Auto-reactive B cells against peripheral antigen, desmoglein 3, escape from tolerance mechanism

Takayuki Ota1,2, Miyo Aoki-Ota1, Kazuyuki Tsunoda1,3, Kouji Simoda4, Takeji Nishikawa1, Masayuki Amagai1 and Shigeo Koyasu2,5

1Department of Dermatology, 2Department of Microbiology and Immunology, 3Department of Dentistry and Oral Surgery and 4Laboratory Animal Center, Keio University School of Medicine, Tokyo 160-8582, Japan
5Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan

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Abstract
To examine the mechanism of B cell tolerance against natural peripheral self-antigen, we generated transgenic mice expressing IgM specific for desmoglein 3 (Dsg3) from AK7 monoclonal antibody which itself does not induce blisters. Dsg3 is mainly expressed on stratified squamous epithelium and is the target antigen of an autoimmune bullous disease, pemphigus vulgaris. Transgenic B cells reactive to Dsg3 were observed in the spleen and lymph node. Although these B cells are autoreactive, they did not develop into B1 B cells. These B cells were functionally competent and anti-Dsg3 IgM was detected in the serum and on the keratinocyte cell surface. These results indicate that auto-reactive B cells against peripheral antigen (Dsg3) are able to develop in the presence of Dsg3 but are ignored by the immune system.

Introduction
Rearrangement of immunoglobulin (Ig) genes during B lymphocyte development leads to the generation of a B cell repertoire containing self-reactive B cells. Controlling such self-reactive B cells is critical in order to avoid detrimental autoimmunity. It is believed that self-reactive B cells are either eliminated or inactivated during lymphocyte development, resulting in self-tolerance (1,2). Mechanisms of B cell tolerance have been examined mostly using Ig transgenic mouse models in which both heavy (H) and light (L) chains of an Ig specific for a known antigen are expressed on the majority of B cells. Such Ig transgenic mice have been further crossed with other transgenic mice expressing the specific antigen systemically or in a tissue-restricted manner.

When immature B cells interact with ubiquitous self-antigens expressed on cell surfaces, they are either eliminated by clonal deletion (3–6) or rescued by receptor editing, which involves further gene rearrangement of the L chain loci, thus replacing the self-reactive receptor with a new receptor (7–11). Immature B cells that encounter circulating self-antigens of low valency, such as small soluble proteins, are inactivated and enter a state of anergy (12–17). In contrast to conventional B cells, CD5+B220low B1 B cells in the peritoneal cavity are often self-reactive as a result of escaping deletion (18,19). Interestingly, evidence has accumulated that these cells are positively selected by self-antigens (20–24).

In contrast to the systemic B cell tolerance mechanisms mentioned above, how tolerance is established against antigen expressed in restricted tissues and organs is still unclear. Peripheral B cell tolerance has also been examined using several transgenic models. B cells expressing IgM specific for H-2Kb MHC class I molecules in 3–83ld transgenic mice are deleted even when the expression of the H-2Kb molecule is restricted to the liver (25). Similarly, when H-2Kb molecules are expressed in the skin under the control of the keratin 4 promoter, transgene positive B cells are also deleted, and reduced numbers of B cells are present in the lymph node and spleen (26). There are additional reports supporting the presence of peripheral deletion mechanisms (27,28). In a different model, transgenic B cells expressing anti-hen egg lysozyme (HEL) specific IgM are neither deleted nor inactivated when antigen is expressed on the surface of thyroid cells (29). Differences in the amount of the antigen or distinct anatomical sites of expression may account for the varied results obtained in distinct model systems, although deletion has been observed for some antigen receptors of extremely low affinity (30,31).

Here we examined the mechanism of B cell tolerance against a native peripheral antigen, desmoglein 3 (Dsg3), a target antigen of pemphigus vulgaris (PV), an IgG-mediated autoimmune blistering disease (32). Dsg3 is a member of the...
cadherin family expressed on stratified squamous epithelium, and mediates keratinocyte cell adhesion as a component of desmosome (33). Although antibody against tissue-specific antigen can be induced relatively easily by immunization of antigen with potent adjuvant (34–36), repetitive immunization of wild-type mice on several different genetic backgrounds with recombinant Dsg3 (rDsg3) emulsified in complete Freund's adjuvant (CFA) failed to induce autoantibody production, implying the presence of systemic tolerance against Dsg3 in vivo. We thus used Dsg3^{−/−} mice in which tolerance against Dsg3 is not established, and developed a PV model by adaptive transfer of splenocytes derived from Dsg3^{−/−} mice into Rag2^{−/−} mice (37,38). The mice show erosions in their oral mucous membranes with typical histological findings of PV. From this PV mouse model, we have established several mAbs reactive to native Dsg3 on keratinocytes (39). Using variable regions of both the H and L chains of one of these mAbs, AK7, we developed anti-Dsg3-IgM transgenic mice. These transgenic mice showed neither deletion nor inactivation of Dsg3-reactive B cells, and IgM deposition was observed on the keratinocyte cell surfaces. Our results indicate that self-reactive cells against peripheral antigens are present at some frequency in a normal repertoire.

**Methods**

**Mice**

Dsg3^{−/−} mice (40) were obtained by mating female Dsg3^{+/−} and male Dsg3^{+/−} mice (Jackson Laboratory, Bar Harbor, ME). These mice have a mixed genetic background of 129/Sv (H-2b) and C57BL/6J (H-2b). In some experiments, we used Dsg3^{−/−} mice on a 129/Sv background that we obtained by backcrossing Dsg3^{−/−} mice to 129/Sv for at least seven generations. C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). All mice were maintained under SPF conditions in our animal facility. All animal experiments were performed in accordance with our Institutional Guidelines.

**Construction of transgenic mice**

cDNA fragments corresponding to variable regions of the H and L chains (V_{H} and V_{L}) of AK7 were cloned from an anti-Dsg3 hybridoma, AK7, by RT-PCR. In brief, total RNA was prepared from AK7 cells and cDNAs for H and L chains were amplified with one step RT-PCR (Qiagen, Hilden, Germany). Subsequently, H and L chains were linked by PCR and subcloned into the pCANTAB5E modified vector (Amersham, Piscataway, NJ). The resulting plasmid was transformed into XL1-Blue (Stratagene, La Jolla, CA) and single chain Fvs (scFvs) were expressed as phage surface molecules. With rDsg3 ELISA, positive clones were obtained (AK7-12) and the sequences determined. The H chain signal sequence and variable region sequence were assembled by PCR. The signal sequence and 3′ region were amplified from an anti-TNP H chain HindIII–HindIII fragment with M86-M87-M88 and M91-M92 as shown (41): (M86) 5′-CCGGAATTCACTCATCGTCTTTGTTCCAGT-3′; (M87) 5′-AGGGTTGCTGAGATTGTTCCAGCAAAATCTAC-CGTGGAAGCTGAAACTAG-3′; (M88) 5′-GCAAGAAGAC-TGACCTATCTCCTGGAGTACAGACAGGGTGGCTAGA-TTGGGTCAGC-3′; (M91) 5′-CCAGCTGGAGCTGAAACGTAG-AG-3′; (M92) 5′-CCGGAATTCCTGCAGCTACCCCGAGCAGGAC-GTGCAGC-3′.

The AK7 L chain variable region of κ isotype was amplified from AK7-12 with M89 and M90 as shown: (M89) 5′-GAGATACGCTACGTTGTCAGC-3′; (M90) 5′-ACGTTTC-AGCTCCAGCTTGCTCCCCCTCCGAACTGTTGAGGCCAGC-GTGCAGC-3′.

The AK7 L chain 3′ region fragments (M86-M87-M88 and M91-M92) and AK7 L chain variable region were fused by amplification with M86 and M90. The 1 kb fragment obtained was digested with EcoRI and subcloned into the pBluescript II SK+ vector. The pMM222 derived 5′ upstream region containing a promoter region of an L chain gene (P_{V_{L}} 3.5 kbp fragment), 1 kb L chain fragment, pMM222 derived 3′ region containing an intronic enhancer, κ constant region and a 3′ enhancer of an L chain gene (P_{kappa} 12 kbp fragment) were fused to obtain the plasmid AK7-L (43).

**Generation of transgenic mice**

The AK7 IgM H and L chain constructs were mixed at equimolar concentrations and microinjected into fertilized eggs of C57BL/6 mice, followed by transfer of viable eggs into the oviducts of pseudo-pregnant C57BL/6 mice. Alternatively, only the H chain construct was injected. For genotyping, tail DNA was amplified with M78 and M81 (H chain) or M89 and M91 (L chain). One H and L chain founder line (AK7-LH1) and five H chain-only founder lines of transgenic mice were established. For the highest expression level of IgMκ, we selected AK7-H18 from the H chain-only founder lines. Mice were used for experiments at 8–16 weeks of age.

**Recombinant Dsg3 (rDsg3)**

rDsg3 was generated as described previously (37,44). We previously developed pEVmod-Dsg3-Ehis to produce recombinant baculoprotein. To stabilize the expressed protein, we introduced the hinge region of human IgG1 (EPKSCDKTHCTPPCP) before the E-tag sequence and produced pEVmod-Dsg3-EhisII. The mouse Dsg3 extracellular region was amplified from AK7-12 with M78 and M79 as shown: (M78) 5′-GGGTGCACCTCAGTGAAAGCAGTGAGCTCGTG-3′; (M79) 5′-GCAATTCTTACCTGGAGAAACGGTG-GACCGTGACGC-3′.

Each amplified fragment was purified and re-amplified with M76 and M77. The 1 kb fragment obtained was digested with XbaI and subcloned into pBluescript II SK+ vector (Stratagene). The anti-TNP H chain EcoRI–EcoRI fragment was digested with XbaI and replaced with the AK7 H chain XbaI fragment. The ~10 kbp constant region of the μ chain (EcoRI–EcoRI) was obtained from pVH167μ (42) and fused to obtain the plasmid AK7-H.
domain was subcloned into pEVmod-Dsg3-EhisII digested by BglII and XhoI to replace human Dsg3 extracellular domain. Recombinant baculoviruses were obtained by cotransfection of rDsg3 with SapphireTM baculovirus DNA (Orbigen, San Diego, CA) into cultured insect Sf9 cells. High Five cells (Invitrogen, San Diego, CA) cultured in serum-free EX Cell 405 medium (JRH Bioscience, Lenexa, KS) were infected with the recombinant viruses and incubated for 3 days, and rDsg3 was produced in the culture supernatant. Baculoprotein was purified and concentrated on TALON (Clontech, Palo Alto, CA) affinity metal resin.

**ELISA**
Total serum IgM was measured with a mouse IgM ELISA Quantization Kit (Bethyl, Montgomery, TX). ELISA using rDsg3 was performed as previously described for human Dsg1 and Dsg3 (44). The ELISA plate was coated with 5 μg/ml of rDsg3 expressing the entire extracellular domain. For anti-Dsg3 IgM, mouse sera at 200-fold dilution were incubated for 1 h on individual ELISA plates.

**Direct immunofluorescence analysis**
Mice were sacrificed and specimens were taken from the hard palate and embedded in OCT compound (Sakura Fine-technical, Tokyo, Japan) for cryostat sectioning. For standard direct immunofluorescence studies, each section was incubated with a 200-fold dilution of Alexa488 anti-mouse IgM antibody (Molecular Probes, Eugene, OR). All sections were examined using a fluorescence microscope (Eclipse E800, Nikon, Tokyo, Japan).

**Flow cytometric analysis**
Two- and three-color flow cytometry was performed on a FACSScan or FACS Calibur (Becton-Dickinson, San Jose, CA) and analyzed using Cell Quest (Becton-Dickinson) or FlowJo (Tree Star, Ashland, OR) software. The following directly conjugated mAbs were purchased from PharMingen (San Diego, CA): phycocerythin (PE)-anti-B220 (RA3-6B2), fluorescein isothiocyanate (FITC)-anti-IgMa (DS-1), FITC-anti-IgM (R6-60.2), FITC–anti-CD21 (7G6), biotin–anti-CD23 (B3B4), biotin–anti-CD5 (53–7.3). To stain AK7 idotype, cells were incubated with 10 μg/ml rDsg3 followed by staining with Alexa488-conjugated anti-E-Tag antibody after washing (Amersham).

**In vivo adoptive transfer experiments**
AK7-LH1 bone marrow cells were collected and 5 × 10⁶ cells were intravenously injected into 9 Gy irradiated wild-type C57BL/6 or 6 Gy irradiated Dsg3−/− mice. Since Dsg3−/− mice on a C57BL/6 background barely survive after birth (data not shown), we used Dsg3−/− mice on a mixed C57BL/6×129/Sv background. After 8 weeks, mice were analyzed.

**Proliferative response**
B220⁺ B cells were purified from AK7-LH1 splenocytes with AutoMACS (Mitenyi Biotech, GmbH, Bergisch Gladbach). 2 × 10⁵ cells were cultured with 200 μl 10% FCS–RPMI in 96-well microtiter plates. LPS (10 μg/ml), anti-mouse IgM F(ab)₂ (10 μg/ml) or rDsg3 (20 μg/ml) were used as a stimulator. After 3 days, proliferation was measured by tritiated thymidine incorporation.

**Calcium mobilization**
Splenocytes were incubated with RPMI1640 containing 1 mM Fluo-3 (Molecular Probes) at 37°C for 30 min. Cells were washed twice with RPMI1640 and resuspended in RPMI1640 containing 10% FCS and PE–anti-B220 and incubated at 4°C for 30 min. Cells were washed twice and resuspended in RPMI1640 containing 10% FCS. Calcium mobilization was measured on a FACS Calibur.

**Results**

**Establishment of AK7 single chain fragment variable (scFv)**
In order to isolate cDNAs encoding H and L chains of anti-Dsg3 mAb, both H and L chain variable genes were amplified by mixed primers from total RNA of AK7 hybridoma cells (39) and subcloned into a pCANTAB5E (Amersham, Picataway, NJ) modified expression vector (45). Resulting scFvs were expressed as fusion proteins of the inserted cDNAs were screened by ELISA for reactivity to mouse Dsg3. For positive clones, the specificity of AK7 scFv was confirmed by staining of a keratinocyte cell line, PAM 212 (Fig. 1A). Sequence analysis of positive clones revealed all clones shared an identical sequence for each V region (Fig. 1B).
Generation of AK7 heavy and light chain transgenic mice

Transgenic constructs encoding the H and L chains of AK7 were generated as shown in Fig. 2(A). Transcription of the transgenes is under control of native Ig gene promoters and enhancers. The H chain transgene contains exons for both membrane-bound and secreted forms derived from an IgMa allotype, so that both forms of IgM should be produced in mice carrying this transgene. The H chain construct alone or a combination of H and L chain constructs was injected into C57Bl/6 fertilized eggs, which have an IgMb allotype. Five founder lines for H chain transgenic mice and one founder line for H and L chain double transgenic mice were established (Fig. 2B). AK7-H18 (H chain alone) and AK7-LH1 (H and L chains) mice were used in the following studies. Quantitative PCR analysis of H chain and L chain revealed that AK7-LH1 mice contain five copies each of the H and L chain constructs and AK7-H18 contain 10 copies of the H chain construct (data not shown).

Presence of Dsg3-reactive B cells in the spleen and bone marrow

Dsg3 is primarily expressed on the cell surface of stratified squamous epithelial cells but is undetectable in the bone marrow (46) (data not shown). Thus, IgMa positive B cells were expected to be present in the bone marrow but reduced or inactivated in the periphery. IgMα positive B cells were readily detected in the bone marrow of AK7-LH1 mice as expected (Fig. 3A). Contrary to our expectation, however, substantial numbers of IgMα positive B cells were detected in the spleen and lymph node of AK7-LH1 mice (Fig. 3A). It was possible that peripheral B cells utilize endogenous L chains associated with the transgenic IgMβ H chain because of incomplete allelic exclusion of the L chain loci, or that the transgenic L chain was once expressed but replaced by endogenous L chains during development by a receptor editing mechanism (7–11). To examine the transgenic L chain expression, we employed rDsg3 to detect the anti-Dsg3 IgM composed of the transgenic H and L chains. As shown in Fig. 3(A), >90% of IgM α positive B cells were reactive with rDsg3, indicating that the L chain is also appropriately expressed. To further confirm the expression of transgenic H and L chains in the periphery, we bred AK7-LH1 with Rag2−/− mice to exclude the expression of endogenous Ig genes. B cells were also detected in the bone marrow and spleen of AK7-LH1-Rag2−/− mice, and such B cells expressed IgM reactive with rDsg3 on their cell surface (Fig. 3B). It was noted that while surface IgM staining of B cells from AK7-LH1 mice shows two peaks, namely IgMαhigh and IgMαlow, B cells from AK7-LH1-Rag2−/− mice consist of only the IgMαlow population.

In AK7-H18 mice, the transgenic H chain would assemble with endogenous L chains, resulting in the expression of IgMα on the cell surface. As expected, IgMα positive cells were detected in the bone marrow and spleen, among which 20% of IgMα positive cells were able to bind rDsg3 (Fig. 3A). These results collectively indicate that B cells expressing Dsg3-reactive IgM are not deleted in the bone marrow or periphery.

Lack of Dsg3-reactive B1 B cells in the peritoneal cavity

The peritoneal cavity is considered to be isolated from many self-antigens, and CDS5/B220low B1 B cells in the peritoneal cavity are often self-reactive as a result of escaping deletion (20–22). In addition, B1 B cells are known to be resistant to Fas-FasL induced apoptosis (46). It was thus expected that B1 B cells expressing transgenic H and L chains be abundant in the peritoneal cavity of AK7-LH1 mice. However, most of the CDS5/B220low B1 B cells expressing IgMα showed little affinity to rDsg3 (Fig. 3C). No B1 B cells were detected in the peritoneal cavity of AK7-LH1-Rag2−/− mice. These results indicate that AK7-LH1 transgenic B cells are unable to differentiate into B1 B cells.

Transgenic B cells in the absence of Dsg3

To examine whether endogenous Dsg3 has any effect on the function of AK7-LH1 transgenic B cells, we transferred bone marrow cells of AK7-LH1 transgenic mice into lethally irradiated Dsg3−/− mice or wild-type mice and analyzed splenocytes of recipient mice 8 weeks after bone marrow transfer. IgMα positive Dsg3-reactive B cells were detected in both Dsg3−/− and wild-type mice as expected. There was little difference in the number of IgMα positive cells and the amount of surface IgM between wild-type and Dsg3−/− recipient mice (Fig. 4). Serum anti-Dsg3 IgM production was comparable between these recipient mice (data not shown). These data further confirm that there is little developmental block of AK7-LH1 B cells in the bone marrow and spleen in the presence of Dsg3.

AK7-LH1 transgenic B cells are not anergic

Although Dsg3-reactive B cells are present in the periphery, such B cells may be inactivated and in an anergic state. To
examine the activity of Dsg3-reactive peripheral B cells, we first checked whether anti-Dsg3 IgM is produced in the sera. As shown in Fig. 5(A), anti-Dsg3 IgM was readily detected in the sera of AK7-LH1 transgenic mice, while only a basal amount of anti-Dsg3 IgM was detected in the sera of AK7-H18 transgenic mice. Anti-Dsg3 IgM was also detected in the sera of AK7-LH1-Rag2\(^{−/−}\)/C255/C255 mice, although the amount of total IgM was much lower than AK7-LH1 mice. Approximately 20% of AK7-LH1 serum IgM were specific for Dsg3. Although not shown, anti-Dsg3 IgM was also detected in the sera of the bone marrow chimeras shown in Fig. 4. In AK7-LH1 mice, a large proportion of CD21\(^{hi}\)/CD23\(^{low}\) marginal zone B cells were observed in the spleen compared to wild-type mice (Fig. 5B). Direct immunofluorescence analysis showed clear deposition of IgM on the hard palates of both AK7-LH1 and AK7-LH1-Rag2\(^{−/−}\)/C255/C255 mice, indicating that the IgM indeed reacts with native antigen (Fig. 5C; data not shown). Interestingly, however, there was no obvious C3 deposition (data not shown).

We next examined the cellular function of transgenic B cells. AK7-LH1 transgenic B cells were purified with anti-B220 conjugated magnetic beads and stimulated with LPS, anti-IgM or rDsg3. Since rDsg3 used here contains the human IgG hinge sequence at the C-terminus and forms a divalent structure, rDsg3 is able to crosslink surface IgM to some extent. As shown in Fig. 6(A) (left panel), stimulation with anti-IgM as well as rDsg3 induced proliferation of transgenic B cells. We also examined anti-Dsg3 IgM production by AK7-LH1 B cells. We noted that AK7-LH1 splenic B cells secreted anti-Dsg3 IgM in culture supernatant without stimulation. When AK7-LH1 splenic B cells were stimulated with LPS, such stimulation greatly enhanced production of anti-Dsg3 IgM by AK7-LH1 B cells (Fig. 6A, right panel). In addition, marked calcium mobilization was observed upon crosslinking of surface IgM (Fig. 6B). We further examined whether the presence of Dsg3 alters functions of AK7-LH1 B cells. To this end, we crossed AK7-LH1 mice and Dsg3\(^{−/−}\)/C255/C255 mice on a 129/Sv background to generate Dsg3\(^{−/−}\)- and Dsg3\(^{+/−}\)-AK7-LH1 mice. We then measured calcium mobilization of AK7-LH1 B cells from Dsg3\(^{−/−}\)/C255/C255 and Dsg3\(^{+/−}\)/C255/C255 mice. As shown in Fig 6(C), we did not observe much difference between AK7-LH1 B cells from Dsg3\(^{−/−}\)/C255/C255 and Dsg3\(^{+/−}\)/C255/C255 mice. These results collectively indicate that the Dsg3-reactive B cells in the periphery of AK7-LH1 transgenic mice are not anergic but functionally competent.

**Discussion**

It is likely that B cells reactive with peripheral antigens are generated but inactivated or simply ignored by native antigen. Such B cell inactivation may be reversed by strong inflammation induced by adjuvant. In fact, it has been demonstrated that, in contrast to systemic antigen, autoimmunity against tissue-specific antigens can be induced relatively easily by immunization with potent adjuvant (34–36). In order to develop a PV mouse model, we have tried extensively to induce an

**Fig. 3.** The analysis of AK7-LH1, AK7-H18 transgenic mice. (A) Cells from bone marrow, spleen and lymph node of AK7-LH1 and AK7-H18 transgenic mice were analyzed by flow cytometry. In AK7-LH1 transgenic mice, IgM\(^{a}\) (transgene) positive B cells are detected in the bone marrow, spleen and lymph nodes. In AK7-H18 transgenic mice, ~20% of splenic B cells showed reactivity against rDsg3. (B) Bone marrow cells and splenocytes were examined for the expression of transgenic IgM\(^{b}\) in AK7-LH1-Rag2\(^{−/−}\)/C255/C255 mice. (C) Analysis of peritoneal cells. Most of the peritoneal cells detected in AK7-LH1 mice were CD5\(^{+}\)/B220\(^{low}\) B1 B cells but they showed little affinity to rDsg3. Few B cells were detected in AK7-LH1-Rag2\(^{−/−}\)/C255/C255 mice.
immune response to Dsg3 in various strains of wild-type mouse without success. Repetitive immunization with rDsg3 emulsified in CFA has failed to induce autoimmunity in wild-type mice, whereas autoantibody is easily produced in Dsg3−/− mice (37). It was thus expected that self-tolerance against Dsg3 is established in wild-type mice. As shown here, however, we observed neither deletion nor inactivation of self-reactive B cells in Dsg3-specific IgM transgenic mice. In AK7-LH1 transgenic mice, a considerable amount of anti-Dsg3 IgM was detected in the sera, and deposition of IgM on the surface of keratinocytes was readily observed without immunization. These results indicate that the self-reactive B cells develop normally in AK7-LH1 mice and the presence of Dsg3 on keratinocyte cell surfaces has little effect on the maintenance of functional B cells reactive to Dsg3. Analysis of AK7-H18 also supports this notion.

In 3–83μa×albumin promoter-Kβ double transgenic mice in which the target antigen, Kβ, is expressed mainly in the liver but not in the bone marrow, peripheral B cells are deleted and no autoantibody is detected in the serum (47). In 3–83μa×keratin 4-Kβ double transgenic mice, deletion is incomplete and a low amount of antibody is detected in the serum. In the HEL-IgM×thyroid mHEL double transgenic mouse model, in which the specific antigen is expressed on cell surfaces in the thyroid gland (29), normal numbers of anti-HEL IgM positive B cells are detected in the spleen.

The lack of deletion or inactivation of Dsg3-reactive B cells may be due to the physical isolation of Dsg3 from B cells, since expression of Dsg3 is mainly restricted to the surface of stratified squamous epithelium in peripheral tissues, as in the case of HEL-IgM×thyroid-mHEL transgenic mice (29). However, it is unlikely that B cells never encounter skin-specific antigen, because it has been demonstrated that self-antigens in the epidermis and dermis are continuously transported to the draining lymph nodes by DCs (48). Such continuous transportation of Dsg3 to the peripheral lymphoid organs would result in elimination or inactivation of Dsg3-reactive B cells. In fact, peripheral deletion has also been observed in 3–83μa×keratin 4-Kβ double transgenic mice where cognate antigen is expressed mainly in the skin (26). As shown here, however, Dsg3-reactive B cells are readily observed in the AK7-LH1 transgenic mice. It is theoretically possible that the affinity between transgenic IgM and Dsg3 is too weak to induce deletion or inactivation. However, this is unlikely to be the case, since AK7 was originally established from PV model
mice where most of the Dsg3-reactive autoantibodies are of a high affinity IgG isotype with somatic hypermutations and affinity maturation (38). In fact, transgenic B cells are able to bind rDsg3 in solution, confirming sufficient affinity between IgM and Dsg3. We noted that while both IgMhigh and IgMlow populations were detected in AK7-LH1 mice, only the IgMhigh population was detected in AK7-LH1 Rag2−/− mice. Since Rag2−/− DCs are reported to be defective in presenting antigen (49), it is possible that antigen-induced down-regulation of surface IgM occurs in AK7-LH1 mice, but such down-regulation is impaired in AK7-LH1-Rag2−/− mice. The IgMlow population was detected when T cells were transferred into AK7-LH1 Rag2−/− mice (data not shown), supporting the above notion. In addition, surface IgM level on B cells from Dsg3−/− mice after bone marrow transfer from AK7-LH1 mice was higher than that of wild-type mice after bone marrow transfer from AK7-LH1 mice, suggesting that the presence of the cognate antigen affects the surface IgM levels but the expression level is insufficient to induce deletion or anergy induction.

In AK7-LH1 as well as AK7-LH1-Rag2−/− mice, anti-Dsg3 IgM was produced in the sera and deposition of IgM on the keratinocyte cell surface was observed, indicating that autoantibody is produced without active immunization. The peritoneal cavity is considered to be isolated from most self-antigens, and the majority of IgM class autoantibodies in normal serum are derived from B1 B cells in the peritoneal cavity (20,21). Recent studies suggest that B cells need to have affinity against self-antigen to develop into B1 B cells (22–24). It was thus possible that the anti-Dsg3 IgM in the sera of AK7-LH1 mice was derived from B1 B cells. In AK7-LH1 mice, however, peritoneal cells contained no CD5+B220low B1 B cells expressing anti-Dsg3 IgM. Similarly, AK7-LH1-Rag2−/− mice had no CD5+B220low B1 B cells in the peritoneal cavity. It is likely from these results that the AK7 antibody reacts only with Dsg3 and no other self-antigen, and B cells expressing the AK7 antibody are unable to differentiate to peritoneal CD5+B220low B1 B cells. Similar results were obtained in ATAJak−/−Thy−1−/− mice where B cells expressing anti-Thy-1 antibody cannot differentiate into CD5+B220low B1 B cells in the absence of Thy-1 (22). Our results indicate that anti-Dsg3 IgM is produced by conventional B cells, not by peritoneal B1 B cells.

It has been shown using VSV-G transgenic mice that B cells do not produce antibodies in response to monomeric antigens without T cell help (50). It is possible that the presence of T cells capable of recognizing Dsg3 is involved in the production of anti-Dsg3 IgM in AK7-LH1 mice. However, anti-Dsg3 IgM was also produced in Dsg3−/− mice after bone marrow transfer from AK7-LH1 mice at a concentration similar to, or less than, that in AK7-LH1 mice, indicating that anti-Dsg3 IgM production does not require Dsg3-reactive T cells generated in Dsg3−/− mice where tolerance against Dsg3 is not established. It has

Fig. 6. Proliferative response and calcium mobilization. (A) (Left panel) Proliferative responses of AK7-LH1 B cells in response to LPS, anti-IgM F(ab)2, and rDsg3 were analyzed by thymidine incorporation. (Right panel) Production of anti-Dsg3 IgM from AK7-LH1 splenic B cells. Purified AK7-LH1 splenic B cells were cultured for 3 days with or without LPS. (B) Calcium mobilization assay. AK7-LH1 B cells were labeled with Fluo-3 and calcium level measured after stimulation by anti-IgM F(ab)2 or rDsg3. Splenic B cells from C57Bl/6 mice were used as a control. (C) Calcium mobilization assay of AK7-LH1 B cells from Dsg3+/− and Dsg3−/− mice after stimulation with anti-IgM F(ab)2 (upper panel) and rDsg3 (lower panel).
also been reported that repetitive, polymeric determinants are able to activate B cells to produce antibodies without T cell help (50). Since Dsg3 is expressed on keratinocyte cell surfaces and thus can form repetitive, polymeric determinants, it is possible that cell surface Dsg3 triggers anti-Dsg3 IgM production. However, anti-Dsg3 IgM was also produced in Dsg3−/− mice reconstituted with AK7-LH1 bone marrow cells in amounts similar to wild-type recipients. Therefore, Dsg3 expressed on keratinocyte cell surface is unlikely to play a role in triggering anti-Dsg3 IgM production.

AK7 is specific to Dsg3 but is not pathogenic (39). Thus, AK7 antibody was unable to induce any blistering or inflammation when produced in vivo. We have recently obtained a pathogenic mAb, AK23, which is able to induce PV phenotype. The epitope of AK23 was identified as the region critical for the homophilic adhesive interaction between two Dsg3 molecules (39). Transgenic mice expressing H and L chains of AK23 may thus show a different phenotype from AK7-LH1. AK23 IgM would induce inflammation, and the development of B cells carrying this IgM may be blocked by deletion. Experiments are currently underway.

To understand the pathophysiology of autoimmune diseases, it is necessary to investigate the mechanisms of tolerance break, preceding disease onset. In the field of pemphigus, populations which have anti-desmoglein IgG autoantibodies without development of an apparent pemphigus phenotype have been described. One such example is the endemic form of pemphigus foliaceus, fogo selvagem, in which the target antigen is desmoglein 1 (Dsg1), a close relative of Dsg3 in the cadherin superfamily (51,52). Unlike sporadic pemphigus foliaceus, which is a disease of mostly middle-aged and older patients, fogo selvagem affects young adults and children of any race exposed to the local ecology in rural areas. In these areas, >50% of normal individuals have anti-Dsg1 IgG autoantibodies, and the onset of the disease is preceded by a sustained antibody response (53). Another example is that a subset of silicosis patients has anti-desmoglein IgG autoantibodies without apparent clinical phenotype (54). Our present results clearly show the presence of self-reactive B cells in the periphery at some frequency, implying that Dsg3-reactive B cells are also present in normal individuals. Together with our findings in mouse models, elucidation of the immunological mechanisms preceding onset of disease will be critical in unveiling the mystery of the tolerance break in autoimmune diseases.

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