Autoreactive B-cell elimination by pathogenic IgG specific for the same antigen: implications for peripheral tolerance

Takayuki Ota¹,⁴, Miyo Aoki-Ota¹,⁴, Kazuyuki Tsunoda¹, Takeji Nishikawa¹, Shigeo Koyasu¹,²,³ and Masayuki Amagai¹

¹Department of Dermatology and ²Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo 160-8582, Japan
³Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan
⁴Present address: Department of Immunology, Scripps Research Institute, La Jolla, CA 92037, USA

Keywords: autoimmunity, B cells, Fas, skin

Abstract

Harmful pathogenic IgG auto-antibodies are produced against desmoglein 3 (Dsg3) in pemphigus vulgaris, an autoimmune blistering disease. Dsg3 is a cadherin-type cell adhesion molecule expressed in desmosomes of the skin and mucous membranes. In AK7-transgenic mice expressing non-pathogenic AK7 IgM against Dsg3, autoreactive transgenic B cells escape from the deletion or inactivation and exist in the periphery. However, when a pathogenic anti-Dsg3 IgG1 mAb (AK23) capable of inducing blisters was injected into AK7-transgenic mice, AK7 B cells were eliminated from the bone marrow (BM) and spleen only when Dsg3 was expressed in the periphery. In contrast, non-pathogenic IgG mAbs (AK7, AK9) failed to eliminate AK7 B cells. Interestingly, the AK23-mediated elimination of mature AK7 B cells in the spleen was significantly diminished in AK7-transgenic mice on a Rag2⁻/⁻ background while BM B cells were still eliminated, suggesting the presence of T-cell-dependent and -independent mechanisms. T cell transfer studies into AK7-Rag2⁻/⁻ mice revealed that autoreactive B-cell elimination in the periphery requires CD4⁺ T cells from wild-type mice but not from gld (FasL mutant) mice. The B-cell elimination was impaired in both BM and periphery when Bcl2 was over-expressed in AK7 B cells. These findings suggest that autoreactive B cells exist unless they are harmful, but once harmful or dangerous events such as tissue destruction are sensed, the mature autoreactive B cells in the periphery are eliminated via a Fas-mediated process in a CD4⁺ T cell-dependent manner.

Introduction

The central B-cell tolerance is maintained by receptor editing and developmental arrest associated with apoptosis (1, 2). Receptor editing in immature autoreactive B cells involves secondary light-chain gene rearrangements that result in the alteration of antigen receptor specificity, allowing the B cells to develop further. In the periphery, successful B cells with T-cell help are subjected to positive selection of high-affinity clones, which promotes differentiation to memory cells and plasma cells. In contrast, lack of T-cell help results in Fas-mediated apoptosis, which is a peripheral mechanism for the elimination of newly formed autoreactive B cells and B cells that have acquired self-reactivity by somatic mutations during the germinal center reaction (3, 4). Indeed, the MRL/lpr mouse, which is deficient in the expression of Fas gene product in combination with undefined MRL background genes, develops a lupus-like disease with auto-antibody production (5–7). Thus, peripheral tolerance mechanisms that involve CD4⁺ T-cell help are important in controlling B-cell autoimmunity.

Pemphigus vulgaris (PV) is an IgG-mediated autoimmune bullous disease, the target antigen for which is desmoglein 3 (Dsg3), a cadherin-type cell–cell adhesion molecule that is present in desmosome (8). Dsg3 is mainly expressed on the skin and mucous membranes but is not expressed in the bone marrow (BM). PV patients continuously produce Dsg3-specific pathogenic IgG antibodies. Several studies have suggested that HLA-DRB1*0402 and HLA-DQB1*0503 are associated with PV in Jewish and non-Jewish populations,
respectively, and appear to recognize several epitopes on the extracellular domain of Dsg3 (9–11). A phage display study of B-cell genes from PV patients has suggested that there are some restricted patterns of heavy (H)- and light (L)-chain gene usage in Dsg3-specific IgG auto-antibodies (12). Epitope mapping using PV patients’ sera against Dsg3 has indicated that Dsg3-reactive B cells are polyclonal and that main isotypes of Dsg3-specific IgGs are IgG1 and IgG4 (13, 14).

To clarify the immunological mechanism of PV, we have developed a PV mouse model by adoptive transfer of immunized or naive Dsg3+ lymphocytes into Rag2−/− mice that express Dsg3 (15–17). In this model, the transferred lymphocytes encounter the native Dsg3 in recipient mice and anti-Dsg3 IgG is stably produced >6 months. The recipient mice develop the characteristic PV phenotypes, including extensive oral erosions with typical loss of cell–cell adhesion of keratinocytes, within 2–3 weeks of transfer.

Using these model mice, we have developed several Dsg3-specific mAbs (18, 19). Most of these mAbs, such as AK7 and AK9, showed no pathogenic activity, while the AK23 mAb was pathogenic and induced the PV phenotype when the hybridoma cells were inoculated into the peritoneal cavities of Rag2−/− mice (19). Subsequently, we developed Dsg3-specific IgM-transgenic mice using the H- and L-chain variable genes from AK7 hybridoma cells (20). In this transgenic mouse, Dsg3-specific B cells develop in the BM and mature B cells are present in peripheral lymphoid organs, such as the spleen and lymph nodes (LNs). AK7-transgenic B cells also secrete Dsg3-specific IgM that binds to native Dsg3 on keratinocytes in vivo. Moreover, the AK7 B cells are not inactivated or anergic in the periphery, since receptor cross-linking with anti-IgM F(ab′)2 or dimerized recombinant desmoglein 3 (rDsg3) induces calcium mobilization and subsequent B-cell proliferation. Therefore, we conclude that these B cells are ignored by peripheral tolerance mechanisms (20).

One possible reason why Dsg3-specific B cells are not eliminated or inactivated even in the presence of Dsg3 in vivo is that the AK7 mAb is non-pathogenic and does not induce any harmful reaction in the body. In the present study, we examined the possibility that a harmful or dangerous event in the body, such as tissue destruction, changes the fate of such non-harmful autoreactive B cells. To this end, we studied the fate of AK7 Dsg3-specific B cells after the injection of AK7-transgenic mice with pathogenic or non-pathogenic IgG mAbs against Dsg3. We found that non-pathogenic AK7 Dsg3-specific B cells were deleted from the BM and spleen by the pathogenic AK23 mAb but not by non-pathogenic AK7 or AK9 mAbs. Furthermore, we show that the elimination of mature autoreactive B cells involves a Fas-dependent apoptotic pathway that is mediated by CD4+ T cells. This mechanism provides a novel feedback system to prevent the activation of autoreactive B cells in the periphery.

Methods

Mice

AK7-LH1-transgenic (AK7-Tg) mice on a C57BL/6 background have been described previously (20). AK7-Tg mice express the membrane bound and secreted forms of IgM (20). AK7-Tg mice do not produce IgG because the transgenic construct lacks the necessary genetic elements for class switching. Dsg3−/− mice (21) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained by mating female Dsg3−/− and male Dsg3−/− mice. Since Dsg3−/− mice on the C57BL/6 background barely survive after birth (data not shown), we used Dsg3−/− mice on a (C57BL/6 × 129/sv) mixed background. B6.129S6-Rag2tm1Flw N12 (C57BL/6-Rag2−/−) mice and C57BL/6-Rag2tm1Flw/TgN(OT-II.2) (OT-II-Rag2−/−) mice were obtained from Taconic (German Town, NY, USA). C57BL/6-Tg (Bcl2) 22Wehil/J (C57BL/6-Bcl2-Tg) mice were obtained from the Jackson Laboratory. C57BL/6J and C57BL/6J Scl-gld (gld) mice were obtained from Sankyo Laboratory (Shizuoka, Japan). AK7-Rag2−/− mice were obtained by mating AK7-Tg and C57BL/6-Rag2−/− mice. OT-II/AK7-Rag2−/− double-transgenic mice on a Rag2−/− background were generated by the mating of AK7-Rag2−/− and OT-II-Rag2−/− mice. Bcl2-AK7 double-transgenic mice were obtained by mating C57BL/6-Bcl2-Tg and AK7-Tg mice. All mice were maintained under specific pathogen free conditions in our animal facility. All animal experiments were performed in accordance with our Institutional Guidelines.

Histological analysis

Mice were sacrificed and biopsy specimens were taken from the hard palate and fixed in a neutral-buffered 10% formalin solution. Samples were processed by standard methods, embedded in paraffin and sectioned at 4–5 μm. Specimens were stained with hematoxylin & eosin. For direct immunofluorescence staining, biopsy specimens from the hard palate were embedded in Tissue-Tek® O.C.T™ Compound (Sakura Finetechnical, Tokyo, Japan) for cryostat sectioning. Each section was incubated with a 100-fold dilution of FITC-conjugated rabbit anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA). The sections were examined under a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan).

Antibodies and flow cytometry

The following directly conjugated mAbs were purchased from BD Biosciences (San Jose, CA, USA): FITC-anti-IgM (Igh-6A), FITC-anti-IgG (R6-60.2), FITC-anti-CD4 (GK1.5), FITC-anti-CD21 (7G6), PE-anti-B220 (RA3-6B2), PE-anti-CD23 (B3B4), PE-anti-CD25 (PC61), PE-anti-C1qRp (AA4.1), PerCP-Cy5.5-anti-B220 (RA3-6B2), allophycocyanin (APC)-anti-CD25 (PC61) and APC-anti-IgM (II/41). To stain for the AK7 idiotype, cells were incubated with 10 μg ml−1 rDsg3 that carries E-tag at its C-terminal, followed by staining with Alexa 488- or Alexa 647-conjugated anti-E-Tag antibody (Invitrogen). The AK7, AK9 and AK23 antibodies were purified with rProtein A FF (GE Healthcare Bio-Sciences, Uppsala, Sweden) from hybridoma cell culture supernatants, as described previously (19). The subclass of AK7, AK9 and AK23 antibodies is IgG1-κ. Three- or four-color flow cytometry was performed on a FACS Calibur (BD Biosciences) and analyzed with the FlowJo software (Tree Star, Ashland, OR, USA).

Injection of mAbs

Purified AK23 mAb (200 μg) or purified AK7 and AK9 mAbs (500 μg) in 0.2 ml PBS were injected intra-peritoneally into
each mouse and the mice were analyzed on day 5 post-injection. Control mice were injected with 0.2 ml of PBS.

Generation of BM chimeras
BM cells (1 × 10^7) from AK7-Tg mice were injected into 6 Gy-irradiated Dsg3^−/− mice. For mixed BM chimeras, 8 × 10^6 AK7-Tg BM cells and 2 × 10^6 wild-type BM cells were mixed and transferred into 9 Gy-irradiated wild-type C57BL/6 mice. After 1 month, the mice were used in the experiments.

Generation of BM chimeras
BM cells (1 × 10^7) from AK7-Tg mice were injected into 6 Gy-irradiated Dsg3^−/− mice. For mixed BM chimeras, 8 × 10^6 AK7-Tg BM cells and 2 × 10^6 wild-type BM cells were mixed and transferred into 9 Gy-irradiated wild-type C57BL/6 mice. After 1 month, the mice were used in the experiments.

Reconstitution of AK7-Rag2^−/− mice with CD4+ T cells
Splenic CD4+ T cells were purified from wild-type, Dsg3^−/− and gld mice using the CD4+ T Cell Isolation Kit (Miltenyi Biotech, Gladbach, Germany), and 1 × 10^7 cells were transferred into AK7-Rag2^−/− mice. The purity of the CD4+ T cells was >90%. One week later, these mice were challenged with the AK23 mAb and analyzed on day 5.

Statistical analysis
Statistical analysis was performed with the unpaired Student’s t-test.

**Results**

Disappearance of AK7 Dsg3-specific B cells from spleen and BM after injection of pathogenic anti-Dsg3 IgG mAb
In AK7-Tg mice, Dsg3-specific AK7 B cells were detected not only in the BM but also in the spleen and LNs without apparent inactivation (20; Fig. 2A, day 0). To address the possibility that AK7 B cells escape from tolerance mechanisms due to their non-pathogenic nature, we explored the effects on Dsg3-specific B cells of pathogenic (AK23) and non-pathogenic (AK7 and AK9) anti-Dsg3 IgG mAbs. The subclass of these antibodies is IgG1-j. As reported previously (19), when hybridoma cells that produced pathogenic AK23 IgG1 were inoculated into the peritoneum of Rag2^−/− mice, the recipient mice developed PV phenotypes such as erosions around the snout and patchy hair loss (Fig. 1A, C and E). In contrast, the mice that received non-pathogenic AK9 hybridoma cells showed no apparent PV phenotype, despite apparent ascites formation and in vivo IgG deposition on the surfaces of keratinocytes (Fig. 1B, D and F).

**Fig. 1.** Administration of pathogenic AK23 IgG1 mAb induces the PV-like phenotype. AK23 (A, C and E) or AK9 (B, D and E) hybridoma cells were inoculated into the peritoneal cavities of Rag2^−/− mice. On day 5, the mice were sacrificed and the hard palate histology (C and D) and IgG deposition (E and F) were analyzed. Arrowheads indicate blister formation just above the basal layers (i.e. suprabasilar acantholysis), but not in mice that received AK9 hybridoma cells (D). Bar = 50 μm.
We injected pathogenic AK23 or non-pathogenic AK7 or AK9 IgG1 mAb into AK7-Tg mice and examined the fate of the Dsg3-specific B cells (Fig. 2A). When AK23 mAb was injected, Dsg3-specific AK7 B cells started to disappear from the spleen on day 2, when the mice started to develop the PV phenotype (Fig. 2A, upper panels; data not shown). Dsg3-specific AK7 B cells were almost completely absent on day 5 (Fig. 2A and B). In contrast, when non-pathogenic AK7 or AK9 IgG was injected, no significant decrease in Dsg3-specific AK7 B cells was observed (Fig. 2A, right panels). The staining with anti-IgM instead of rDsg3 showed essentially same results (data not shown). The effects of AK23 mAb were transient because Dsg3-specific AK7 B cells were detected again 6 weeks after the injection of AK23 IgG, at which time point the PV phenotype had subsided (data not shown). A similar fate for AK7 B cells was observed in the BM compartment (Fig. 2A, bottom panels).

Requirement for interaction of pathogenic IgG with Dsg3 for the elimination of Dsg3-reactive B cells

To determine whether the presence of target antigen of AK23 mAb, Dsg3 on keratinocytes, is required for the elimination of Dsg3-reactive B cells, we generated BM chimeras. First, we transferred BM cells from AK7-Tg mice into Dsg3<sup>−/−</sup> mice and injected AK23 mAb 4 weeks after BM transfer. In these chimeric mice, the skin and mucous membranes lacked Dsg3 expression and were not damaged by AK23 mAb injection. As shown in Fig. 3(A), the deletion of AK7 B cells was not observed and AK7 B cells were readily detected in the spleens of the chimeric Dsg3<sup>−/−</sup> mice, indicating that the elimination of AK7 B cells is not due to cross-reactivity between AK23 mAb and unrelated molecules on B cells and that the binding of AK23 mAb to Dsg3 on keratinocytes of the skin and mucous membranes is necessary for the elimination of Dsg3-reactive B cells in vivo.

Most of the B cells (>80%) in the AK7-Tg mice were Dsg3 reactive. To further determine whether B-cell deletion is an antigen-specific event, we generated mixed BM chimeras by transferring BM cells from both AK7-Tg and wild-type mice into irradiated wild-type mice. When AK23 mAb was injected into the chimeric mice, the AK7 B cells were deleted by day 5, while the B220<sup>+</sup>IgM<sup>+</sup> B cells that were not reactive with Dsg3 remained in the spleen (Fig. 3B). These data indicate that the AK23-mediated elimination of mature AK7 B cells is an antigen-specific event and unlikely due to cytokine- or immune complex-mediated non-specific events.

Requirement of CD4<sup>+</sup> T cells for the deletion of mature Dsg3-reactive B cells

To explore further the deletion mechanism of Dsg3-specific B cells, we crossed AK7-Tg mice with Rag2<sup>−/−</sup> mice, which lack mature T or B cells (22). The presence of the AK7 transgene allowed Dsg3-specific B cells to develop in these mice. When AK23 mAb was injected into the AK7-Rag2<sup>−/−</sup> mice, the deletion of AK7 B cells in the spleen was markedly impaired when compared with AK7-Tg mice on a non-Rag2<sup>−/−</sup> background, although the number of remaining splenocytes of the AK23 mAb-injected AK7-Rag2<sup>−/−</sup> mice were lower than the control mice (Fig. 4A, upper panels, and Fig. 4B). On the other hand, most of the AK7 B cells in the BM were eliminated by day 5 even in AK7-Rag2<sup>−/−</sup> mice. In particular, B220<sup>high</sup>IgM<sup>high</sup> mature B cells were nearly absent in BM (Fig. 4A, bottom panels). The deletion of BM cells may lead to the reduction of peripheral B cells in AK23 mAb-treated AK7-Rag2<sup>−/−</sup> mice. The lack of supply from BM explains the reduction of the number of splenocytes in AK23 mAb-treated AK7-Rag2<sup>−/−</sup> mice. However, significant number of B cells remained in the spleen of AK23 mAb-treated AK7-Rag2<sup>−/−</sup> mice. As shown previously, Dsg3-specific B cells can develop into mature B cells in AK7-Tg
mice (20). Even if Dsg3 is expressed in the skin, AK7-Tg mice B cells developed to IgMloAA4.1+/C0 mature B cells but not IgMloCD23+/AA4.1 B cells, which have recently been identified as T3 anergic B cells. (Fig. 4C) (23, 24). In AK7-Rag2−/− mice, B cells in the spleen were mostly immature B cells as demonstrated by the high expression of AA4.1 (Fig. 4D).
However, upon AK23 mAb injection, the AA4.1 expression level (Fig. 4D) and the surface IgM expression level (Fig. 4A) were reduced in the B cells, indicating that the remaining B cells in the spleen were considered as mature B cell. These results suggest that the deletion mechanisms in the spleen and BM are different. Mature B cells, which were depleted by AK23 mAb injection in AK7-Tg mice, but not in AK7-Rag2−/− mice, require some cellular components that are missing in Rag2−/− mice, i.e. mature T cells.

To examine the involvement of mature T cells in the B-cell deletion process in the spleen, we reconstituted AK7-Rag2−/− mice with CD4+ T cells. The reconstitution of AK7-Rag2−/− mice with wild-type CD4+ T cells restored the elimination of the AK7 B cells from the spleen upon AK23 mAb injection (Fig. 5A and C). In contrast, reconstitution with CD4+ T cells from Dsg3−/−/− mice only partially restored the elimination of AK7 B cells (Fig. 5A and C). These findings collectively suggest that CD4+ T cells are required for the elimination of mature AK7 B cells by AK23 mAb and that the effector CD4+ T cells are impaired or absent in Dsg3−/−/− mice, suggesting the involvement of antigen specificity.

To further examine the requirement for antigen specificity of CD4+ T cells, we used CD4+ T cells from OT-II-transgenic mice specific for chicken ovalbumin peptide fragment

![Fig. 5. Restoration of B-cell deletion in AK7-Rag2−/− mice by wild-type CD4+ T cell transfer.](image-url)

(A) CD4+ T cells were purified from wild-type (WT), Dsg3−/− or gld mice and transferred into AK7-Rag2−/− mice. Five days later, AK23 mAb (0.2 mg per mouse) was administered and the splenocytes were analyzed on day 5. No cells were transferred into the control mice. Results shown are representative of eight (control and wild-type CD4+ T cells), three (Dsg3−/− CD4+ T cells) and four (gld CD4+ T cells) independent experiments. (B) The AK23 mAb (0.2 mg per mouse) was administered to OT-II/AK7-Rag2−/− double-transgenic mice and the splenocytes were analyzed on day 5. Results shown are representative of four independent experiments. (C) The frequency of Dsg3-specific AK7 B cells in the spleen was examined 5 days after injection of AK23, AK7-Rag2−/− mice without injection (AK7 Rag2, n = 8); AK7-Rag2−/− mice (AK23, n = 8); AK7-Rag2−/− mice reconstituted with wild-type CD4+ T cells (B6 CD4 + AK23, n = 8); AK7-Rag2−/− mice reconstituted with gld CD4+ T cells (gld CD4 + AK23, n = 4); *P < 0.05. (D and E) PBS or 0.2 mg of AK23 mAb was administered to AK7-Tg mice and on day 5 the frequencies of CD4+CD25+ T cells in spleens were evaluated by flow cytometry and statistical analysis (n = 4 in each group).
323–339 presented in MHC class II molecule I-A^b (25). We bred OT-II-Rag2^−/− mice and AK7-Rag2^−/− mice to derive OT-II/AK7-Rag2^−/− mice. In these double-transgenic Rag2^−/− mice, all the peripheral CD4^+ T cells were specific for the ovalbumin peptide and all the peripheral B cells were specific for Dsg3. When AK23 mAb was administered to these mice, mature B cells in the spleen remained as in the AK7-Rag2^−/− mice although the numbers were slightly reduced (Fig. 5B).

Critical role of Fas-mediated apoptotic pathway in the elimination of autoreactive B cells

Since it is likely that the elimination of mature B cells involves apoptosis, we examined the involvement of the Fas-FasL pathway, which is considered to play an important role in the process of B-cell maturation as well as the deletion of autoreactive B cells (26). To this end, AK7-Rag2^−/− mice were reconstituted with CD4^+ T cells from FasL mutant gld mice (27, 28). When the mice were challenged with AK23 mAb, the deletion of AK7 B cells was impaired and a significant number of mature AK7 B cells remained in the spleen, as in the AK7-Rag2^−/− mice (Figs 4A and 5A), indicating that FasL on CD4^+ T cells is crucial for the deletion of mature AK7 B cells. One of FasL^+ CD4^+ T cell populations is a subset of CD4^+CD25^+ regulatory T cells (Tregs) (29, 30). Interestingly, AK23 mAb injection into AK7-Tg mice increased the frequency of the CD4^+CD25^+ Treg population in the spleen (Fig. 5D and E).

We also tested the involvement of Bcl2, which is a regulator of lymphocyte survival and highly expressed in long-lived lymphocytes (31). The over-expression of Bcl2 allows the continued survival of cells that should be eliminated through apoptosis (32–34). We used Bcl2-transgenic mice, which express Bcl2 mainly in the B-cell lineage (35), and generated Bcl2-AK7 double-transgenic mice. In Bcl2-AK7 double-transgenic mice, the number of Dsg3-specific B cells increased compared with AK7 mice (compare the panels of Fig. 2A, day 0, and Fig. 6, PBS), suggesting that AK7 B cells are not totally neglected and deleted to some extent by apoptotic process. However, when the mice were challenged with AK23 mAb, the AK7 B cells were found to be resistant to deletion and remained in the periphery (Fig. 6). Moreover, the AK7 B cells in the BM were also unaffected by AK23 mAb injection.

These data collectively suggest that Fas-dependent apoptotic signaling is involved in the peripheral deletion of Dsg3-specific B cells upon AK23 mAb injection and that anti-apoptotic Bcl family members, such as Bcl2, counteract the Fas-mediated apoptotic pathway. Bcl-2 also blocks the T cell-independent B-cell deletion in the BM.

Discussion

Patients with autoimmune diseases carry both pathogenic and non-pathogenic auto-antibodies (19, 36). Normal individuals also carry some non-pathogenic auto-antibodies without any clinical symptoms (37). It has also been shown in mice that the developmental block of autoreactive B cells is sometimes insufficient and that autoreactive B cells are released into the peripheral lymphoid organs without inactivation (20, 33, 38), which suggests that the body can ignore self-reactive lymphocytes unless they are harmful. To investigate whether a harmful or dangerous event, such as tissue destruction, can affect the fate of non-harmful B cells, we established in this study a unique system to examine the effects of pathogenic IgG directed against an auto-antigen on the fate of benign non-pathogenic B cells that are reactive with the same antigen. In AK7-Tg mice, Dsg3-specific B cells exist in peripheral lymphoid organs without inactivation (20), which is consistent with the findings of Warren et al. (37). As shown in the present study, a pathogenic AK23 IgG1 mAb, but not a non-pathogenic AK7 or AK9 mAb, induced the deletion of Dsg3-reactive B cells from the BM as well as the spleen of AK7-Tg mice (Fig. 2A). The deletion of Dsg3-specific B cells was not due to cross-reactivity between the AK23 mAb and unrelated molecules on B cells, since the Dsg3-specific B cells were unaffected in Dsg3^−/− mice (Fig. 3A). It is thus likely that the binding of the pathogenic AK23 mAb to the native Dsg3 on keratinocytes induces tissue destruction, which is necessary for the initiation of deletion of Dsg3-reactive B cells. Our present results suggest the presence of two different mechanisms for the deletion of autoreactive B cells. The deletion of peripheral antireactive B cells is mediated by FasL-C4^+ T cells. This finding is consistent with previous reports that Fas-deficient animals express markedly elevated levels of serum auto-antibodies and that transgenic B cells that express antigen egg lysozyme (HEL) receptors recognizing autologous soluble HEL are deleted through a Fas-dependent pathway (4,39–41). In contrast, autoreactive B cells in the BM are eliminated by a T cell-independent manner. Immune complexes may induce the deletion of mature cells, since...
immune complexes are known to interact with FcγRIIB, leading to B-cell apoptosis (42). Or some cytokines produced during inflammation may lead to the deletion of BM B cells. The importance of the apoptotic pathway in both BM and periphery is underlined by our finding that Bcl2 over-expression almost completely blocked the effect of AK23 mAb in the BM and spleen (Fig. 6), which is consistent with a previous observation made by Lang et al. (33).

The developmental stage of B cells is altered in the spleen in AK7-Rag2\(^{2/-}\) mice. Almost all the B cells were considered as AA4.1\(^{+}\) immature B cells in AK7-Rag2\(^{2/-}\) mice without any treatment (Fig. 4D). However, these B cells seem to be able to develop into mature B cells under certain circumstances. As seen in Fig. 5(B), two population of B cells (IgM\(^{lo}\) and IgM\(^{hi}\)) were observed in the spleen of OTII-AK7-Rag2\(^{2/-}\) mice, which suggests that in the presence of T cells immature B cells can develop to mature B cells in AK7-Rag2\(^{2/-}\) mice. When AK23 IgG mAb was injected, the reduction of AA4.1 and IgM expression on B cells was apparent (Fig. 4A and D). This finding suggested that maturation of B cells could occur probably by some cytokines triggered by AK23 IgG mAb injection and skin inflammation, although the detail mechanism remains to be elucidated. Therefore, when CD4\(^{+}\) T cells were transferred into AK7-Rag2\(^{2/-}\) mice, B cells may have developed into mature B cells in the spleen, and those B cells were eliminated upon AK23 IgG mAb injection (Fig. 5A).

Intriguingly, the CD4\(^{+}\) T cells from Dsg3\(^{-/-}\) mice were impaired for B-cell deletion caused by the AK23 mAb (Fig. 5A). The AK23 mAb did not delete B cells in OT-II/AK7 double-transgenic mice on a Rag2\(^{2/-}\) background (Fig. 5B). These data collectively suggest the involvement of antigen specificity. Dsg3-specific T cells are expected to be deleted in wild-type mice but survive in Dsg3\(^{-/-}\) mice. In fact, the development of the PV phenotype in our PV model mice requires both Dsg3\(^{-/-}\) B cells and Dsg3\(^{-/-}\) T cells (17). If Dsg3-reactive effector T cells are involved in the antigen-specific mature B-cell deletion, one would predict the complete deletion of AK7 B cells in AK7-Rag2\(^{2/-}\) mice transferred with CD4\(^{+}\) T cells from Dsg3\(^{-/-}\) mice. However, the results were opposite. Recent studies have shown that Tregs distinguished by the expression of CD25 and Foxp3 among CD4\(^{+}\) T cells develop in the thymus and express TCR with relatively high affinity to self-Ag/MHC complex and that the presence of cognate antigen promotes the differentiation and survival of Tregs in an antigen-specific manner (43–46). One possibility is the involvement of Dsg3-reactive Tregs that may develop in wild-type mice that express Dsg3 in the thymus. It is noteworthy that OT-II/Rag2\(^{2/-}\) mice lack Tregs (data not shown). It has been shown that a CCR4\(^{+}\) subset of Tregs expresses FasL (29, 30). In addition, CD4\(^{+}\)CD25\(^{+}\) Tregs have been shown to kill other activated cells including B cells (47, 48) by the action of granzyme A in humans and granzyme B in mice (49–51). Our results suggest that Tregs are important not only in suppressing autoreactive T cells but also in deleting autoreactive B cells through the Fas-FasL system. It is of interest that AK23 mAb injection increases the frequency of CD4\(^{+}\)CD25\(^{+}\) Tregs (Fig. 5D). It is unclear at the moment if the effector phase deleting Dsg3-reactive B cells involves Dsg3-specific cognate interaction between Dsg3-reactive B cells and Dsg3-specific Tregs. It is possible that the increase in the number of Tregs in peripheral organs enhanced the Fas-Fasl-mediated deletion independent of Dsg3. The fact that only Dsg3-reactive B cells were deleted in the mixed BM chimeras (Fig. 3B) seems to support the involvement of antigen-specific cognate interaction. Immune complex consisting of Dsg3 and AK23 mAb may be involved in the antigen presentation by both dendritic cells (DCs) and B cells. Precise mechanisms including antigen presentation remain to be determined in future studies.

Autoreactive T cells reactive with identical Dsg3 epitopes are detected in both patients and healthy donors (52). Interestingly, the numbers of IL-10\(^{+}\) Tregs are much lower in PV patients than in healthy individuals (53). These data suggest that Dsg3-specific Tregs are involved in the maintenance of peripheral tolerance in healthy individuals, despite the presence of autoreactive T cells. In another auto-antibody-mediated disease, myasthenia gravis, the number of Tregs in the peripheral blood is significantly lower in untreated patients than in age-matched healthy individuals, whereas it is normal or elevated in patients on immunosuppressive therapy (54). A separate study has reported functional defects in Tregs in patients with myasthenia gravis (55).

A scenario emerges from our data and previous studies: once harmful IgG develops and causes some damage to tissues, the immune system can sense the danger and shuts off the development of antigen-specific B cells. This mechanism prevents harmful autoimmune reactions and provides a reasonable feedback system for the control of B-cell development. Our data also suggest that the dysregulation of this feedback system, e.g. the failure of the Fas-mediated killing of autoreactive B cells, leads to the expansion of autoreactive B cells and the onset of autoimmune diseases, such as PV.

**Funding**


**Acknowledgements**

We thank Ms Hiromi Itoh for excellent animal care and Ms Yoshiko Fujii for the preparation of recombinant proteins and mAbs.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK7-Tg</td>
<td>AK7-LH1-transgenic</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>Dsg3</td>
<td>desmoglein 3</td>
</tr>
<tr>
<td>H-chain</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HEL</td>
<td>hen egg lysozyme</td>
</tr>
<tr>
<td>L-chain</td>
<td>light chain</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>PV</td>
<td>pemphigus vulgaris</td>
</tr>
<tr>
<td>Tregs</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4 T cells</td>
</tr>
<tr>
<td>CD25</td>
<td>CD25 T cells</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Foxp3 T cells</td>
</tr>
<tr>
<td>Dsg3</td>
<td>desmoglein 3</td>
</tr>
<tr>
<td>PV pemphigus vulgaris</td>
<td></td>
</tr>
</tbody>
</table>
Pathogenic IgG eliminates autoreactive B cells

References


