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Supplemental Data

Expression of Immunoglobulin Receptors with Distinctive Features Indicating Antigen Selection by Marginal Zone B Cells from Human Spleen

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SUPPLEMENTARY RESULTS

Search for Sequences Related to Those of the MZ B Cells in FM and GC B Cells

In order to search for sequences related to one of the MZ B cell clonal families in other B cell subsets, we used a qRT-PCR with VH CDR3 clone-specific oligos (Supplementary Figure S2). There was no amplification for these specific VH CDR3 rearrangements in FM, GC B cells or in placental tissue (used as control), while amplification was specifically observed in MZ B cells (Supplementary Figure S2), possibly indicating that the clones investigated were present only in MZ B cells.

When sequenced, all the molecular clones from FM and GC B cells were found to contain *IGHV1-69* genes, proving the specificity of the methodology. The clones were either unique or recurrent according to the definition provided above. When we looked for sequences related to those found expanded in the MZ, 2 groups only of such clones were found in the GC, but not in the FM B cells from spleen 1, whereas only 1 and 2 groups of MZ-related sequences were detected in the FM and GC B cells respectively from spleen 4 (Supplementary Table S2). These sequences shared some (but not all) of the mutations of the clones from MZ B cells (in Supplementary Figure S3 is shown one of these groups) and the pattern of mutations was consistent with less diversification in GC- than in MZ-derived clones. However, because of the mutation pattern and the paucity of clones detected no progenitor/effector relationship could be established.

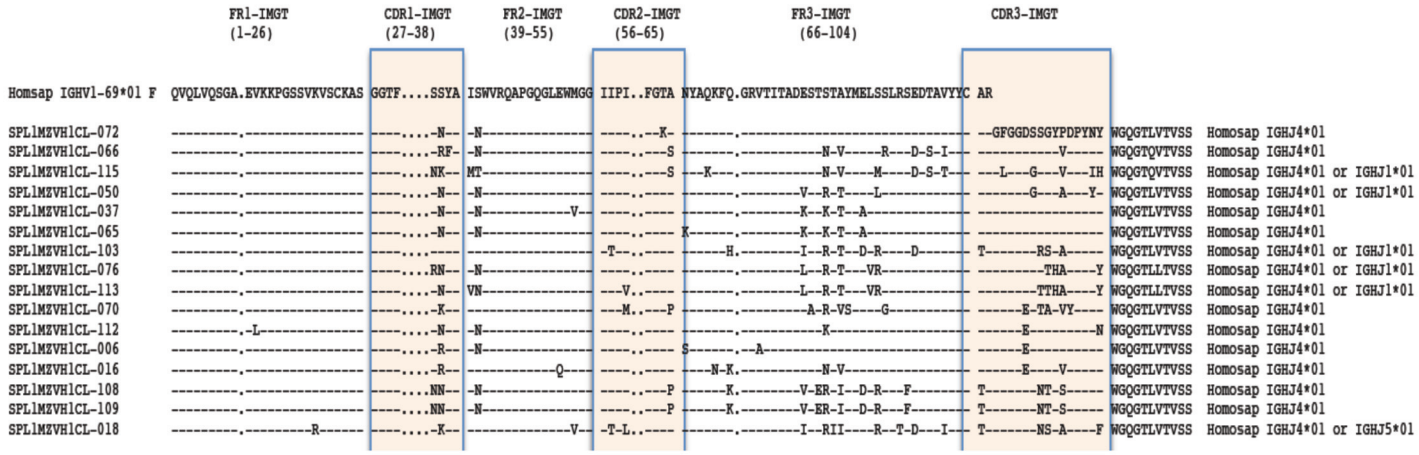
Supplementary Table S1. IGHV gene features of MZ, GC and FM B cells.

B cells subsets	N. Seq.	Average n. mutations (Min-Max)	IGHV gene diversity (%)	Mean transitions (Min-Max)	Mean transversions (Min-Max)
All sequences					
MZ	469	11.76 (0-48)	4.0	6.71 (0-26)	5.05 (0-22)
FM	419	3.58 (0-30)	1.2	2.01 (0-19)	1.57 (0-18)
GC	300	10.23 (0-35)	3.7	5.97 (0-24)	4.26 (0-16)
Mutated only					
MZ	361	14.80	5.06	8.43 (1-26)	6.36 (0-22)
FM	123	10.61	3.62	5.8 (1-19)	4.8 (0-18)
GC	225	13.44	4.86	7.81 (0-24)	5.64 (0-18)

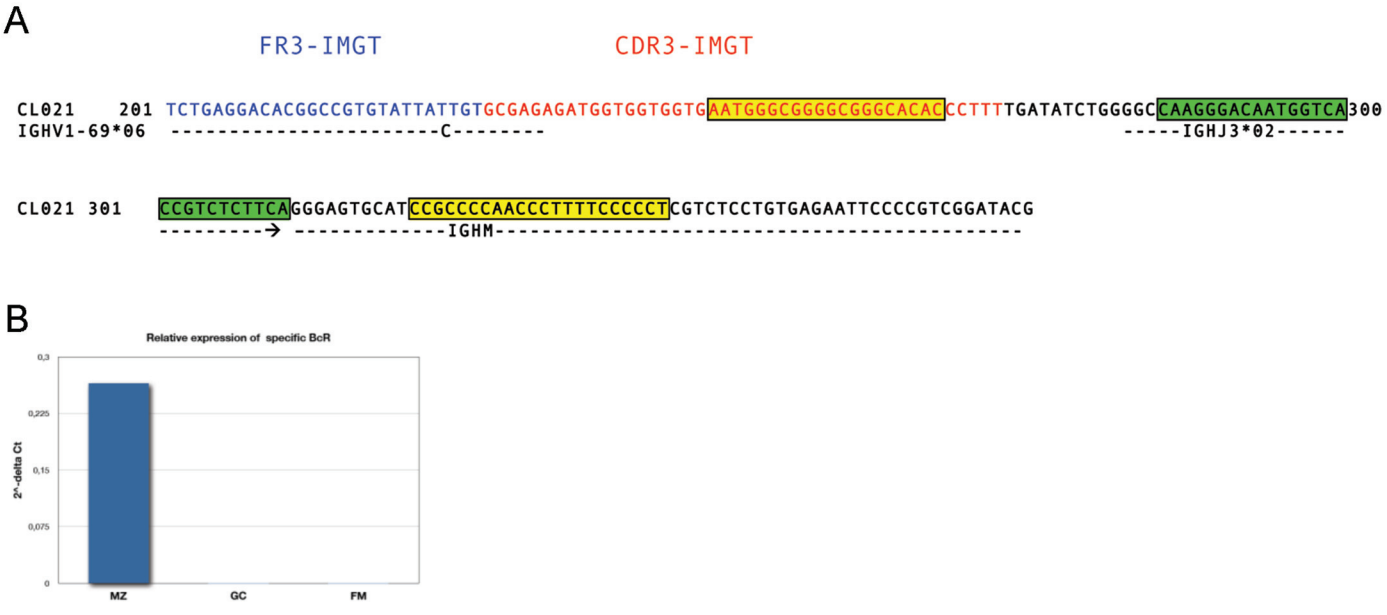
Supplementary Table S2. Summary of the results obtained following IGHV1-69 gene-specific nested PCR.

Spleen	B cell subset	IGHV gene	Sequences analyzed	Unique rearrangements (% TOT)	Recurrent rearrangements	Groups of shared clones[a]	N. of sequences[b]
1	FM	*1-69*	29	29/29 (100%)	0		
1	GC	*1-69*	59	26/59 (44%)	33	2	3 9
4	FM	*1-69*	46	23/46 (50%)	23	1	2
4	GC	*1-69*	51	8/51 (15.6%)	43	2	5 8

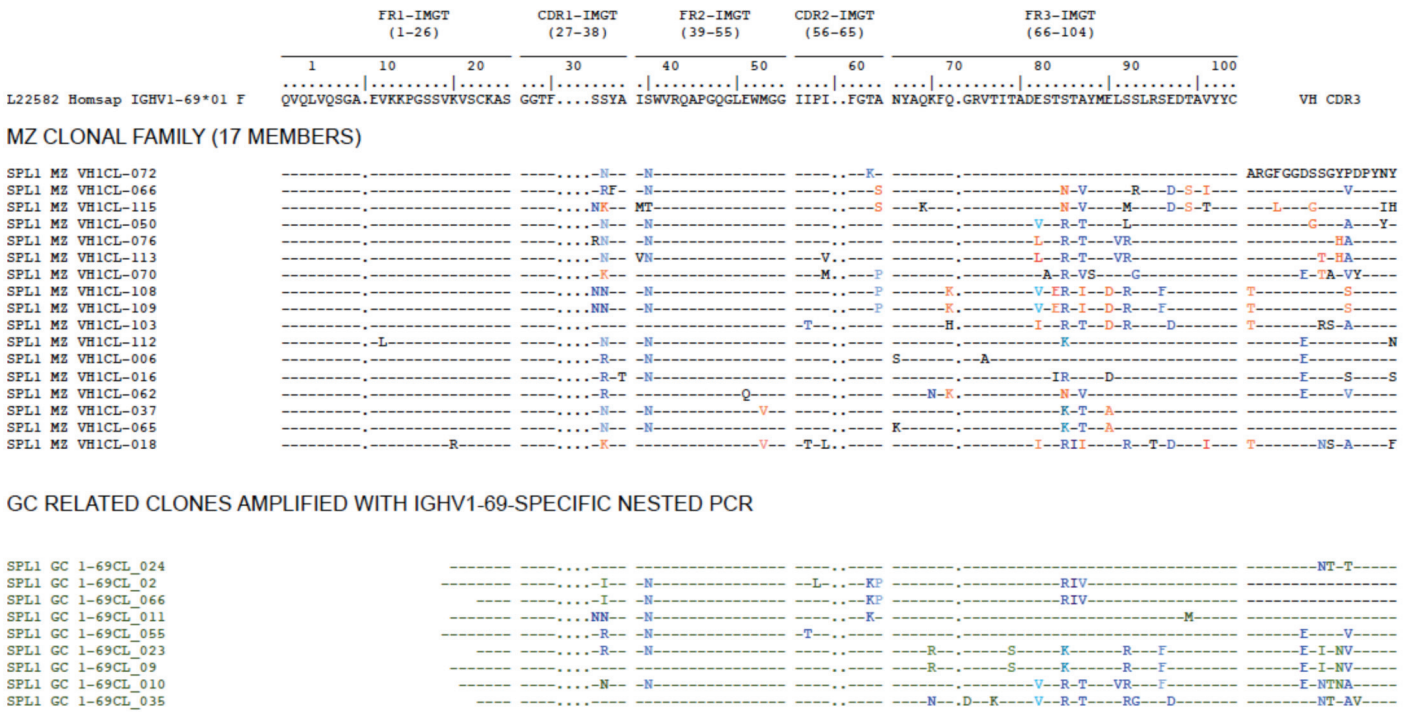
[a] Indicate the number of clonal families that are related to the clones found in the MZ B cells.
[b] Indicate the number of sequences included in each group of clones related to MZ.



Supplementary Figure S1. Intraclonal diversification in MZ B Cells. An example of related sequences isolated from MZ B cells of spleen 1 is reported. Amino acids sequences of related clones are aligned with the closest germ line gene sequence (*IGHV1-69*). In certain cases, IMGT software indicates two possible IGHJ germ line genes. The first has the highest identity. The presence of shared and unique mutations in the different clones is indicative of intraclonal diversification.



Supplementary Figure S2. Clone specific qRT-PCR. (A) Partial nucleotide sequence of a marginal zone clonal family member that uses IGHV1-69*04/ IGHD2-21*01 /IGHJ3*02 rearrangement is shown. Forward specific primer (yellow) was designed based on sequence of VH CDR3 and used in conjunction with primer specific for IGHM gene (yellow). Hybridization probe selectively bind the IGHJ3 gene (green). (B) Relative expression of specific VH CDR3 rearrangement was calculated by normalization with POL2 housekeeping gene. No amplification was obtained in the FM and GC B cell subsets while amplification was specifically observed in MZ B cells, possibly indicating that the clones investigated were present only in MZ B cells. Relative expression is calculated as $2^{-\Delta Ct}$.



Supplementary Figure S3. Shared sequences between B cell subsets with IGHV1-69-specific PCR. Related clones isolated from the corresponding GC B cell subsets by using IGHV1-69 leader specific primer in conjunction with IGHM specific primer followed by semi-nested PCR using FR1-VH1 gene subgroup specific primer are shown. Shared mutations are indicated by blue letters; subset specific mutations are in orange and green for MZ and GC B cells, respectively.

METHODS

List of Fluorescent Antibodies Used in FACS Sorting and Analyses:

FITC conjugated	polyclonal anti-δ and anti-α (Dako, Milan, Italy), CD71 (BD);
PE conjugated	anti-CD80 (BD), polyclonal anti-μ (Dako) and anti-FcRL-2 (R&D systems, Minneapolis, MN, USA);
PE-Cy5-conjugated	anti-CD24, anti-CD27 (Beckman Coulter, Milan, Italy), anti-μ (BD);
PE-Cy7-conjugated	anti-CD38 (BD); APC-conjugated anti-CD21, anti-CD22, anti-CD23, anti-γ, anti-CD43 (BD), anti-CD1c (Miltenyi Biotec, GMBH-Germany), anti-FcRL-4 (R&D);
APC-H7-conjugated	anti-CD19 (BD);
PE-CF594 –conjugated	anti-CD27 (BD).

List of Fluorescent Antibodies Used in Immunofluorescence Microscopy:

Unconjugated:	rabbit polyclonal anti-μ Ab(Ventana) (1:20)
	mAb (IgG1) anti- δ (Dako) (1:10)
	goat polyclonal anti- α Ab from Southern Biotechnology (Birmingham, Alabama, USA) (1:150)
	rat anti-AID (1:5) plus donkey-anti-rat CY3 (1:50) (Cell Signaling/Merk Millipore, Milano,Italy)
FITC conjugated:	rabbit polyclonal anti-antibody γ from Dako (1:10)
PE-conjugated:	rabbit polyclonal anti-μ Ab and anti δ-(Dako) (1:10)
	anti-CD38(BD) (1:5)
PE-Cy5-conjugated:	anti-CD27(Beckman Coulter) (1:50)
PerCP-conjugated:	anti-AID(1:2) (R&D)