Published in final edited form as:
Autoimmunity. 2013 February ; 46(1): 6–13. doi:10.3109/08916934.2012.710859.

Autoimmunity to isomerized histone H2B in systemic lupus erythematosus

HESTER A. DOYLE[1], **DANA W. ASWAD**[2], and **MARK J. MAMULA**[1]

[1]Department of Rheumatology, Yale University School of Medicine, New Haven, Connecticut, USA

[2]Department of Molecular Biology and Biochemistry, University of California, Irvine, California, USA

Abstract

Histone H2B is a common target of autoantibodies in both spontaneous and drug-induced systemic lupus erythematosus (SLE). Recent studies demonstrate that Asp^{25} of histone H2B (H2B) spontaneously converts to an isoaspartic acid (isoAsp) *in vivo*. Our laboratory has demonstrated that the posttranslational modification of an aspartic acid to an isoaspartic acid within self-peptides renders otherwise ignored peptides immunogenic. Analysis of serum from lupus-prone mice and histone antibody positive SLE patients revealed antibodies specific to the Asp and isoAsp $H2B_{21-35}$ peptide, and that the expression of these antibodies is dependent on TLR9. IsoAsp $H2B_{21-35}$ is immunogenic in non-autoimmune prone mice and mice lacking the ability to repair isoAsp have significantly reduced levels of antibodies to H2B. Asp $H2B_{21-35}$ incubated at physiological temperatures and pH acquires the isoAsp modification, demonstrating that $H2B_{21-35}$ is prone to spontaneous isoAsp formation *in vivo*. Autoimmunity to isoAsp H2B suggests that this form of the autoantigen may be critical in the induction of anti-histone autoantibodies in human SLE and in murine models of disease.

Keywords

Isoaspartyl; *B cells*; *drug-induced lupus*; *autoantigen*; *autoantibodies*

Introduction

The ability of the immune system to distinguish self from non-self is the result of numerous cellular interactions and immunologic checkpoints. Positive and negative selection in the thymus and bone marrow, clonal deletion and anergy are just a few of the mechanisms in place to prevent the immune system from mounting a response against self-tissues. However, the presence of autoimmune diseases demonstrates that these mechanisms are not perfect in maintaining tolerance. One way in which immune tolerance can be broken is through the modification of self-antigens. Protein modifications occur during numerous physiologic processes including infection, apoptosis, and cellular stress.

Correspondence: Mark J. Mamula, Section of Rheumatology, Yale University School of Medicine, P.O. Box 208031, New Haven, Connecticut 06520-8031, USA. mark.mamula@yale.edu.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Protein modifications create novel “self-antigens” that are essentially not present during the development of immune tolerance. Our laboratory has demonstrated that the conversion of aspartic acid (Asp) to an isoaspartic acid (isoAsp) is one such modification that triggers B and T cell autoimmunity to otherwise inert self-peptides (Figure 1) [1]. Unlike other modifications, isoAsp residues arise spontaneously at physiological pH and temperatures in the absence of any enzymes. A repair pathway, catalyzed by protein-L-isoaspartate *O*-methyltransferase (PIMT; E.C. 2.1.1.77), evolved to convert isoAsp residues back to Asp residues and is found in both prokaryotes and eukaryotes [2].

In a recent search for proteins that contain naturally elevated levels of isoAsp proteins, we found that histone H2B (H2B) is highly susceptible to isoAsp formation since H2B accumulates relatively high levels of isoAsp when the PIMT repair system is blocked [3]. H2B is the only histone that has the isoAsp modification, as increased isoAsp content is not detected in the other histones [3,4]. In H2B, Asp^{25} is followed by a Gly residue, a motif that makes Asp^{25} particularly prone to undergo isomerization to an isoAsp residue [5].

What makes this observation of interest is that patients with systemic lupus erythematosus (SLE) display multiple autoantibody reactivity to nuclear, cytoplamic and cell membrane antigens. Among the most prominent autoantibodies are those directed against the nucleosome, which consist of a DNA helix wound around an octomeric complex of two histone molecules each of H2A, H2B, H3, and H4. It is estimated that 24–95% of spontaneous SLE patients and 67–100% of drug-induced lupus (DIL) patients have histone specific antibodies [6]. Histone H2B, in particular, is a principle target of autoimmunity in SLE [7,8].

It is unclear why high titers of autoantibodies develop against histones in SLE and related connective tissue diseases. Indeed, histones are among the most highly conserved intracellular proteins, making them more likely to have conferred immune tolerance over the evolution of species. Nonetheless, histones undergo numerous posttranslational modifications, such as acetylation, ubiquitination, and ADP ribosylation [6,9]. Although these and other modifications presumably are involved in regulating DNA-histone interactions, as well as DNA repair and replication, they can also potentially make histones immunogenic. Under certain stimuli, histones become acetylated or deiminated, and antibodies to these modifications are found in autoimmune prone mice [10,11].

Based on these observations, we hypothesize that isoAsp plays a role in B cell recognition of H2B, thus providing one explanation as to why antibodies develop to this particular histone. The present studies were undertaken to examine the immunogenicity of isoAsp-modified histone H2B and determine if this modification is a target of spontaneous autoimmunity in human lupus and murine models of disease.

Methods

Peptides and reagents

L-Asp and L-isoAsp p21–35 (AQKKDGKKRKRSRKE) were synthesized by AnaSpec (San Jose, CA) to greater than 98% purity as determined by mass spectrometry. L-Asp mouse cytochrome C p81–104 (MIFAGIKKKGERADLIAYLKKATN) was synthesized by the W. M. Keck Biotechnology Resource Laboratory at Yale University and used as a control peptide. Peptides were reconstituted in sterile water prior to use and stored at − 80°C. Histone H2B and whole histone preparations for use in ELISA were purchased from ImmunoVison (Springdale, AR).

Animals and immunizations

All animal protocols were consistent with accepted guidelines of the National Institutes of Health for the care and use of laboratory animals as well as approved by the Yale University Institutional Animal Care and Use Committee. Animals were housed at the animal facility at the Yale University School of Medicine. MRL *lpr* mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred at the animal facility at Yale University. Female B10.A mice were purchased from the National Cancer Institute (Frederick, MD).

Mice that express the isoAsp repair enzyme protein-L-isoaspartate *O*-methyltransferase only in the brain (Tg PIMT KO mice) on the C57BL/6 background were a generous gift of Dr. Steven Clarke (UCLA) [12] and were bred at the Yale Animal Facility. Sera from 3H9 heavy chain transgenic mice on the MRL *lpr* background [13], with and without TLR9, were a kind gift from Mark Schlomchik, Yale University.

To determine antibody responses against each H2B peptide isoform, female B10.A mice (6–10 weeks old, National Cancer Institute) were immunized with 50 μg of either Asp or isoAsp peptide antigen emulsified in CFA (Sigma, St. Louis MO) administered i.p. and in one footpad. Twenty-one days later, the mice were boosted with the same peptide antigen but emulsified in IFA (Sigma, St. Louis, MO). Seven days after boosting, mice were bled via the retro-orbital vein and serum collected for use in ELISA.

Patient population

Patient sera samples were obtained from the Yale University Rheumatology Diagnostic Laboratory. Patient sera selected were positive for reactivity to histones based on a commercial ELISA (Inova Diagnostics, San Diego, CA). Normal control sera were from healthy individuals.

ELISA

ELISA plates (Nunc) were coated with 100 μl of a 50 μg/ml solution of either isoform of H2B peptide or control peptide in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6; Sigma) and incubated overnight at 4°C. Wells were washed with PBS + 0.05% Tween 20 (PBST), and blocked with 3% BSA-PBST for 1 h at room temperature. Sera samples were diluted in 0.3% BSA-PBST, 100 μl added to each well and incubated 2 h at room temperature. After the wells were washed with PBST, alkaline phosphatase labeled-goat anti-mouse IgG, alkaline phosphatase labeled-goat anti- human IgG or alkaline phosphatase labeled-goat anti-mouse IgM (Southern Biotech, Birmingham, AL) was added to the wells at a 1:1000 dilution and incubated at room temperature for 1.5 h. Wells were washed one last time, and developed by the addition of the substrate pNPP (Sigma, St. Louis, MO). Wells were read on a SpectraMax 450 ELISA reader (Molecular Dynamics, Sunnyvale, CA) at 405 nm.

Histone H2B and whole histone ELISAs were performed in a similar manner as described above for the H2B peptide ELISA, with the exception that histone H2B or whole histones were coated onto ELISA plates at a concentration of 50 μg/ml. A commercially available dsDNA ELISA (DiaSorin, Stillwater, MN) was used to assess dsDNA IgM in mouse sera.

IsoAsp determination

Asp $H2B_{21-35}$ was incubated in PBS pH 7.4 at 37°C for 14 or 26 days. Negative controls included the Asp $H2B_{21-35}$ peptide that had been stored at − 80°C. The pmol amounts of isoAsp in each peptide preparation were determined using the ISOQUANT Isoaspartate Determination Kit per manufacturer's instructions (Promega, Madison, Wisconsin). The

internal positive control for the ISOQUANT kit was the isoAsp delta sleep inducing peptide (DSIP; WAGGDASGE) that contains exactly 1 pmol of isoAsp per pmol of peptide.

Statistical analysis

All statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA). Results were considered significant if the p value was < 0.05.

Results

Autoimmune prone mice have antibodies that react to Asp and isoAsp H2B$_{21-35}$

Previous studies demonstrated H2B undergoes isomerization *in vivo* [3]. Since both lupus patients and lupus-prone mice develop autoantibodies to H2B, we wanted to determine first if lupus prone mice, specifically MRL *lpr* mice, naturally develop antibodies to H2B$_{21–35}$. Sera from MRL *lpr* mice between 5 and 26 weeks of age were tested for the presence of IgG antibodies to both Asp and isoAsp H2B$_{21–35}$. As early as 5 weeks, mice have detectable levels of IgG against both Asp (Figure 2A) and isoAsp (Figure 2B) H2B$_{21-35}$.

The IgG levels against both H2B$_{21-35}$ isoforms also increase as the mice age (Figures 2A and 2B). Individual sera strongly positive for H2B$_{21–35}$ bound both peptide isoforms with similar intensity, and this binding was specific for H2B$_{21-35}$ isoforms as sera from 16- and 20-week old mice had relatively low binding to another murine self-peptide, cytochrome c$_{81–104}$ (OD 405 nm < 0.2). Control sera from both young (4–5-week-old) and old (20–26-week-old) non-autoimmune prone B10.A mice also had low levels of anti-H2B$_{21-35}$ antibodies (OD 405 nm < 0.2).

Anti-DNA 3H9 Tg mice have antibodies that react to Asp and isoAsp H2B$_{21-35}$

As histones are in close association with DNA in nucleosomes structure, it is not unreasonable to believe that antibodies against histones arise in conjunction with anti-DNA antibodies and due to similar mechanisms as anti-dsDNA antibodies. In order to test this hypothesis, we examined the serum profile of 3H9 immunoglobulin transgenic MRL *lpr* mice. 3H9 Tg mice express a DNA-specific heavy chain variable region (3H9) derived from a MRL *lpr* mouse with disease [14]. The 3H9 μ heavy chain pairs with endogenous light chains, resulting in a polyclonal B cell repertoire enriched for B cells expressing only IgM that are specific for ssDNA and dsDNA [13,14].

The 3H9 Tg MRL *lpr* mice have significant levels of serum IgM against isoAsp H2B$_{21–35}$ as compared to serum IgM against a control peptide ($p = 0.0108$) (Figure 3A), in addition to having significant levels of anti-dsDNA IgM (Figure 3B). TLR9 is needed for the development of anti-dsDNA antibodies in MRL *lpr* mice, as mice lacking TLR9 have significantly reduced levels of anti-dsDNA [15]. The 3H9 Tg MRL *lpr* TLR9−/− mice have reduced serum IgM levels against isoAsp H2B$_{21–35}$ as well as the expected reduction in anti-dsDNA antibodies (Figure 3A, $p = 0.003$ and Figure 3B, $p \leq 0.001$, respectively).

isoAsp H2B$_{21-35}$ is immunogenic in non-autoimmune prone mice

Clearly, autoimmune prone mice develop antibodies against H2B$_{21–35}$. Because the presence of isoAsp in self-peptides can render otherwise immunologically ignored proteins immunogenic [1], we wanted to know if the isoAsp H2B peptide isoform was also immunogenic in non-autoimmune prone mice. To test this, non-autoimmune prone B10.A mice were immunized with Asp or isoAsp H2B$_{21–35}$ peptide or injected with PBS-CFA as a control. After 27 days, serum was collected for use in histone H2B and whole histone ELISAs. Serum from mice immunized with isoAsp H2B$_{21–35}$ had higher levels of anti-H2B

(intact molecule) and anti-whole histone antibodies as compared to mice immunized with Asp $H2B_{21-35}$ or the control PBS-CFA injected mice (Figure 4).

Mice lacking the ability to repair isoAsp do not recognize isoAsp $H2B_{21-35}$

Transgenic PIMT knockout mice have the *Pimt* gene behind a rat neuron specific-enolase promoter so that PIMT is only expressed in the brain of these mice [12]. As the average life span of non-Tg PIMT knockout mice is 42 days, the presence of the PIMT transgene greatly extends the life span of these mice (up to 400 days), allowing for the accumulation of isoAsp in proteins. Theoretically, since the immune system of these mice is constantly exposed to isoAsp residues, immune tolerance should develop to isoAsp residues. Indeed, sera from Tg PIMT knockout mice have significantly less reactivity to isoAsp $H2B_{21-35}$ than do sera from Tg PIMT wild-type mice ($p = 0.0247$) (Figure 5). The Tg PIMT knockout mice also have a significant reduction in autoantibodies to Asp $H2B_{21-35}$ (Figure 5; $p = 0.0071$).

Histone antibody positive SLE patients also have antibodies that react to Asp and isoAsp $H2B_{21-35}$

Because autoimmune prone mice have naturally occurring antibodies against Asp and isoAsp $H2B_{21-35}$, we next determined if isoAsp H2B is a target of antibody responses in patients with anti-histone antibodies. Patient sera samples from the Yale Rheumatology Diagnostic Laboratory were analyzed for antibody responses to isoAsp $H2B_{21-35}$ based on the presence or absence of anti-histone antibodies as previously determined by commercial ELISA. Patients who had previously tested positive for anti-histone antibodies had significantly higher amounts of antibody against both Asp and isoAsp $H2B_{21-35}$ ($p = 0.0068$ and 0.0031, respectively) as compared to normal human sera (Figure 6).

Spontaneous conversion of Asp^{25} to isoAsp in $H2B_{21-35}$

One interesting aspect of the antibody response in MRL *lpr* and lupus patient sera is that antibodies exist to both Asp and isoAsp $H2B_{21-35}$ (Figure 2 and Figure 6). Typically, we have found immune tolerance is maintained to Asp forms of self-peptides [1,16]. A possible explanation for the presence of anti-Asp $H2B_{21-35}$ antibodies is that Asp^{25} of H2B undergoes spontaneous conversion to the isoAsp isoform when at physiological solutions and at temperatures of 4°C or greater. The presence of a Gly residue following the Asp^{25} of H2B suggests that this site is especially prone to isoAsp formation, as peptides with the Asp-Gly sequence are biochemically labile [5].

Thus, we tested the possibility of the spontaneous conversion of Asp^{25} to isoAsp in the H2B peptide. We found a 3-fold increase in the amount of isoAsp in Asp $H2B_{21-35}$ incubated for 14 days under physiological conditions (pH 7.4 and 37°C) as compared to Asp $H2B_{21-35}$ stored at minus 80°C (Figure 7). After 26 days at physiological pH and temperature, the amount of isoAsp in Asp $H2B_{21-35}$ further increased by 15-fold as compared to Asp $H2B_{21-35}$ stored at − 80°C (Figure 7). This data demonstrates that Asp $H2B_{21-35}$ spontaneously converts to isoAsp $H2B_{21-35}$ under physiological conditions. As described previously, conversion of Asp to isoAsp occurs more frequently and quickly at physiologic temperature and pH [5,17]. Considering the half-life of H2B in mouse tissues is 223 days [18], there is ample time for isoAsp to accumulate within H2B.

Discussion

A key question with regard to autoimmune diseases is how tolerance is lost to self-antigens. One possibility is that self-proteins become post-translationally modified and thus immune tolerance never develops to these “new antigens.” Among all the histone proteins, H2B has long been recognized as a target of autoantibody responses in both spontaneous and drug-

induced lupus. The data presented here suggest that the immunogenicity of H2B is due to the posttranslational modification of Asp^{25} via the spontaneous isomerization to an isoAsp residue.

Both lupus prone MRL *lpr* mice and histone antibody positive lupus patients have antibodies specific to both Asp and isoAsp $H2B_{21-35}$. This may not be unexpected, especially in our human sera studies, because the $H2B_{21-35}$ peptide maps to the area of a previously reported T cell epitope of H2B [19].

Further proof that the autoimmune prone background influences the antibody response to H2B comes from our studies in the 3H9 Tg MRL *lpr* mouse. Although the 3H9 Tg on the C57BL/6 background does not have increased anti-DNA antibody titers above those seen in normal mice [14], 3H9 expression in the MRL *lpr* background exhibit greatly increased levels of anti-DNA antibodies [13]. Although the hallmark of the 3H9 Tg MRL *lpr* mouse is anti-DNA antibody production, it appears that anti-H2B IgM also arises from the close association of H2B with DNA in nucleosomes.

Although previous studies demonstrated 3H9 sera binding to chromatin [20], this is the first demonstration of 3H9 sera binding to a specific histone H2B determinant. The antibody response to $H2B_{21–35}$ is also dependent on TLR9, as 3H9 Tg MRL *lpr* TLR9 −/− sera contains not only reduced levels of anti-dsDNA, previously described as a consequence of TLR9 deletion [15] but also have reduced levels of anti-isoAsp $H2B_{21–35}$. IsoAsp-modified H2B as a component of chromatin structure triggers both TLR and autoantibodies to this altered site on H2B protein. Perhaps not unexpectedly, TLR triggering acts as an accelerant to histone specific autoimmunity.

One question that arises is why there is a lack of tolerance in autoimmune prone mice or lupus patients to this modified H2B peptide. First, we have demonstrated an abundance of intracellular isoAsp modified proteins in tissues and cells of MRL *lpr* mice especially T cells [21]. The increase in isoAsp in MRL *lpr* cells is not due to non-functional PIMT (the enzyme that repairs isoAsp residues) as PIMT isolated from both non-autoimmune and MRL *lpr* mice repair isoAsp residues with the same efficiency [21]. During disease, isoAsp formation of histone H2B may exceed the ability of PIMT to repair this modification, leaving a large pool of “neoantigens” available for recognition by the immune system. Similar studies are underway to determine if isoAsp residues are increased in the cells of lupus patients and the efficiency of PIMT-based repair in humans.

The excess amount of isoAsp self-proteins in MRL *lpr* cells, and specifically that found in H2B, could arise during inflammatory processes known to occur during lupus disease progression. For example, histone deimination has been shown to occur in neutrophils in response to inflammatory stimuli [11]. MRL *lpr* mice and lupus patients also have increased amounts of apoptotic cells, believed to be a reservoir of autoantigens in SLE [22–25]. Nucleosomes have been shown to be exposed on the cell surface of apoptotic cells [26] with acetylation arising in histone H2A, H2B, and H4 and methylation within histone H3 [10,27,28]. In a similar manner, isomerization (Asp to isoAsp) is enhanced in cells undergoing stress and/or apoptosis [29,30]. Indeed, we found increased isoAsp levels in murine B cells undergoing apoptosis (Doyle, unpublished data). More importantly, these posttranslationally modified histones are recognized by autoantibodies in lupus patient sera [10,27,28]. Based on the above studies, it is not unreasonable to assume that H2B becomes isomerized in apoptotic cells and is exposed on the surface of these cells.

Further support that isoAsp-modified H2B is immunogenic arises from our results in the PIMT deficient mice. Because these mice cannot repair isoAsp modification, high tissue levels of isoAsp self-proteins likely result in immune tolerance to this modification. This

certainly appears to be the case, since PIMT-deficient mice have significantly less anti-Asp and anti-isoAsp $H2B_{21–35}$ peptide antibodies as compared to Tg PIMT wild type mice. MRL strains generate very high titers of autoantibodies to the $H2B_{21–35}$ determinant, thus 1/1000 sera dilutions were utilized (Figure 2 and 3) with the same 1/1000 dilution used as control, haplotype matched B10.A serum. In contrast, non-autoimmune mice make significantly less antibodies to $H2B_{21–35}$.

Figure 5 utilizes wild-type and PIMT deficient C57BL/6 strains in parallel and utilizing 1/100 serum dilutions to detect weaker autoantibody responses that may differ between two non-autoimmune prone strains (in the presence or absence of PIMT repair). We believe this is a relevant approach since `normal' non-autoimmune humans also often have detectable, but low titer, autoantibodies to a number of intracellular lupus autoantigens (approximately 5% of the population) [31]. The observation suggests that the inability to repair isoAsp modifications, leading to high tissue and extracellular isoAsp protein concentrations, confers statistically significant differences in immune tolerance (or lack of immune tolerance). Again, this system resembles that found in humans since three known polymorphisms in the PIMT gene confer slightly different enzymatic activities which may lead to subtle, but statistically relevant, differences in immune tolerance [32,33]. The latter observation may be a nidus for the amplification of autoimmunity in humans.

Despite the immunogenicity of the isoAsp H2B peptide, we still see antibodies to Asp $H2B_{21–35}$. In a similar manner, studies in our laboratory using snRNP D as an antigen demonstrate that mice immunized with isoAsp snRNP $D_{65–79}$ developed antibodies against not only isoAsp snRNP $D_{65–79}$, but also developed antibodies to Asp snRNP $D_{65–79}$, native snRNP, and dsDNA [1]. This demonstrates that the immune response to isoAsp snRNP $D_{65–79}$ diversified to include Asp snRNP $D_{65–79}$ as well as other nuclear antigens. van Bavel and colleagues described a similar situation with regard to an acetylated H2B peptide. Plasma from pre-diseased lupus mice reacted more strongly with H2B peptide (amino acids 1–18) acetylated at lysine 12 as compared to the non-acetylated H2B peptide.

However, once these mice developed full-blown disease, plasma reactivity to acetylated H2B peptide was similar to that seen with the non-acetylated H2B peptide, indicating that the antibody response has diversified to include reactivity to the non-acetylated H2B [27]. We expect that a similar scenario of antibody diversification occur in that the antibody response to isoAsp $H2B_{21–35}$ spreads to include an antibody response to Asp $H2B_{21–35}$. Most likely there is a preponderance of isoAsp $H2B_{21–35}$ due to the Asp^{25}–Gly^{26} sequence in H2B that is known to be an especially labile sites for isoAsp formation [5]. In fact, when we incubated Asp $H2B_{21–35}$ at physiological temperature and pH, there was an increase in the amount of isoAsp compared to the same peptide stored at – 80°C. This is not surprising as Young and colleagues first described H2B as undergoing significant levels of isomerization at Asp^{25} [3,4].

Autoantibody production to H2B is also notable in the context of drug-induced lupus (DIL) [34]. In particular, procainamide and hydralazine are among a list of pharmacologics that trigger lupus-like pathology and anti-H2B autoimmunity. Procainamide and hydralazine are known methyltransferase inhibitors and have been most studied as inhibitors of DNA methyltransferases, in particular DNA methyltransferase 1 (Dnmt1) [35]. Although procainamide directly inhibits Dnmt1, hydralazine inhibits the ERK signaling pathway in T cells, and subsequently Dnmt1 mRNA and activity [36]. Interestingly, Cournoyer and colleagues have shown that PIMT expression is dependent on ERK signaling [37]. Therefore, one possible link between hydralazine and isoAsp accumulation is in the inhibition of PIMT expression via the ERK pathway. Ongoing studies from our laboratory are examining PIMT repair function in models of drug-induced lupus.

In conclusion, the presence of an isoAsp residue at position 25 of histone H2B renders histone H2B a target of autoimmunity in both mice and humans. The presence of a posttranslationally modified Asp residue may explain why autoantibodies develop histone H2B in both spontaneous and drug-induced lupus.

Acknowledgments

We thank Dr. Steven Clarke for providing the Tg PIMT mice and Dr. Mark Schlomchik and Kevin Nickerson for providing the 3H9 Tg MRL *lpr* sera. This work was supported by an Arthritis Investigator Award from the Arthritis Foundation (to H.A.D.), by NIH grants NS-17269 (to D.W.A.), AI-48120 (to M.J.M.), and AR-41032 (M.J.M.) and a grant from the Alliance for Lupus Research (to M.J.M.).

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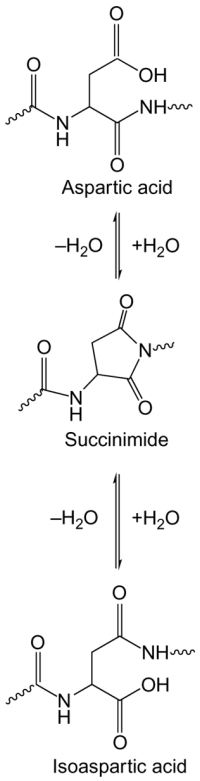

Figure 1.
Structure and formation of isoaspartic acid. Initial nucleophilic attack of the peptide bond nitrogen leads to the formation of a succinimide intermediate, which after hydrolysis under physiological conditions yields isoaspartic acid.

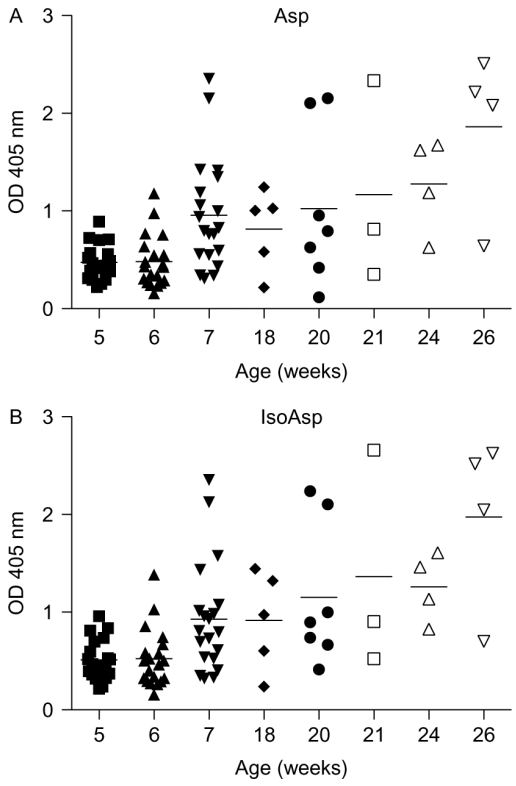

Figure 2.

MRL *lpr* sera contain high titers of antibodies that react against Asp and isoAsp $H2B_{21–35}$. Sera from MRL *lpr* mice were diluted 1:1000 and IgG against (A) Asp and (B) isoAsp $H2B_{21–35}$ measured by ELISA. Horizontal line represents the mean. Results represent 4–20 mice per time point.

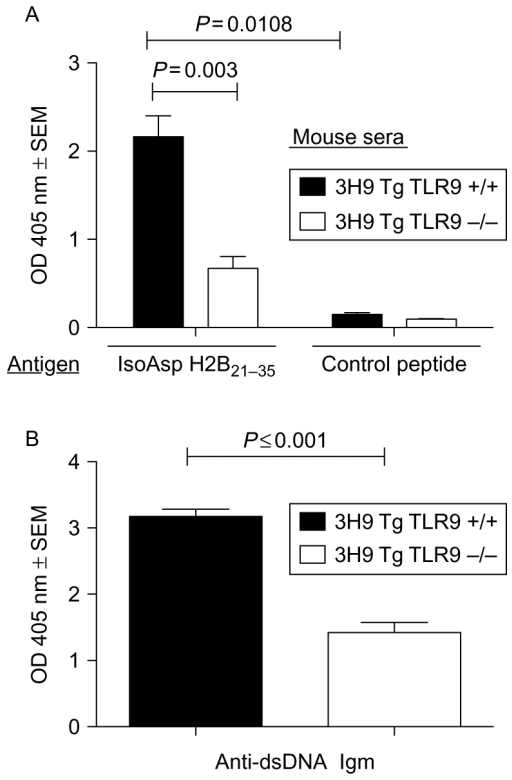

Figure 3.

3H9 Tg MRL *lpr* mice have IgM antibodies against isoAsp $H2B_{21–35}$ that are regulated by TRL9. Sera from 3H9 Tg MRL *lpr* TLR9 wild-type or knockout mice were diluted 1:1000 and IgM against (A) isoAsp $H2B_{21–35}$ or a control peptide and (B) dsDNA measured by ELISA. Mice were 17–20 weeks of age with results representing 4 mice per group. Statistics were calculated with a 2-tailed Students *t*-test.

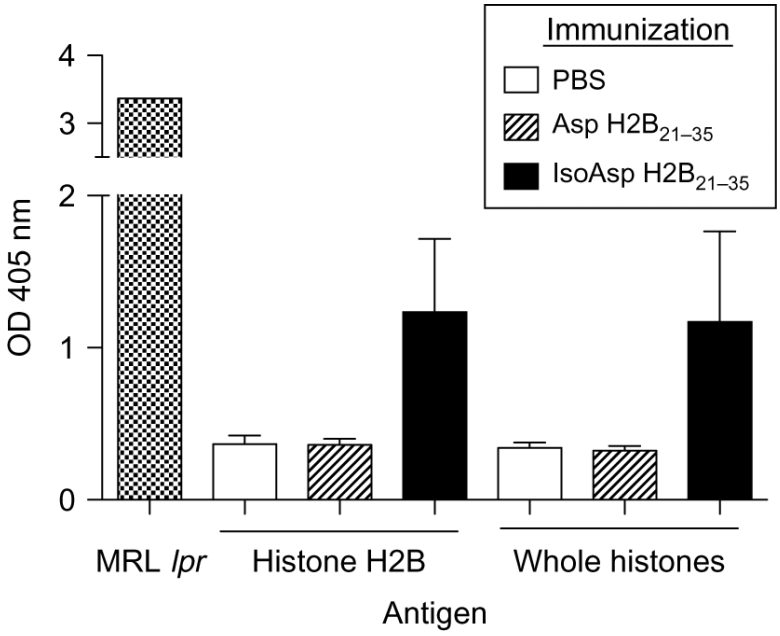

Figure 4.

Mice immunized with isoAsp $H2B_{21-35}$ develop antibodies against histone H2B and whole histones. Sera from B10.A mice immunized with either Asp $H2B_{21-35}$, isoAsp $H2B_{21-35}$ or injected with PBS-CFA were diluted 1:100 and IgG against histone H2B and whole histones measured by ELISA. Sera from MRL *lpr* mice served as a positive control. Results represent four mice per group.

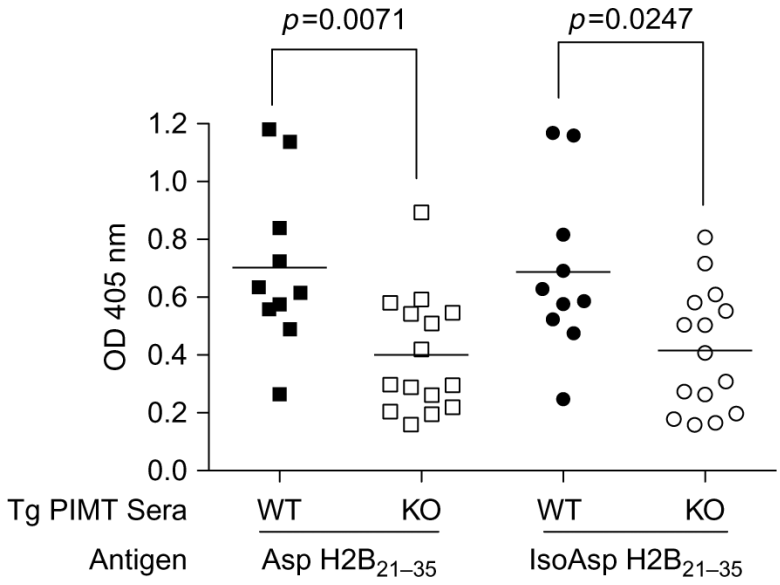

Figure 5.

Mice unable to repair isoAsp have reduced levels of antibody against $H2B_{21–35}$. Sera from mice lacking the isoAsp repair enzyme PIMT (Tg PIMT knockout) or Tg PIMT wild-type mice were diluted 1:100 and used to measure IgG against both isoforms of $H2B_{21–35}$ by ELISA. Horizontal line represents the mean. Results represent 10–15 mice per group. Statistics were calculated using the Mann-Whitney *U*-test.

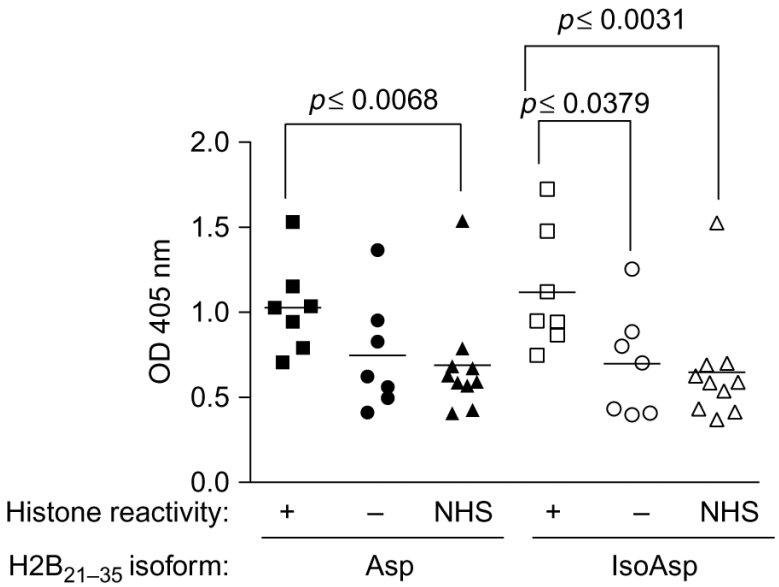

Figure 6.

Sera from individuals who have antibodies against histones react against Asp and isoAsp $H2B_{21–35}$. Sera (1:100 dilution) from histone antibody positive individuals, histone negative individuals and normal healthy individuals (NHS) were analyzed for IgG responses to both Asp and isoAsp $H2B_{21–35}$ by ELISA. Results are representative of 7–10 individuals per group. Experiment was repeated 3 times. Statistics were calculated by the Mann-Whitney *U*-test.

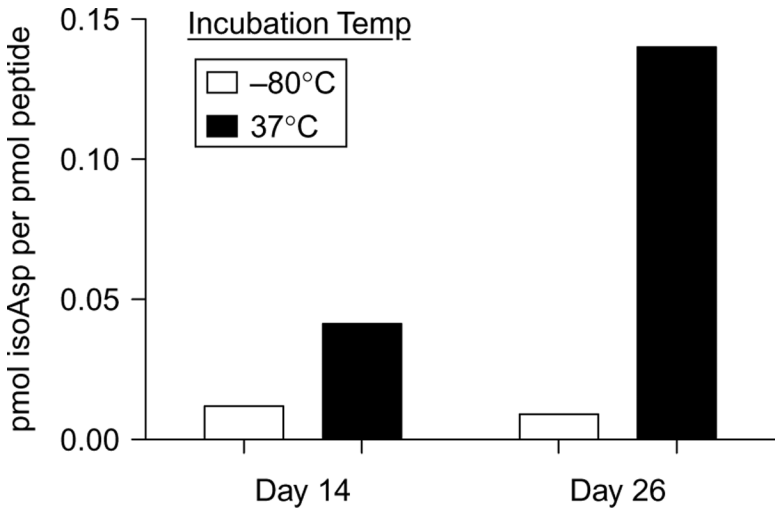

Figure 7.

Asp $H2B_{21–35}$ undergoes spontaneous isomerization at Asp^{25}. The isoAsp content of Asp $H2B_{21–35}$ was determined using the ISOQUANT Isoaspartate Determination kit after incubation in PBS pH 7.4 at 37°C for either 14 or 26 days. Asp $H2B_{21–35}$ stored at – 80° C was used to determine the baseline amount of isoAsp within the peptide.