Cellular Immunology 281 (2013) 76–84

Contents lists available at SciVerse ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Innate autoreactive B cells as antigen-presenting cells in the induction of tolerance to conserved keratin polypeptide

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ARTICLE INFO

Article history:
Received 6 September 2012
Accepted 24 January 2013
Available online 10 February 2013

Keywords:
Innate B cells
Autoreactivity
Antigen presentation
Keratin

ABSTRACT

Innate B cells account for a substantial proportion of total B lymphocytes and express autoreactive B cell receptors directed against self-constituents. However, whether innate autoreactive B cells present autoantigens to T cells, and if so, whether they trigger an autoimmune response, are unclear. In this study, we have characterized splenic keratin-reactive B cells from naïve mice and investigated their roles in keratin antigen presentation. We observed that splenic keratin-reactive B cells expressed germline encoded V_H and V_K genes based on Igs from anti-keratin hybridomas. Moreover, they frequently utilized gene segment of DFL16.2 and J_K2 in the CDR3 regions of heavy and light chain, suggesting that these cells are probably selected on the basis of the specificity of their BCRs. In the presence of keratin antigen, splenic keratin-reactive B cells stimulated significant IL-2 productions from keratin-specific T hybridomas, which were augmented by increasing the concentration of keratin and the numbers of keratin-reactive B cells. By contrast, keratin-reactive B cells failed to stimulate the proliferations of freshly isolated keratin-specific T cells from lymph nodes. The phenotypic analysis of splenic keratin-reactive B cells indicated that low expressions of B7-1 and B7-2 might be the underlying mechanisms for this incomplete function of B cell presentation. Our experiments indicate that splenic keratin-reactive B cells are ineffective in activating freshly isolated T cells from lymph nodes, suggesting a role for innate autoreactive B cells as antigen-presenting cells in tolerance to self-antigens.

1. Introduction

Innate B cells comprise a significant fraction of B lymphocytes and exhibit characteristics that distinguish them from conventional B cells. The most predominant feature of innate B cells is the recurrent expression of a restricted set of germ-line encoded B cell receptors (BCRs), which express very few or no mutations at all [1]. Although significant advances that have been made in the investigation of innate B cells, their biology and functions appear to be more complex than what has been thought. Innate B cells express germ-line encoded B cell receptors (BCRs) and are characterized by autoreactivity [2]. A series of autoantigens, whether expressed on the cell surface or in the cytoplasm, have been revealed to be the targets of BCRs on innate autoreactive B cells [3–5]. Thus, the interaction of autoantigens with innate autoreactive B cells is an inevitable event in normal individuals because these autoantigens are exposed in the process of maintaining the body homeostasis. Moreover, the current views of B cells biology are also enhanced by the realization that in addition to their well-defined role in antibody production, innate B cells also play a variety of immunoregulatory roles through their antigen-presentation ability and through cytokine and chemokine production [6–10]. B1a cells were found to be either expanded in NZM2410 lupus-prone mice or accumulated in the target organs in aged BWF1 mice. In both murine models of systemic lupus erythematosus, B1 cells had the functional capability to serve as

Abbreviations: BCRs, B cell receptors; APC, antigen presenting cells; AK auto-Ab, anti-keratin autoantibody; LN, lymph nodes; TCR, T-cell antigen receptor; Ig, immunoglobulin; CDR, complementary determining region; FR, framework region; NHSB, N-hydroxysuccinimido-biotin; HEL, hen egg lysozyme; OVA, ovalbumin; NOD, non-obese diabetic.

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http://dx.doi.org/10.1016/j.cellimm.2013.01.009

APC, which contribute to disease pathogenesis by augmenting the presentation of autoantigens to pathogenic T cells [11,12]. Thus, unique and fascinating questions about the contributions of innate autoreactive B cells to autoimmunity were raised. However, to our knowledge, whether innate autoreactive B cells could also present autoantigens to T cells, and if so, whether they trigger an autoimmune response, remains to be unclear. Since innate autoreactive B cells represent a frequent component of the normal immune system, it is of great importance to evaluate the significance of this kind of innate autoimmunity.

Keratins comprise the largest subgroup of intermediate filament proteins and are the most abundant component in epithelial cells. Whole-genome sequencing has confirmed that keratin genes are highly conserved in the genomic organization and sequence among mammals [13]. Anti-keratin autoantibody (AK auto-Ab), which is the circulating antibody against epidermal keratins, was detected in all normal human sera and are mostly IgM isotypes [14–16]. Considering that AK auto-Ab arises independent of deliberate immunization, it was thought to be the main component of so-called natural antibodies. In previous studies, we detected the presence of keratin-reactive B cells in murine spleen and peritoneal cavity B cell populations, although the frequency in the spleen was significantly lower than that in the peritoneal cavity B cells [17]. In this study, we have characterized genetic sequences of BCRs on splenic keratin-reactive B cells by analyzing the variable genes of four strains of anti-keratin hybridomas that were generated from B cells of unimmunized mice. We also investigated the function of keratin-reactive B cells in autoantigen presentation by incubating them with keratin-specific hybridomas or T cells. We sought to determine the role of innate autoreactive B cells in autoimmunity.

2. Materials and methods

2.1. Extraction of keratin polypeptides

Keratins polypeptides were extracted from BALB/c mouse epidermis according to standard methods [14]. The skin from newborn BALB/c mice was digested overnight with Dispase (Gibco, USA) at 4 °C. The epidermis was then tore off and cut into pieces. In a Tris–HCl buffer (25 mM Tris–HCl, pH 7.4), the epidermis was pulverized with an ultrasonic pulverizer under ice bath, and centrifuged (12,000*g*) at 4 °C for 30 min. The supernatant was drained, and the sediment was again treated using the same procedure. The obtained sediment was immersed in Tris–HCl buffer, 8 M urea and 0.1 M β-mercaptoethanol at 4 °C for 50 h to depolymerize the keratin peptides. After centrifugation (12,000*g*) at 4 °C for 30 min, the supernatant was sucked out and immediately dialyzed against distilled water at 4 °C to repolymerize the keratin peptides. The depolymerization-repolymerization cycle was repeated three times so as to purify the extracted keratins. The content of keratin peptides was measured with an ultraviolet spectrophotometer (Beckman, CA). The extracted keratins were analyzed using 10% SDS–PAGE, and were compared with commercial keratins (Sigma, USA).

2.2. Generation of hybridomas

The splenocytes from unimmunized 10-week-old female BALB/c mice were fused with the SP2/0 myeloma cells, followed by selection in the hypoxanthine/aminopterin/thymidine (HAT) medium (Sigma). The hybridomas that produce keratin-reactive IgM were screened by ELISA using mouse keratin as an antigen, and were sub-cloned by limited dilution.

The female BALB/c mice were immunized by intra-dermal injection of 100 μg of keratin emulsified with Freund's complete adjuvant (Sigma) at the hind footpads and at the base of tail to generate keratin-specific T-cell hybridoma. After one week, the axillary, inguinal, and popliteal lymph nodes (LN) were collected and pooled. The LN cells were cultured in the presence of 4 μg/mL keratin and 20 units/mL recombinant human IL-2 (Roche Diagnostics, Mannheim, Germany) for 4 days. The LN cells were then fused with BW5147.G.1.4 (ATCC No. TIB-48) thymoma cells using standard methods [18]. The hybridomas were screened by IL-2-producing ability in the presence of syngeneic spleen cells and keratin. The surface markers (T-cell antigen receptor [TCR], CD3, CD4) were also examined by flow cytometry. One clone with a $TCR^+CD3^+CD4^+$ phenotype and responds to keratin stimulation (in the presence of syngeneic spleen cells) was obtained and was named as HKZ.

2.3. Sequencing of V_H and V_K cDNA

Total cellular RNA was extracted from 10^7 hybridoma cells using the TRIzol reagent (Gibco-BRL, Life Technologies, Gaithersburg, MD). Reverse transcription was performed using a kit from Gibco-BRL, with total RNA as template and oligo-dT as a primer. The resulting cDNA was used to amply V_H and V_L genes following the method described by Orlandi et al. [19]. The amplified V_H and V_L fragments were cloned and sequenced. The variable region genes of Ig were compared the germline sequences in EMBL-GenBank (http://www.ncbi.nlm.gov/igblast) and International ImMunoGeneTics database (http://imgt.cines.fr/cgibin) to find the most homologous sequence. Base differences between the Ig gene sequences and the corresponding germline genes were analyzed and scored as mutations. The CDR and FR locations and the numbering system are according to Kabat et al. [20] .

2.4. Antigen-specific cytokine release assay

The B cells were isolated from the spleens of 10-week-old female BALB/c mice by negative selection using magnetic sorting with an enrichment cocktail (Stem Cell Technologies Inc, Vancouver, British Columbia, Canada). The isolated B cells were then incubated with the biotinylated keratin, which was prepared by conjugating mouse keratin with N-hydroxysuccinimido-biotin (NHSB). After incubation with avidin-conjugated immuno magnetic beads, the keratin-binding B-cells were separated using a magnet (IMagnet, BD Pharmingen). The FACS staining of the FITC-conjugated keratin and the B cell marker B220 were performed to control cell purity. This procedure normally yielded B cells that had a keratin binding of >96 and B220 of >95% (keratin-reactive B cells). The eluted cells were also generated as splenic B cells that are depleted of keratin-reactive B cells (keratin-nonreactive B cells).

The keratin-reactive B cells were irradiated (γ irradiation, 3000 rad) and incubated in 96-well plates with the hybridoma HKZ cells at a ratio of 5:1 in the presence or absence of keratin for 24 h. The γ-irradiated keratin-nonreactive B cells were used as the control groups. OVA was also used as an irrelevant antigen. An irrelevant T cell hybridoma (HEL-specific, 3A9) was also incubated with γ-irradiated keratin-reactive B cells in the presence of keratin or keratin plus HEL to exclude the stimulation caused by keratin linking BCRs. The supernatants (0.1 mL) from each well were transferred into 96-well plates containing 1×10^4/well of IL-2-dependent CTLL-2 cells. After 18 h, ^{3}H-thymidine (1 μCi/well) (Amersham, Arlington, IL) was added to each well. After incubation for an additional 12 h, the CTLL-2 cells were harvested using glass fiber filters. The radioactivity of the incorporated ^{3}H-thymidine was counted using a β-ray counter. The proliferation index was calculated as follows: average cpm of cells exposed to keratin/average cpm of cells not exposed to keratin. Each group of experiments was