneity of B cells with a MZ-B cell phenotype, by providing evidence for the existence of class-switched memory MZ-B cells in the rat. In essence, we observed IGHV5 encoded Cγ transcripts, among FACS-purified MZ-B cells, defined as HIS24^{low}HIS57^{bright} cells. Furthermore, we found that most IgG encoding transcripts are mutated. There is no significant difference in IGHV5 repertoire and subclass usage of these IgG encoding transcripts collected from B cells with a MZ-B cell phenotype and B cells with a follicular (FO) B cell phenotype. However, the IGHV5 genes encoding for IgG antibodies of MZ-B cells exhibited significantly fewer mutations, compared to those with a FO-B cell phenotype. In one rat we found a clonally related set of IgG encoding sequences, of which one was derived from the MZ-B cell fraction and the other from the FO-B cell fraction. We speculate that these two subpopulations of class-switched B cells are both descendants from naive FO-B cells and are generated in germinal centers. Class-switched memory cells with a MZ-B cell phenotype may provide the animal with a population of IgG memory cells that can respond rapidly to blood-borne pathogens.

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1. Introduction

The marginal zone (MZ) represents a distinct anatomical B cell compartment in the spleen located at the outer areas of the white pulp, at the border of the red pulp (for review see e.g. Steiniger et al., 2006). The circulatory system of the spleen ensures an intimate contact of blood and cells of the MZ. Most of the cells in this compartment are B cells, but macrophages and dendritic cells (and in humans also CD4$^+$ T cells) are also present. MZ-B cells have unique characteristics (for reviews see e.g. Martin and Kearney, 2002; Pillai et al., 2005; Weill et al., 2009). In rodents the vast majority of MZ-B cells expresses high levels of IgM and low levels of IgD (IgM^{hi}IgD^{lo}) in combination with high levels of CD21 and low levels of CD23 (CD21^{hi}CD23^{lo}) (Oliver et al., 1997). This unique phenotype distinguishes them from the majority population of mature, naive B cells, i.e. follicular (FO) B cells, which are IgM^{lo}IgD^{hi}CD21^{lo}CD23^{hi}. Rat FO-B cells can also be defined as mature (i.e. CD90$^-$ (Kroese et al., 1995)) small-sized, HIS24^{high}HIS57$^{neg/low}$ B cells whereas MZ-B cells are slightly larger cells and can be distinguished as CD90$^-$HIS24^{low}HIS57^{high} cells (Dammers et al., 1999; Kroese et al., 1990, 1995). Importantly, MZ-B cells also have different functional characteristics, such as their pre-activated status and their proliferative and stimulatory requirements (Oliver et al., 1997, 1999). Rodent MZ-B cells appear to be biased towards T cell-independent (TI-2) immune responses against micro-organism-derived polysaccharide antigens (Guinamard et al., 2000; Martin et al., 2001; Vinuesa et al., 2003). These properties in combination with their topographical localization in spleen, allow them to respond rapidly to blood-borne pathogens by the generation of massive numbers of antibody secreting cells during the first few days after infection (Martin et al., 2001).

MZ-B cells are a heterogeneous population of cells, and comprise both naive and memory cells. In rats and mice, the majority (up to

Abbreviations: FO, follicular; GC, germinal center; IGHV, Ig heavy chain V region genes; IGHD, Ig heavy chain D region genes; IGHJ, Ig heavy chain J region genes; HEL, hen egg lysozyme; MZ, marginal zone; TI-2 antigen, T cell independent type 2 antigen.

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80%) of MZ-B cells are naive cells which express germline encoded V region of the Ig genes (Dammers et al., 2000; Makowska et al., 1999). Occurrence of memory B cells in the MZ was first demonstrated by Liu et al. (1988), showing the appearance of hapten-binding, IgM^+ memory cells with a MZ-B cell phenotype in the MZ of spleens from immunized rats. Hapten-binding MZ-B cells were also demonstrated by flow-cytometry and/or immunohistology in several subsequent studies in immunized normal and Ig-gene targeted mice (Gatto et al., 2004, 2007; Obukhanych and Nussenzweig, 2006; Pape et al., 2003; Phan et al., 2005). In rodents, memory cells constitute a minority MZ-B cell population. Up to 20% of the rodent MZ-B cells might be IgM memory B cells as indicated by the presence of mutated Ig H chain V gene (IGHV) trancripts encoding for IgM antibodies (IGHV-Cμ transcripts) among purified $sIgM^+$ MZ-B cells (Dammers et al., 2000; Makowska et al., 1999). There are some data that suggest that, in addition to these unswitched IgM-expressing memory MZ-B cells, also some class-switched (memory) B cells are found among the MZ-B cell population in rodent spleens after immunization (Gatto et al., 2004; Liu et al., 1988; Obukhanych and Nussenzweig, 2006; Pape et al., 2003). For example, Gatto et al. (2004) observed the presence of IgG^+ phage (Qβ)-specific B cells with a MZ phenotype (i.e. $CD21^{hi}CD23^{low}$ B cells), up to 21 days upon immunization of normal mice. Whether these class switched MZ-B cells were indeed "classical" memory cells with their characteristic mutated high affinity BCR's was, however, not investigated. The presence of mutated, antigen-specific (memory) MZ-B cells was subsequently demonstrated in mice 12 days after immunization with viral particles (Gatto et al., 2007). The isotype of these cells was, however, not known, but the authors speculated that these mutated MZ-B cell sequences were derived from class-switched cells. In humans, a relative large proportion (30%) of the MZ-B cells (defined as $CD21^+CD23^-CD27^+$ cells) appear to express IgG (Ettinger et al., 2007); the mutational status of these isotype-switched IGHV genes is also not known.

Thus, although both IgM^+ and IgG^+ memory cells appear to be present among the pool of MZ-B cells in both rodents and humans, direct evidence for presence of MZ-B cells with mutated IgG encoding genes is currently lacking. Furthermore, the origin of the IgG^+ memory MZ-B cells is enigmatic. This prompted us to analyze in detail the nucleotide sequences of IgG encoding (IGHV-Cγ) transcripts from purified rat MZ-B cells, defined in a sIg independent fashion. We show that indeed naturally occurring (i.e. without deliberate antigenic stimulation) MZ-B cells express mutated IGHV genes encoding for IgG antibodies. The repertoire of the MZ-B cell derived IgG encoding transcripts does not differ from that obtained from class-switched B cells with a FO-B cell phenotype, albeit that MZ-B cell derived IgG encoding transcripts exhibit lower numbers of mutations.

2. Materials and methods

2.1. Animals

Male PVG rats were purchased from Harlan (Horst, The Netherlands) at the age of 6–8 weeks. Animals were maintained until use under clean conventional conditions at the central animal facility of the University Medical Center Groningen. Experiments were approved by the Animal Ethics Committee of the University of Groningen.

2.2. Flow-cytometry

Spleens were taken from 4.5 to 8 months old animals. Single-cell suspensions were prepared from spleen and labeled with mAb as described previously (Dammers et al., 1999). Briefly, spleen cell suspensions from 4 animals were stained for flow-cytometry with the following two sets of mouse monoclonal antibodies: FITC conjugated anti-rat IgM (HIS40; eBioscience, San Diego, CA, USA) and biotinylated anti-rat IgD (MaRD3; AbD Serotec, Oxford, UK) or FITC anti-rat MZ-B cell marker (HIS57; BD Pharmingen, San Diego, CA, USA; Dammers et al., 1999) and biotinylated anti-rat CD45R (HIS24; Ebioscience). Biotinylated mAb were revealed with streptavidin conjugated to the tandem fluorochrome PE-Cy5.5 (Ebioscience). The two sets of antibodies were used in combination with a mixture of PE conjugated anti-rat TCRαβ (R73; eBioscience); TCRγδ (V65; eBioscience), CD90/Thy1.1 (HIS51; eBioscience) and CD161a/NKR-P1a (10/78; BD Pharmingen). The PE channel was used as a "Dump" channel; only PE negative ($Dump^-$) cells were sorted. Herewith, we were able to exclude immature B cells (i.e. CD90 positive B cells: Kroese et al., 1995), T cells and NK cells from our sorts. Cell analysis and cell sortings were performed on a MoFlo flow cytometer (Cytomation, Ft Collins, CO). Dead cell, plasma cell, monocyte/macrophage, and erythrocyte contamination was excluded from sorting by using forward and side scatter profiles. Sorted cells were collected in sterile FACS tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) containing 500 μl of newborn calf serum (PAA laboratories GmbH, Pasching, Austria). At least one million cells per B cell subset were sorted. B cell subsets were obtained with >95% purity. FlowJo software (Tree Star, San Carlos, CA) was used for flow cytometry data analysis.

2.3. Molecular cloning of rearranged IGHV5-Cγ transcripts

Total RNA was extracted from sorted cells using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA) according to instructions of the manufacturer. Briefly, sorted cells were pelleted by $300 \times g$ centrifugation for 10 min at 4 °C and then resuspended in a total volume of 350 μl lysis buffer containing β-mercaptoethanol (Stratagene). First strand cDNA was synthesized using an oligo-$(dT)_{12-18}$ primer (Invitrogen, Breda, The Netherlands) and SuperScript™ II reverse transcriptase (200 U/μl; Invitrogen) as described in the manufacturer's protocol. Rearranged immunoglobulin IGHV5-Cγ transcripts were amplified in a 50 μl reaction mixture, containing 2 μl cDNA and 0.6 pmol/μl IGHV5 (PC7183) family specific primer (5′-CTTAGTGCAGCCTGGAAGGT-3′; Dammers et al., 2000), 0.6 pmol/μl universal Cγ constant region primer (5′-GACAGGGATCCAGAGTTCCA-3′) and 2.5 U Taq DNA Polymerase (Invitrogen). The universal Cγ region primer was designed on the basis of a conserved sequence found in exon 1 of all rat IgG subclasses. To assess the amount and quality of the cDNA, PCR was also performed for β-actin, using β-actin specific primers as described by Stoel et al. (2008). The PCR program for amplification of IGHV5-Cγ transcripts and β-actin consisted of 35 cycles of 30 s at 94 °C (2 min in first cycle), 1 min at 58 °C and 1 min at 72 °C, respectively. This program was followed by an additional incubation period of 25 min at 72 °C to allow extension of all IGHV5-Cγ products. The quality and size of the PCR products was evaluated by agarose gel electrophoresis. PCR products were subsequently cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was isolated from randomly picked colonies with the Nucleospin Plasmid QuickPure kit (Clontech, Mountain View, CA, USA). Plasmids containing an insert of approximately 600 bp were sequenced in both directions at our local sequence facility (Department of Pathology and Laboratory Medicine, Division of Medical Biology, University Medical Center Groningen, Groningen, The Netherlands). Sequence processing was performed using ClustalW from the European Molecular Biology Laboratory and Chromas software (Digital River GmbH, Cologne, Germany).

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