The Normal Counterpart of IgD Myeloma Cells in Germinal Center Displays Extensively Mutated IgVH Gene, Cµ–Cd Switch, and λ Light Chain Expression

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Summary

Human myeloma are incurable hematologic cancers of immunoglobulin-secreting plasma cells in bone marrow. Although malignant plasma cells can be almost eradicated from the patient’s bone marrow by chemotherapy, drug-resistant myeloma precursor cells persist in an apparently cryptic compartment. Controversy exists as to whether myeloma precursor cells are hematopoietic stem cells, pre-B cells, germinal center (GC) B cells, circulating memory cells, or plasma blasts. This situation reflects what has been a general problem in cancer research for years how to compare a tumor with its normal counterpart. Although several studies have demonstrated somatically mutated immunoglobulin variable region genes in multiple myeloma, it is unclear if myeloma cells are derived from GCs or post-GC memory B cells. Immunoglobulin (Ig)D-secreting myeloma have two unique immunoglobulin features, including a biased λ light chain expression and a Cµ–Cd isotype switch. Using surface markers, we have previously isolated a population of surface IgM⁺IgD⁺CD38⁺GC B cells that carry the most impressive somatic mutation in their IgV genes. Here we show that this population of GC B cells displays the two molecular features of IgD-secreting myeloma cells: a biased λ light chain expression and a Cµ–Cd isotype switch. The demonstration of these peculiar GC B cells to differentiate into IgD-secreting plasma cells but not memory B cells both in vivo and in vitro suggests that IgD-secreting plasma and myeloma cells are derived from GCs.

Materials and Methods

A assay for Sµ–αδ R combination. Genomic DNAs were extracted from 3 × 10⁷ EBV transformed cells or 10⁵ fresh purified cells, according to the standard procedure. Genomic DNA was submitted to PCR amplification using the 5′ primer P3 (5′-CG-GCAATGAGATGGCTTT-3′) and the 3′ primer P4 (5′-GGCAAATCATGCGTTT-3′), as shown in Fig. 1A. All PCR reactions were performed with Taq polymerase (Perkin-Elmer Corp., Norwalk, CT) using the reaction buffer provided by the manufacturers and a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT).

Abbreviations used in this paper: CDR, complementarity determining region; GC, germinal center; s, surface.
Corps) with 35 cycles of 1 min denaturation at 94°C, 2 min primer annealing at 60°C, and 3 min extension at 72°C. Complete extension of the products was then performed by a final 10-min incubation at 72°C. For DNA sequencing, PCR products were cloned using the TA cloning kit (Invitrogen, Carlsbad, CA) on both strands. Individual bacterial colonies were randomly picked and extracted using primers specific for each VH family and C\textsubscript{D} regions from IgM, IgG, IgA, IgM, IgE, Ig\textalpha, IgD measurement, flat-bottomed 96-well plates were coated with monoclonal anti-IgD antibodies (Nordic Immunological, Tiburg, The Netherlands) overnight at 4°C. Cloning was performed by culturing 1 cell/tube No. 1 being the first 3' of the VDJ region. (D) Schematic representation of the break points.

Figure 1. C\textmu–C\textalpha switch recombination in IgM→IgD→CD38+GC B cells. (A) Schematic representation of the S\textgamma–\alpha and \alpha/\mu–\Sigma/\mu recombinations (14–16). (B) PCR amplification of S\textgamma–\alpha recombination. Lane A, IgM→IgD→CD38+GC founder cell; lane B, IgM→IgD→CD38+GC B cells; lanes C, D, and E, EBV clones from IgM→IgD→CD38+GC cells; lanes F, G, and I, EBV clones from IgM→IgD→CD38+ cells. (C) DNA sequences of S\textgamma–\alpha junctional regions from IgM→IgD→CD38+ cells. Upper strings, germline sequences of the S\textgamma region; lower strings, germline sequences of the \alpha/\mu–\Sigma/\mu intron. The central string represents the sequences of cloned PCR products. Homologous nucleotides are linked by vertical bars. A rowed figures, base numbers base \textalpha, 1 being the first 3' of the VDJ region.

Table 1. Percentage and Number of Clones Expressing Ig\textkappa and Ig\textlambda Derived from Three CD38+ GC B Cell Subsets

| Clones derived from | Percentage of clones expressing Ig\textkappa | Number of clones |
|--------------------|---------------------------------------------|-----------------|
| Ig\textgamma→IgD→CD38+ | 47%                                         | 83              |
| Ig\textdelta→CD38+    | 32%                                         | 53              |
| Ig\textgamma→IgD→CD38+ | 99%                                         | 71              |
Figure 2. Differentiation of IgM⁻⁻⁻⁻IgD⁺⁻⁺⁻CD38⁺⁺⁺⁺ B cells into IgD⁺⁺ plasma cells in vitro. IgD, IgG, IgA, and IgM secretion (A) and IgD immunostaining (B and C) of IgM⁻⁻⁻⁻IgD⁺⁺⁻⁻⁻⁻CD38⁺⁺⁺⁺ B cells cultured for 2 wk in the presence of IL-10, IL-2, and CD40 ligand transfected murine fibroblasts. Ig contents were measured by ELISA and results are given in μg/ml. Original magnifications of microphotographs are 100 (B) and 1,000 (C).

Figure 3. Ig heavy chain expression by tonsillar plasma cells. Immunoenzymatic staining of tonsillar tissue sections (A, B, and C) or plasma cell cytospin preparations (D and E). (A) Double red anti-IgD and blue anti-CD38 staining showing purple IgD⁺ plasma cell within a GC and red IgD⁺ follicular mantle (FM) B cell. Original magnification: 400. (B and C) Double red anti-IgD and blue anti-Ki67 staining showing red IgD⁺⁺ plasma cells under the tonsillar epithelium (EP). Original magnifications are 100 (B) and 400 (C). (D) Single red anti-IgD staining showing IgD⁺ plasma cells among purified tonsillar plasma cells. Original magnification: 1,000. (E) Double blue anti-IgD and red anti-IgM staining showing exclusive expression by plasma cells of each isotype. Original magnification: 1,000.
son, Mountain View, CA). They were permeabilized by an overnight incubation with 1% paraformaldehyde at 4°C. Intracellular IgD was stained with a mouse anti-IgD antibody-FITC (Dako Corp., Carpinteria, CA). CD38+ plasma cells were finally separated into intracellular IgD+ or IgD− plasma cells by cell sorting.

Sequence Analysis of the VH5 Transcripts. This was done according to our established methods (35–37). In brief, messenger RNA was extracted from 2.5 × 10⁶ plasma cells and cDNA was obtained by reverse transcription. Full-length VH5–δ transcripts were amplified with 5’ LH5 primer (5’-CCCAGATTCTAGGCTCAACCCGCATCCT-3’) with 3’ primer HCoδ (5’-GGCCGCTGGCCAGCGGAAGATCTCCTT-3’), HCoμ (5’-GGGTCAACCGCCATCCT-3’), or HCoγ (5’-CAGGGAGAGACGACGATT-3’) with Taq polymerase (Perkin-Elmer Corp.). PCR reaction was 35 cycles of 1 min denaturation at 94°C, 2 min of primer annealing at 60°C, and 3 min at 72°C. The frequency of Taq error in our lab is <2%. The PCR products were cloned, using the TA cloning kit (Invitrogen). Plasmids extracted from individual bacterial colonies were sequenced.

Results

Hypermutated sglgM−IgD−CD38+GC B cells have undergone Cμ−Cδ switch by recombination between Sμ and the Pentamer-rich Cδ region. We have previously identified a population of sglgM−IgD+CD38−GC B cells that contain extensively mutated Ig variable region genes (36). An intriguing link between these B cells and IgD-secreting myeloma cells is the rare single expression of Igδ isotype of Igs. Such a phenotype can only be explained by either Cμ− deleted as observed in IgD+ plasma cell. To clarify this issue, PCR primers were designed for probing recombination events between the 442-bp α/μ region and the 443-bp Σ/μ region or between Sμ and the pentamer-rich region Cδ (Fig. 1A). In three tonsillar samples, Sμ−α/μ recombination but not α/μ−Σ/μ recombination was detected in sglgM−IgD−CD38+GC cells and their derived EBV transformed clones, but not in sglgM−IgD−CD38+GC founder cells (37) and their EBV-derived clones (Fig. 1B presents the result from one tonsil sample). To determine the Sμ−α/μ break points, PCR-generated DNA products were cloned and sequenced. Fig. 1C shows the sequences of four Sμ−α/μ junctions obtained from freshly isolated sglgM−IgD−CD38+GC B cells and their EBV clones. The four break points, which are presented in a schematic diagram in Fig. 1D, demonstrate that the Cμ−Cδ switch had occurred in sglgM−IgD−CD38+GC B cells.

Hypermutated sglgM−IgD−CD38+GC B cells express λ light chains. Since the second feature of IgD secreting myeloma was its preferential Igδ light chain expression (11, 12), we analyzed the light chain expression of a panel of EBV transformed clones derived from discrete B cell subsets of two tonsil samples (Table 1). Although 39 out of 83 EBV clones from sglgM−IgD−CD38+GC founder cells and 17 out of 53 EBV clones from sglgD−CD38+GC B cells express λ light chains, 75 out of 76 EBV clones from sglgM−IgD−CD38+GC B cells express λ light chains. VH sequence analysis showed that most clones were clonally independent (see Materials and Methods). These data demonstrate that sglgM−IgD−CD38+GC B cells display the second feature of IgD myeloma cells: preferential expression of λ light chain.

Figure 4. Analysis of Ig heavy chain variable region genes of IgD+ plasma cells. (A) Schematic representation of VH5–Cδ sequences from plasma cells of one representative tonsil. Leader, CDR1, CDR2, and DJ/Leader regions are boxed. Sequence names are listed on the left (Seq.) and boxed names represent clonally related sequences. Total mutation numbers are listed on the right. Germline sequences of D regions were not assigned. Mutations are represented as replacement (circled stem) and silent (stem). (B) Nucleotide sequences from the largest clone. The upper string gives the nucleotides of the VH5–1 germline sequence, the D region from the least mutated sequence (192), and the JH6 germline sequence. CDR1, CDR2, and CDR3 are boxed. D arrows, matches to the first string. Mutated bases are indicated. (C) Genealogical tree from this clone. Each analyzed sequence is indicated by its circled name, and silent (stem) and replacement (circled) mutations are within brackets.

Table 2. Tonsillar PC s Secrete IgD

| Experiment No. 1 | Experiment No. 2 |
|------------------|------------------|
|                  | IgM              | IgD              | IgG              | IgA              | IgE              |
| PCs              |        |        |        |        |        |
| GCs              | <40    | 11 ± 1 | 501 ± 64 | 205 ± 18 | <0.4          |

Table 3. IgD Sequences from PC Show a High Rate of Somatic Mutations as Well as a Strong Clonal Relationship

| VDJ associated with | Mutations (number per VDJ segment) | Clonality (percent of related sequences) |
|---------------------|-----------------------------------|----------------------------------------|
| Cμ                  | 4 ± 2 (1–9, n = 19)               | 21 (4/19)                              |
| Cγ                  | 10 ± 8 (1–31, n = 62)             | 53 (3/62)                              |
| Cδ                  | 21 ± 12 (1–65, n = 52)            | 83 (43/52)                             |

CD20−CD38+ PCs and CD20−CD38− GCs were isolated by FACS sorting. 2 × 10⁶ cells were cultured overnight in 200 μl Iscove medium. Ig contents were measured by ELISA assay. Data are given in ng/ml (mean ± SD).
Hypermutated sIgM - IgD - CD 38 + GC B cells display poor clonal relatedness. The sIgM - IgD - CD 38 + GC B cells had lost a major part of the S regions after Cμ - Cδ switch (Fig. 1 D), it was anticipated that they would not undergo further isotype switch. Indeed, sIgM - IgD - CD 38 + GC B cells differentiated mainly into IgD - secreting cells after 10 d of culture on CD40 transfected L cells with IL-2 and IL-10 (Fig. 2), a culture condition under which naive B cells under isotype switch to IgG and differentiate into IgG - secreting cells (38, 39). Thus, sIgM - IgD - CD 38 + GC B cells display two common features with IgD - secreting myeloma cells: (i) the Cμ - Cδ isotype switch and the preferential λ light chain expression, and they could differentiate into normal IgD - secreting cells in vitro.

IgD - secreting Plasma Cells Represent a Major Population of Plasma Cells in Human Tonsils. We have previously demonstrated that hypermutated sIgM - IgD - CD 38 + GC B cells could not give rise to circulating memory cells in blood (36). However, IgD + plasma cells were previously identified in human tonsils by immunohistology (40, 41), suggesting that sIgM - IgD - CD 38 + GC B cells may differentiate into IgD - secreting plasma cells. An immunohistological analysis performed on four randomly selected tonsillar samples with anti-IgD showed that IgD + plasma cells represent an average of 16% (range 6–20%) of total CD38 + plasma cells. They were found either within GCs (Fig. 3 A) or within mucosal epithelium (Fig. 3, B and C), as reported earlier for IgA + plasma cells (42). To further characterize IgD + plasma cells, tonsillar plasma cells were isolated by cell sorting according to their CD38 - phenotype as previously described. In agreement with the immunohistological analyses on tissue sections, plasma cells isolated from five tonsil samples contains 17% (3–48%) IgD + and only 2–5% IgM + cells (Fig. 3 D). Double anti-IgD and anti-IgM staining showed that plasma cells contain either IgM or IgD, but never both isotypes (Fig. 3 E). Furthermore, IgG, IgA, and IgD were the major Ig isotypes secreted by these plasma cells during overnight cultures (Table 2). The question is do IgD - secreting plasma cells in tonsils indeed represent the progeny of IgM - IgD + GC B cells and the normal counterpart of IgD - secreting myeloma cells?

IgD - secreting Plasma Cells Have Undergone Extensive Somatic Mutation and Display Striking Clonal Relatedness. The first important feature of the VH5–Cμ - Cδ switch recombinations in IgD + plasma cells of three tonsil samples (A): PCR amplification of Sμ - α - δ recombinations of genomic DNA from IgD + (lanes D +) and IgD - (lanes D -) plasma cells. (B) DNA sequences of Sμ - α - δ junctional regions from IgD + plasma cells Upper strings, germline sequences of the Sμ region; lower strings, germline sequences of the α/μ - δ - α intron. The central string represents the sequences of cloned PCR products. Homologous nucleotides are linked by vertical bars. A rowed figures: the base numbers: base No. 1 being the first 3' of the VDJ region. (C) Schematic representation of the break points.
lated sequences (Fig. 4, B and C) indicate that somatic mutations have been accumulated during the extensive clonal expansion of IgD plasma cell precursors, the sIgM−IgD+ CD38+ GC B cells. Since an average of 16% of tonsillar plasma cells secrete IgD and only ~2–5% of human B cells use VH5 genes, each tonsil sample may contain only 30–80 IgD-VH5–expressing plasma cells. These cells may represent the descendents of a single GC and may explain the observed restricted V gene usage.

IgD-secreting Plasma Cells Have Undergone C\textsubscript{m}–C\textsubscript{d} Switch. To determine whether IgD-secreting plasma cells have undergone C\textsubscript{m}–C\textsubscript{d} switch, CD38\textsuperscript{2} total tonsillar plasma cells were separated into intracellular IgD\textsuperscript{1} and intracellular IgD\textsuperscript{2} subsets by a two-color immunofluorescence cell sorter. Sm–sd junctions were amplified from DNA of 10,000 cells of each subset. Fig. 5 A shows that Sm–sd junction can be amplified from IgD\textsuperscript{+} plasma cells of three tonsil samples, but not from IgD\textsuperscript{−} plasma cells. Fig. 5 B shows the sequences of three examples of Sm–sd junctions from IgD\textsuperscript{+} plasma cells. The corresponding break points are depicted in Fig. 5 C.

IgD-secreting Plasma Cells Preferentially Express \(\lambda\) Light Chains. To determine the light chain expression of normal IgD-secreting plasma cells, double staining with anti-IgD (blue) and anti-Ig\(\kappa\) (red) as well as anti-IgD (blue) and anti-Ig\(\lambda\) (red) were performed on serial sections of three tonsil samples. Although few IgD\textsuperscript{+} plasma cells expressed Ig\(\kappa\) light chain (most cells are single stained blue; Fig. 6, A and B), >90% were shown to express Ig\(\lambda\) light chain (double stained purple; Fig. 6, C and D).

IgD-secreting Myeloma Cells Have Undergone Somatic Mutation in Their Ig Variable Region Genes. A previous study by Kiyoi et al. showed that four cases of IgD-secreting myeloma contained somatically mutated IgV genes (32). We analyzed the VH sequences of two well-characterized human IgD-secreting myeloma. VH sequence from patient 1 (15) displays 40 nucleotide differences from the three closest germline sequences (VH 3-33/DP50, VH 3-30.3/DP46,
Patient 1: VH versus VH3-30.3/DP46 germline

| CDR1 | CDR2 |
|------|------|
|      |      |

Patient 2: VH versus VH1-69/DP10 germline

| CDR1 | CDR2 |
|------|------|
|      |      |

Figure 7. Analysis of Ig heavy chain variable region genes of IgD myeloma cells. Schematic representation of VH sequences from IgD myeloma cells of two samples. Patient 1 (15) shows 40 nucleotide substitutions, and patient 2 (14) shows 85 nucleotide differences plus one 9-bp deletion (between brackets). Mutations are represented as replacement (circles) and silent (stems).

Discussion

IgD was first discovered by Rowe and Fahey as a unique myeloma protein (33). IgD-secreting myeloma cells were found to display two unusual features that could not fit into the current model of antigen-driven B cell development (44). First, while the membrane IgD on B cells shows a predominance of the Igk type, more than two thirds of all known IgD myeloma proteins were shown to belong to the lambda type (11, 12). Second, IgD-secreting myeloma cells had undergone a unusual Cμ-Cδ switch. This raises the question of whether the features of IgD-secreting myeloma cells represent only a malignant event or reflect a normal B cell maturation pathway.

Here we demonstrate that hypermutated sgM=IgD+CD38+ B cells and IgD+ plasma cells could be found in the same GCs. Second, CD40-activated sgM−IgD+CD38+ GC B cells were shown to directly differentiate into IgD-secreting cells when cultured with IL-2 and IL-10. Third, both sgM=IgD+CD38+ GC B cells and normal IgD-secreting plasma cells displayed a similar somatic hypermutation rate. Fourth, both sgM=IgD+CD38+ GC B cells and normal IgD-secreting plasma cells had been originated from a few cells that had undergone impressive clonal expansion and somatic mutation within GCs. Fifth, like IgD-secreting myeloma cells, both cell types preferentially expressed the IgA light chain and had undergone Cμ-Cδ switch.

Previous studies have shown that IgVH and IgVL genes of IgG, IgA, and IgD myeloma contain extensive somatic mutation (23–33). These findings strongly suggest that IgD-secreting myeloma cells are not derived from the transformation of stem cells (19) or pre-B cells (20), but from GC B cells or post-GC memory B cells. Our previous study demonstrated sgM−IgD+CD38+ GC B cells did not mature into blood memory B cells. This, together with the present finding that sgM−IgD+CD38+ GC B cells differentiate into IgD-secreting plasma cells, suggests that IgD-secreting myeloma cells are derived from B cells at the GC B cell stage, but not at the post-GC memory B stage.

The identification of sgM=IgD+CD38+ GC B cells and IgD+ plasma cells defines a novel GC B cell development pathway in human, characterized by (a) a nonclassical iso-type switch from Cμ to Cδ, (b) a light chain shift from κ to λ, (c) the impressive oligoclonal expansion and somatic hypermutation, and (d) generation of IgD-secreting plasma cells. The molecular triggers and functional implications of the Cμ−Cδ switch, the κ−λ light chain shift, and the enormous clonal expansion and somatic mutation in sgM=IgD+CD38+ GC B cells are currently unknown. The κ−λ light chain shift may result from a secondary light chain rearrangement (receptor editing; references 45, 46) in GCs, as recently demonstrated in mouse GC B cells (47–50).

The identification of a significant number of IgD+ plasma cells in human tonsils also challenges the previous hypothesis that IgD functions simply as an antigen receptor, but not as a secreted antibody. This, together with recent identification of IgD+ memory B cells in human bone marrow (51) and virus-specific IgD-secreting plasma cells in the spleen of mice (52), strongly suggests that IgD plays an important role in certain types of humoral immune responses.
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