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A model for the development of human IgD-only B cells: Genotypic analyses suggest their generation in superantigen driven immune responses

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ABSTRACT

Human peripheral blood (PB) B cells expressing only IgD and tonsillar IgD-secreting plasma cells carry highly mutated V_H genes and show preferential Igλ usage. To further characterize these peculiar cells and gain insight into their generation, we analysed rearranged V_H and V_L genes of single IgD-only λ^+ PB B cells and IgD^+ plasma cells from four individuals each. We demonstrate that the high somatic hypermutation activity in these cells is not restricted to V_H genes but also present in V_L genes. Moreover, not only PB IgD-only B cells, as reported earlier, but also IgD-expressing plasma cells often belong to very large clones. Surprisingly, the V_H3-30 gene segment was used in each PB donor by >30% of IgD-only cells and in 2 tonsils by >50% of IgD plasma cells, whereas it was used less frequent in other B cells. All these features fit to a model in which IgD-only cells develop in superantigen-driven germinal center reactions, in which B cells are activated by binding of antigens to constant parts of Cδ and often λ light chains and the V_H3-30 segment, and are selected for deletion of Cμ. IgD-only B cells may hence represent a unique B lineage subset generated in response to particular antigens.

1. Introduction

Mature naive B cells coexpress the IgM and IgD isotypes of the B cell receptor (BCR). This coexpression of the Cμ and Cδ genes is mediated by differential splicing. The specific function of IgD is still obscure. IgD knockout mice have a normal development of the immune system and a largely normal antibody response (Nitschke et al., 1993). These mice only show a moderate decrease in mature B cell numbers and a delay in affinity maturation, indicating some stimulatory effects of IgD. In IgM-deficient mice, IgD can largely replace the function of IgM, suggesting similar signalling capabilities of the two receptors (Lutz et al., 1998). When B cells are activated by binding of antigen to their BCR, IgD is usually down-regulated (Monroe et al., 1983), and the cells can be driven into T cell-dependent immune responses and establish germinal centers (GC) (MacLennan, 1994). GC B cells modify their rearranged V genes by somatic hypermutation and are then selected for high affinity-binding to the antigen (Küppers et al., 1993; Rajewsky, 1996). Many GC B cells perform class switch recombination, a process by which the Cμ and Cδ genes are deleted and replaced by one of the seven other IgH constant region genes (Cγ1–4, Cα1–2 or Cε) (Rajewsky, 1996). Class switching involves DNA cleavage in switch regions located upstream of the Cμ, Cγ, Cα and Cε genes (Coffman et al., 1993). Selected B cells finally differentiate into plasma cells or memory B cells. Three main subsets of human memory B cells were identified: "classical" class switched memory B cells, $IgM^+IgD^{low/-}$ (IgM-only) B cells and a subset of IgM^+IgD^+ B cells with somatically mutated V genes (Klein et al., 1997; Klein et al., 1998b). All three subsets express the memory B cell marker CD27, a member of the tumor necrosis factor receptor superfamily (Agematsu et al., 1997; Klein et al., 1998b).

First indication for an additional human B cell subset characterized by sole expression of IgD came from analysis of B cell malignancies. In about 2% of multiple myelomas and 10% of hairy cell leukemias, the malignant cells express only IgD (Fibbe and Jansen, 1984; Fine et al., 1974; Kluin et al., 1995; Rowe and Fahey, 1965; Vaandrager et al., 1998). Later, a GC B cell population expressing only IgD and carrying the most highly mutated V_H genes ever seen in normal B cells was described (Liu et al., 1996). These IgD-

Abbreviations: PB, peripheral blood; BCR, B cell receptor; FR, framework region; CDR, complementarity determining region; GC, germinal center; R/S, replacement versus silent mutations; MID, *Moraxella* IgD binding protein.

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only cells were restricted to few GC where they represented a major fraction of cells (Liu et al., 1996). The Cμ gene was found to be deleted in these cells due to class switching between Sμ and a pentamer-rich σ/δ region upstream of Cδ (Cδ lacks a classical switch region) (Liu et al., 1996). Nearly all IgD-only GC B cells express λ light chains, while human B cells usually show a κ/λ ratio of 60/40 (Arpin et al., 1998). These unusual GC B cells can differentiate into post-GC cells, as 1–20% of tonsillar plasma cells secrete IgD (Arpin et al., 1998; Brandtzaeg et al., 1978; Ferrarini et al., 1976; Surjan et al., 1978). Indeed, IgD-secreting plasma cells are characterized by an exceptionally high mutation load, deletion of Cμ, and expression of λ chains by more than 90% of cells (Arpin et al., 1998). Also IgD in human serum shows a preferential λ usage, indicating that serum IgD is largely produced by IgD-only plasma cells (van Nieuwkoop and Radl, 1985).

We identified IgD^+IgM^- memory-type B cells at low frequency (0.5–1% of B cells) in peripheral blood (PB) of humans (Goossens et al., 2001; Klein et al., 1998b). These IgD-only cells are likely also descendents of IgD-only GC B cells as they show a similar high mutation load, a preferential λ usage, and expression of CD27 (Klein et al., 1998b). A considerable fraction of these cells showed clonal relatedness with a high degree of intraclonal V gene diversification (Goossens et al., 2001).

It was proposed that the preferential λ usage of IgD-only cells could reflect light chain revision in the GC (Arpin et al., 1998), but detailed analysis of the light chain loci of normal IgD-only B cells and IgD-only multiple myelomas argues against this idea (Goossens et al., 2001; van der Burg et al., 2002). Notably, IgD-switched B cells are not detectable in mice, most likely due to the lack of a pseudo-switch region 5′ of Cδ in these animals. Hence, IgD-only B cells cannot be studied in mice.

Since the differentiation pathway and the function(s) of IgD-only cells are still enigmatic, we further characterized these cells by V gene analysis of single IgD-only PB B cells and tonsillar IgD^+ plasma cells. We wanted to find out whether these populations show a restricted V gene usage, as possibly indicated by the predominant λ chain expression. Moreover, as the previous analysis of IgD plasma cells was unsuitable to determine the clonal composition of this population (only a single V gene or V genes of a single V_H family were analysed) (Arpin et al., 1998; Zheng et al., 2004), we studied this issue by analysis of single plasma cells using primers for all V_H and most V_L families.

2. Experimental procedures

2.1. Molecular single cell V gene analysis of human naive, IgG^+ and IgD-only PB cells

The isolation of single IgD-only memory B cells ($IgM^-IgD^+Ig\lambda^+$) from PB of three healthy adult donors by fluorescence-activated cell sorting was reported previously (Goossens et al., 2001). To exclude contaminating naive B cells from the analysis, unmutated sequences from donors 1 to 3 were neglected. For donor 4, CD27 was used as a fourth marker to exclude naive B cells, and IgD-only B cells were sorted from B cells enriched by CD19-MACS as $IgM^-IgD^+Ig\lambda^+CD27^+$ B cells. Cells were sorted into PCR tubes and their heavy and light chain gene rearrangements were analysed by seminested PCR and direct sequencing of amplificates (Goossens et al., 2001). Cells from donor 4 were amplified without an initial primer extension preamplification step, but with primers for all V_H and V_λ families multiplexed in the first round of PCR (Küppers, 2004). From donor 1, also naive and class switched λ-expressing B cells were analysed (as previously described) (Goossens et al., 2001), and from donor 4 additionally IgG^+ λ-expressing B cells. Naive B cells were sorted as $CD27^-IgD^+Ig\lambda^+$, and class switched as $IgM^-IgD^-Ig\lambda^+$ (donor 1) or $CD27^+IgG^+Ig\lambda^+$ (donor 4) B cells.

2.2. Isolation of tonsillar IgD and IgG plasma cells

Tonsils were obtained from tonsillectomies of children and adolescents suffering from hyperplastic tonsils or recurrent tonsillitis. All samples were collected with informed consent of the donors. The study was performed with prior approval by the institutional review board. Directly after surgery tonsils were placed in medium on ice and minced. The suspensions were pressed through 100 μm meshes (Becton Dickinson, Heidelberg, Germany) and subjected to Ficoll-Hypaque/Isopaque (GE Healthcare, Freiburg, Germany) density centrifugation. Tonsillar mononuclear cells were resuspended in cooled phosphate buffered saline (PBS) and used for either preparation of cytospins (tonsils A, B and C) or single cell sorting (tonsil D). Cytospins were dried overnight, fixed in acetone, air dried, and stained with antibodies against CD38, IgD, IgM, IgG or IgA (all from Dako, Hamburg, Germany) and the Dako Envision system with alkaline phosphatase and Fast Red as substrate. No staining for IgE was performed, as IgE plasma cells are very rare in the tonsil (Brandtzaeg et al., 1978; Hoefakker et al., 1993). For the enumeration of plasma cells, only intensively stained cells were considered. For each immunochemical staining three cytospins with 1 to 7×10^4 cells per cytospin were evaluated. Cells showing an intensive IgD or IgG staining were micromanipulated with the help of micropipettes fixed to a hydraulic micromanipulator (Küppers et al., 1993). From tonsil D, tonsillar cells were stained for CD38 and intracellular IgD or intracellular IgG (anti-CD38-APC, anti-IgD-PE, anti-IgG-PE from Becton Dickinson) to sort single IgD-only plasma cells ($CD38^{high}$, cytosolic IgD^+) and class switched plasma cells ($CD38^{high}$, cytosolic IgG^+), respectively. For intracellular stainings of IgD and IgG, the Fix and Perm-kit from Caltag (Caltag, Hamburg, Germany) was used.

2.3. PCR and sequence analysis of V_H, V_κ and V_λ rearrangements of single cells

Single cells were digested with 0.25 mg/ml proteinase K (Roche, Mannheim, Germany) for two hours at 50 °C. The proteinase was inactivated by incubation for 10 minutes at 95 °C. IgH, Igκ and Igλ rearrangements were amplified in a seminested approach, using family-specific primers for V_H leader regions or FR I of human V_H genes, and FR I-specific primers for V_κ and V_λ genes together with two sets of nested primers for the respective joining regions as described (Bräuninger et al., 1999; Küppers et al., 1993). No $V_\kappa J_\kappa$ joints were amplified from cells of donor 4 and tonsil D. Amplificates were gel-purified and directly sequenced using the BigDye Deoxy sequencing kit (Applied Biosystems, Weiterstadt, Germany) and an automatic sequencer (ABI3100, Applied Biosystems). Sequences were analysed using the EMBL IMGT and GenBank databases and DNASTAR Lasergene v6 software (Dnastar Inc., Madison, WI). The V gene sequences were submitted to the EMBL database (see notes to Tables 2 and 4).

2.4. Calculation of the size of IgD-only B cell and plasma cell clones and statistical evaluation

The frequency of IgD-only B cells among CD19-enriched B cells for the four blood donors was about 0.2%. Assuming a total blood volume of 5 l per donor, a frequency of 1×10^5 B cells/ml PB, and that the PB B cells isolated from 50 ml PB are a random sample of the total PB, a clone with 5 members among 50 PCR-positive IgD-only B cells would have a total size of about $10^5 \times 5000 \times 0.002 \times 0.1 \approx 100{,}000$ cells in the PB. A similar calculation can be performed for tonsillar IgD plasma cells: From the tonsillar tissue biopsies about 1×10^9