Intracellular Protein Modification Associated with Altered T Cell Functions in Autoimmunity[1]

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Posttranslational protein modifications influence a number of immunologic responses ranging from intracellular signaling to protein processing and presentation. One such modification, termed isoaspartyl (isoAsp), is the spontaneous nonenzymatic modification of aspartic acid residues occurring at physiologic pH and temperature. In this study, we have examined the intracellular levels of isoAsp residues in self-proteins from MRL$^{+/+}$, MRL/*lpr*, and NZB/W F_1 mouse strains compared with nonautoimmune B10.BR mice. In contrast to control B10.BR or NZB/W mice, the isoAsp content in MRL autoimmune mice increased and accumulated with age in erythrocytes, brain, kidney, and T lymphocytes. Moreover, T cells that hyperproliferate to antigenic stimulation in MRL mice also have elevated intracellular isoAsp protein content. Protein L-isoaspartate *O*-methyltransferase activity, a repair enzyme for isoAsp residues in vivo, remains stable with age in all strains of mice. These studies demonstrate a role for the accumulation of intracellular isoAsp proteins associated with T cell proliferative defects of MRL autoimmune mice. *The Journal of Immunology,* **2006, 177: 4541–4549.**

Many posttranslational modifications of cellular proteins are often critical to their biologic functions. Some modifications can significantly compromise the biologic function of proteins and cellular metabolism. Moreover, we and others have shown that modifications also alter the immunogenicity of such proteins and peptides and create new self-Ags, which induce autoimmune responses and/or tissue pathology (1, 2). Indeed, a number of posttranslational modifications are associated with autoimmune disease. For example, hydroxylation and glycosylation of type II collagen can generate T cell determinants that are specifically recognized by distinct T cell subsets in rodent models of arthritis (3, 4). Human myelin basic protein, an autoantigen in multiple sclerosis, has been shown (5) to consist of a number of charge isomers generated by deamidation, phosphorylation, methionine sulfoxide, and deimination. Similarly, autoimmune responses characteristic of systemic lupus erythematosus (SLE)[3] can be elicited with an isoaspartyl (isoAsp)-modified form of self-Ag, cytochrome *c*, and small nuclear ribonucleoprotein particles (snRNP) (6).

L-Asparagine and L-aspartate are among the most labile amino acids subject to spontaneous degradation reactions. isoAsp formation arises spontaneously from an isomerization of Asp residues or deamidation of Asn residues under physiologic conditions of both pH and temperature (7–11). The existence of such abnormal residues can significantly change protein structure and compromise function as has been identified for epidermal growth factor, calmodulin, bacterial phosphocarrier protein (Hpr), Alzheimer's β-amyloid protein, prion protein, tissue plasminogen activator, calbindin, and others (12–19). To keep the structural and functional integrity of proteins, the widely distributed protein L-isoaspartate *O*-methyltransferase (PIMT) catalyzes the repair of isoAsp proteins (20).

The role of isoAsp modifications in SLE was examined in a PIMT-deficient mouse model. It was found that PIMT-deficient mice have 2- to 6-fold higher levels of isoAsp residues in their brain, heart, liver, erythrocytes, thymus, spleen, and lymph nodes than are observed in wild-type tissues (21, 22). Moreover, wild-type mice reconstituted with PIMT$^{-/-}$ bone marrow develop high titers of anti-DNA autoantibodies and kidney pathology typical of that found in SLE (22). This observation supports the notion that this spontaneous protein modification may be an important feature of lymphocyte function in autoimmunity.

There have been a number of defects described in lymphocytes from patients with SLE. In particular, enhanced TCR/CD3-mediated T cell proliferation was found from patients with SLE (23) and in lupus-prone mice (24, 25). In an analogous manner, a lower threshold of T cell activation and higher threshold for apoptotic death were found in lupus-prone T cells (25, 26). Relevant to the present study, nonautoimmune strains of mice deficient in PIMT have hyperreactive T cells virtually identical to those observed in human SLE and in murine lupus models (22). Taken together, these studies implicate isoAsp modifications in altered functions of lymphocytes in a manner that may affect the expression of disease. The present study was undertaken to identify the roles, whether any, of isoAsp-modified intracellular proteins in a spontaneous murine model of lupus autoimmunity.

Herein, we demonstrate that the amount of isoAsp-modified proteins are significantly higher and accumulate with age in erythrocytes, brain, kidney, and T cells from MRL lupus-prone mice compared with levels observed in normal mice. In contrast, the specific activity of the isoAsp repair enzyme PIMT is similar and remained

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; isoAsp, isoaspartyl; PIMT, protein L-isoaspartate *O*-methyltransferase; snRNP, small nuclear ribonucleoprotein particle; PCC, pigeon cytochrome *c*; CD62L, CD62 ligand.

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stable with age in both normal and lupus-prone mice. The levels of isoAsp-modified protein increased dramatically in lupus prone $CD4^+$ T cells that spontaneously activate in vivo or are stimulated with Ag in vitro. No PIMT genetic polymorphisms are evident in lupus-prone vs normal mice that may influence PIMT function in either strain. Taken together, our study identifies an increased production of intracellular isoAsp modifications in lupus-prone animals contributing to the onset of autoimmune B and T cell responses and autoimmune pathology in the MRL model of lupus.

Materials and Methods

Mice

C57BL/6, B10.BR, B10.A, MRL/Mp ($MRL^{+/+}$), MRL/Mp-Fas^{lpr} (MRL/*lpr*), and New Zealand Black/White F_1 hybrid (NZB/W F_1) mice were obtained from The Jackson Laboratory. AND-transgenic mice expressing an $\alpha\beta$ TCR ($V\alpha11^+$, $V\beta3^+$), which recognizes the aa 88–104 of pigeon cytochrome *c* (PCC 88–104) were maintained and screened as described previously (27). All animals were bred and maintained in specific pathogen-free facilities at the Yale Animal Resources Center.

Preparation of mouse tissue homogenates, erythrocyte lysates, and cell lysates

Brain and kidney were removed immediately from sacrificed animals and placed in ice-cold buffer (5 ml/g wet weight) containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM disodium EDTA (pH 7.4), and the protease inhibitor PMSF (25 μM). The tissues were disrupted in a glass homogenization tube and centrifuged at 20,800 $\times$ *g* for 10 min. The resulting supernatant fractions contained both cytosolic proteins and microsomes (21).

Whole blood (100–200 μl) collected at the indicated time points was combined with 200 μl of 2 mg/ml disodium EDTA, 10 mM sodium phosphate, and 137.9 mM sodium chloride (pH 7.4). Pelleted and washed erythrocytes were lysed in 5 volumes of 5 mM sodium phosphate, 1 mM disodium EDTA (pH 7.4), and 25 μM PMSF. The lysates were centrifuged in 1.5-ml conical tubes at 20,800 $\times$ *g* for 10 min to remove the membrane ghosts (21).

Cell lysates were prepared by freezing and thawing cells at a concentration of 2×10^8 cells/ml in a buffer consisting of 10 mM sodium phosphate (pH 7.2), 1 mM EDTA, 100 mM NaCl, and 0.2% Triton X-100 as previously described (28).

Protein assay

A modified Lowry assay was used to determine protein concentrations in the extracts by the DC Protein Assay (Bio-Rad). Samples were assayed in duplicate with BSA as a concentration standard.

Measurement of PIMT activity

Either 30 μg (20 μl) of brain and kidney extract protein, 40 μg of cell lysate, or 0.6–1 mg of erythrocyte lysate was incubated with 10 μl of 5.45 μM isoAsp-DSIP (ISOQUANT isoaspartate detection kit; Promega), 10 μl of 0.1 mM *S*-adenosyl-L-methionine (1 μCi [*methyl*-^{3}H]*S*-adenosyl-L-methionine; 10–15 Ci/mmol; Amersham Biosciences), and 10 μl of 5$\times$ reaction buffer (0.5 M sodium phosphate (pH 6.8), 5 mM EGTA, 0.8% Triton X-100, and 0.02% NaN_3) at 30°C for 30 min. The enzyme reaction was stopped and the reaction mixture was immediately spotted onto a sponge insert in a scintillation vial cap and incubated with scintillation mixture (Ready-Safe; Beckman Coulter). After incubation, the sponge insert was removed, and the diffused [^{3}H]methanol was measured for radioactivity. The radioactivity of the blank was subtracted from the total for the determination of methyltransferase activity. Specific activity was presented as picomoles of methyl groups transferred to isoAsp-DSIP per minute per milligram of protein. Data represent the average $\pm$ SD for at least five individual samples.

Measurement of isoAsp residues

The amount of isoAsp in erythrocyte lysate (each 0.6–1 mg), tissue extracts (each 30 μg), or cell lysates (each 15 μg) was measured with a commercial-ISOQUANT isoaspartate detection kit according to the manufacturer's protocol (provided by Promega).

T and B cell purification

Pooled axillary, inguinal, brachial, and popliteal lymph nodes, and spleens from age-matched female mice were used for purification of B cells by positive selection with CD19 microbeads T cells were purified by negative selection using MHC class II and CD11b microbeads in the MACS system (Miltenyi Biotec). The resulting B and T cell populations were >95% pure as determined by flow cytometry.

$CD4^+$ T cells were isolated by negative selection using anti-CD8 (clone 56-6.7), anti-CD45R/B220 (RA3-6B2), anti-CD16/32 (2.4G2), anti-$I\text{-}E^k$ (14–4-4S), anti-CD11b (M1–70), anti-$\gamma\delta$ (GL3) biotinylated Abs (BD Pharmingen), and streptavidin microbeads (Miltenyi Biotec). In some experiments, the purified $CD4^+$ T cells were stained with allophycocyanin-conjugated anti-CD4 (RM4-5), FITC-conjugated anti-CD44 (IM7), and PE-conjugated anti-CD62 ligand (CD62L, MEL-14) for sorting the naive ($CD44^{low}CD62L^{high}$), activated ($CD44^{high}CD62L^{high}$), and memory ($CD44^{high}CD62L^{low}$) $CD4^+$ T cells subsets using flow cytometry (FACS Aria; BD Biosciences). Typical purities as determined by sorting were >98%.

$CD4^+$ T cell proliferation assays

Cells were cultured in Click's medium containing 5% FCS, 2 mM L-glutamine, 0.1 mM 2-ME, and antibiotics (100 U/ml penicillin/streptomycin and 50 μg/ml gentamicin). In brief, purified $CD4^+$ T cells (2×10^5) and mitomycin C-treated CH 27 B lymphoblastoid cells (4×10^5) were plated in 96-well flat-bottom microtiter plates with PCC 88–104. Cells were incubated for 48 h, after that the cells were pulsed with 1 μCi [^{3}H]thymidine (Valeant Pharmaceuticals) for 18 h before being harvested onto filters with a semiautomatic cell harvester. Radioactivity was counted with a Betaplate liquid scintillation counter (Wallac).

Measurement of homocysteine concentration

The amount of total homocysteine in heparinized plasma was measured with a Homocysteine Microplate Assay kit according to the manufacturer's protocol (Diazyme Laboratories). The assay allows accurate measurement of 2.5–60 μM homocysteine from plasma samples.

Statistical analysis

All statistics were performed using a Student's unpaired two-tailed *t* test. A value of $p < 0.05$ was regarded as significant.

Results

Self-proteins are highly isoAsp modified in erythrocytes from lupus-prone mice and accumulate over time in vivo

We have previously (22) demonstrated that T cells from normal strains of mice unable to repair intracellular isoAsp proteins hyperproliferate and elicit lupus-like autoimmunity. To examine the role of isoAsp proteins in spontaneous autoimmune disease, erythrocytes were first used to screen the amounts of isoAsp residues in $MRL^{+/+}$, MRL/*lpr*, and NZB/W F_1 lupus-prone mice and in $H\text{-}2^k$-matched B10.BR mice, as a nonautoimmune strain. As shown in Fig. 1*A*, isoAsp concentrations in 8- to 10-wk-old $MRL^{+/+}$ erythrocytes were significantly enhanced (1.6-fold increase) compared with B10.BR nonautoimmune mice. By comparison, the isoAsp concentration in B10.BR erythrocytes was not only significantly lower but also did not change between the ages of 8 and 22 wk. In contrast, isoAsp levels increased over the same time period in $MRL^{+/+}$ mice. In a similar manner, the isoAsp levels were also higher in MRL/*lpr* compared with B10.BR mice in the same time span and accumulated over time (Fig. 1*A*). Although Fas-mutated MRL/*lpr* mice exhibit earlier and more severe lupus-like symptoms than Fas-intact $MRL^{+/+}$ mice, the amounts of isoAsp residues were not different between $MRL^{+/+}$ and MRL/*lpr* mice at any of the time points examined. This observation suggests that isoAsp formation was not affected in the absence or presence of Fas protein, but instead is linked to the expression of lupus autoimmunity.

PIMT enzyme activity in erythrocytes is similar between normal and lupus-prone mice and remains stable over time

It is believed that PIMT-catalyzed carboxyl methylation of isoAsp sites is the major mechanism in either the repair or degradation of damaged self-proteins. It was, therefore, reasonable to assume that altered isoAsp levels in cellular proteins may be due to aberrant

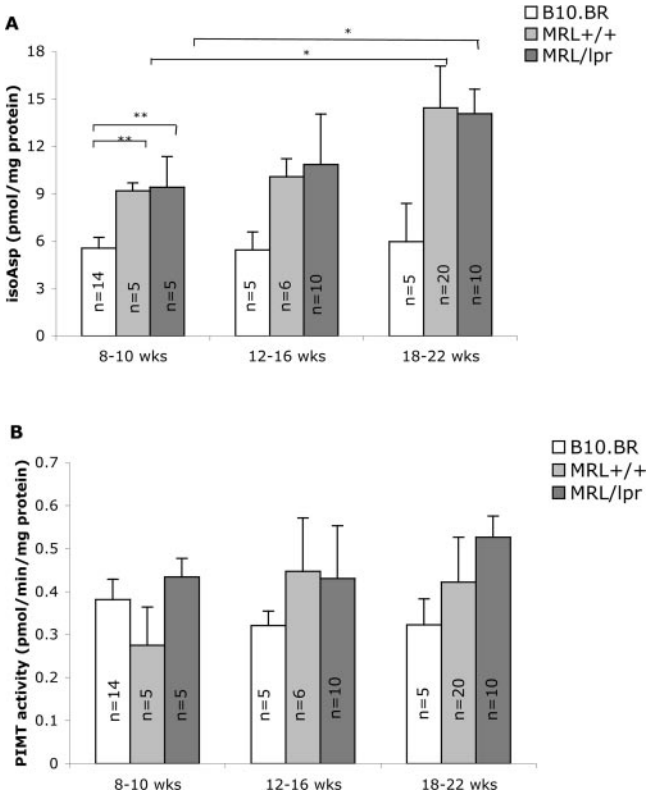

FIGURE 1. Concentration of isoAsp residues are higher in erythrocytes of lupus-prone mice and become elevated with age, but PIMT activity remains stable in both normal and lupus-prone mice. *A*, L-isoAsp residues were measured in erythrocyte lysates prepared from B10.BR, MRL$^{+/+}$, and MRL/*lpr* mice of different ages, as indicated. *B*, Erythrocyte lysates from different ages of the indicated mouse strains were assayed for PIMT-specific activity. *A* and *B*, The number of mice analyzed is indicated at the bottom of each bar and the error bars indicate SD of the mean. *, $p < 0.001$; **, $p < 0.0001$.

repair mechanisms of PIMT in MRL mice. Next, we evaluated the PIMT repair enzyme activity in erythrocytes from B10.BR and lupus-prone mice of different ages. As shown in Fig. 1*B*, we found no statistically significant difference in PIMT enzyme activity between these strains and at any of the ages examined. Although a trend of increased PIMT activity appears in MRL mice with age, no quantitative significance exists and no reported inducible increases in PIMT activity have ever been described. Therefore, the accumulation of isoAsp proteins in MRL mice was not due to the inability of PIMT to efficiently repair protein modifications. Possibly, the normal PIMT activity cannot keep up with the overproduction or decreased removal of intracellular isoAsp proteins.

In repairing isoAsp proteins, PIMT converts the methyl donor AdoMet to *S*-adenosylhomocysteine and then to adenosine and homocysteine. Extracellular homocysteine levels in the serum are an indirect reflection of methyl transferase reactions within the cell. Indeed, homocysteine is a known powerful in vivo inhibitor of methyltransferase reactions (29). Therefore, we next asked whether plasma homocysteine concentrations were altered in lupus-prone mice. As shown in Fig. 2, the plasma homocysteine concentration was not significantly different between 8- and 10-wk-old B10.BR and MRL$^{+/+}$ or MRL/*lpr* mice. However, the plasma homocysteine concentration was significantly lower in older lupus-prone mice compared with B10.BR mice. This difference was observed at all ages beyond 14 wk. Therefore, the altered isoAsp levels are not likely due to the inhibition of methyltransferase by elevated levels of homocysteine.

There are three known single nucleotide polymorphisms in the human PIMT gene identified on exons 2, 5, and 7a. One of these polymorphisms results in two major isoforms with either an Ile residue or a Val residue at amino acid position 119 (30). Analysis of the enzymatic activities of the position 119 variants show that the Ile^{119} isoform is associated with higher specific activity and thermostability, whereas the Val^{119} isoform is associated with higher substrate affinity (31). PIMT is a highly conserved protein repair enzyme in both prokaryotes and eukaryotes (32). However, no detectable polymorphisms or mutations were found in these coding regions from five separate strains of mice, including three normal mice strains (C57BL/6, B10.BR, and B10.A) and two strains of lupus-prone mice (MRL$^{+/+}$ and MRL/*lpr*; data not shown). This suggested that the increased isoAsp-modified protein content in cells from lupus-prone mice (Fig. 1*A*) was not due to decreased PIMT enzyme activity (Fig. 1*B*), higher content of methyltransferase inhibitor (Fig. 2), or from PIMT gene polymorphisms within sequences from exons 2, 5, or 7.

Accumulation of isoAsp-modified protein in brain and kidney from older MRL$^{+/+}$ mice

PIMT enzyme activity is highest in testis, brain, and heart with lower activity found in kidney, erythrocytes, and liver (21). The highest amounts of isoAsp-modified proteins are found in brains of

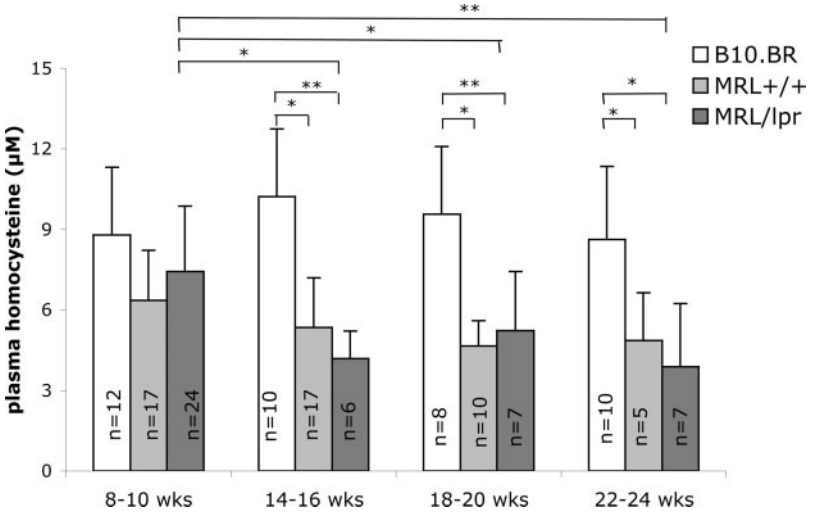

FIGURE 2. Plasma concentration of homocysteine is decreased with age in lupus-prone mice. Homocysteine levels (μM) were measured in heparinized plasma collected from B10.BR, MRL$^{+/+}$, and MRL/*lpr* mice of different ages. The number of mice analyzed is indicated at the bottom of each bar and the error bars indicate SD of the mean. *, $p < 0.01$; **, $p < 0.001$.

Table 1. *Amount of isoAsp residues and PIMT activity in brain and kidney of B10.BR and MRL$^{+/+}$ mice of different ages*[a]

Tissue Extract	isoAsp Residue[b] (pmol/mg protein)		PIMT Activity[c] (pmol/min/mg protein)	
	8- to 10-wk-old	18- to 20-wk-old	8- to 10-wk-old	18- to 20-wk-old
B10.BR brain	329.45 ± 57.94	335.28 ± 65.9	195.9 ± 42.27	199.87 ± 18.62
B10.BR kidney	147.58 ± 22.82	172.04 ± 23.07	122.02 ± 15.03	137.91 ± 28.47
MRL$^{+/+}$ brain	300 ± 50.4	372.95 ± 16.27[d]	209.93 ± 11.37	206.8 ± 14.99
MRL$^{+/+}$ kidney	137.49 ± 34.61	200.95 ± 14.68[d]	161.59 ± 33.5	156.08 ± 47.8

[a] $n = 5$.
[b] L-isoAsp residues were measured in tissue extracts prepared from different ages of B10.BR and MRL$^{+/+}$ mice.
[c] Brain and kidney extracts from mice of different ages of the indicated mouse strains were assayed for PIMT activity.
[d] $p < 0.05$.

PIMT$^{-/-}$ mice (21). Coincidentally, the kidney is one of the major target organs of autoimmune pathology in SLE. We hypothesized that brain and kidney might be the most sensitive organs for accumulating isoAsp-modified proteins in lupus-prone mice. As shown in Table I, the isoAsp-modified proteins from brain and kidneys accumulated over time in MRL$^{+/+}$ mice. Importantly, there are no apparent differences in brain or kidney isoAsp levels between B10.BR and MRL in aged mice (18–20 wk). This observation suggests that simple increases in tissue isoAsp do not reflect the pathology found (or lack of pathology) in these tissues. In a similar manner as observed in erythrocytes (Fig. 1*B*), PIMT enzyme activity remained stable in brain and kidney from B10.BR and MRL$^{+/+}$ mice over time (Table I).

SLE is marked by the appearance of autoantibodies to a variety of intracellular macromolecules including nucleosomes, dsDNA, and ribonucleoproteins. Therefore, we next determined whether the existence of antinuclear Abs and anti-snRNP Ab were coincident with the higher levels of isoAsp modifications. Sera from 18- to 20-wk-old MRL$^{+/+}$ mice showed positive antinuclear Ab responses by indirect immunofluorescence and production of anti-snRNP autoantibodies as determined by ELISA (data not shown).

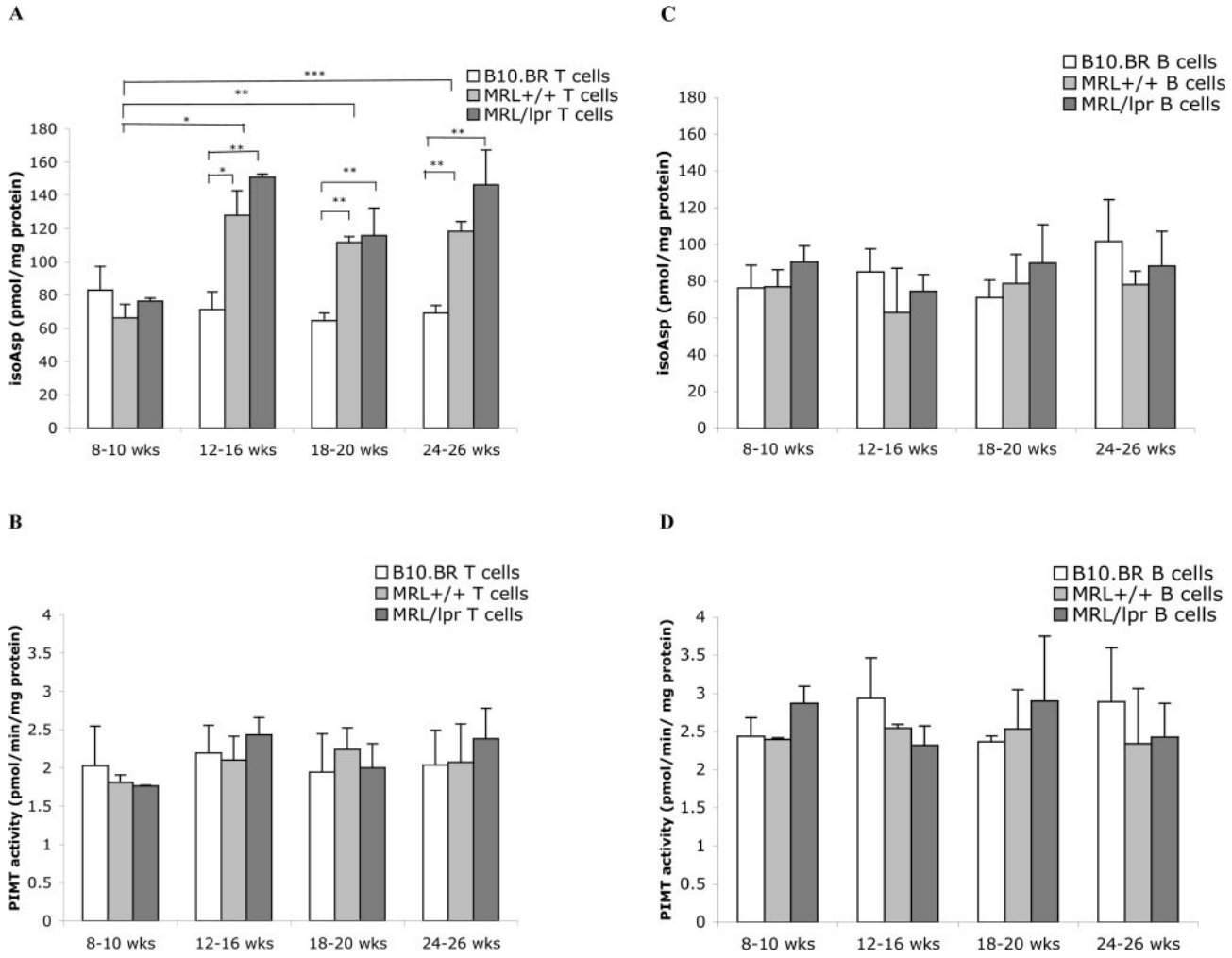

FIGURE 3. Concentration of intracellular isoAsp residues are elevated in T cells of lupus-prone MRL mice but are unchanged in B cells. The purified T cells (*A* and *B*) and B cells (*C* and *D*) from different ages of B10.BR, MRL$^{+/+}$, and MRL/*lpr* mice were assayed for the amount of isoAsp residues (*A* and *C*) and PIMT-specific activity (*B* and *D*). Error bars, SD of the mean ($n = 3$ at least for each bar). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Accumulation of intracellular isoAsp-modified proteins with age in lupus-prone T cells

Next, we examined the intracellular isoAsp content in purified T and B cells from lupus-prone mice. As illustrated in Fig. 3*A*, significant differences in intracellular isoAsp were observed beginning at 12 wk of age and at all later time points examined. Levels appeared to plateau at 12–16 wk of age and did not change throughout the life of the animal. There was not a significant difference in the amount of isoAsp residues between Fas-intact MRL$^{+/+}$ T cells and Fas-mutated MRL/*lpr* T cells. Interestingly, the increase in isoAsp content occurred simultaneously with the presence of spontaneous activation of MRL CD4$^+$ T cells at 12–16 wk of age as previously reported (33). As found in Fig. 3*C*, there was no apparent difference in isoAsp modification between lupus-prone B cells and normal B cells throughout the course of the study. Finally, PIMT enzyme activity in T and B cells of normal and lupus-prone mice remained stable between 8 and 26 wk of age (Fig. 3, *B* and *D*). However, there is no difference in either isoAsp modification or PIMT enzyme activity in another lupus-prone mouse, NZB/W F_1, from erythrocytes, brain, kidney, or T cells (Table II).

Increase of isoAsp-modified proteins upon receptor-mediated or antigenic stimulation of lupus-prone T cells

Given the association of spontaneous T cell activation with the rise in intracellular isoAsp content in vivo, we addressed the question of whether or not the accumulation of intracellular isoAsp in T cells was effected by TCR engagement in vitro. As shown in Fig. 4*A*, the intracellular isoAsp-modified proteins in stimulated T cells following anti-CD3 and anti-CD28 mAb treatment were not significantly increased compared with unstimulated T cells isolated from young (8–10 wk) or older (18–20 wk) B10.BR mice. In contrast, the isoAsp-modified proteins were significantly increased in stimulated MRL/*lpr* T cells isolated from young or older mice (Fig. 4*B*).

T cells were then stimulated by PMA and ionomycin to help distinguish the signaling molecules important in isoAsp-mediated stimulation in MRL/*lpr* T cells. The amounts of isoAsp-modified proteins were increased in stimulated B10.BR and MRL/*lpr* T cells by PMA and ionomycin (Fig. 4*C*). This result suggests a TCR proximal signaling defect that leads to the increase of isoAsp modification of MRL-prone T cells upon TCR activation.

Vratsanos and colleagues (25) reported that CD4$^+$ T cells from MRL lupus-prone mice have a lower threshold of activation in response to Ag, a feature also found in CD4$^+$ T cells from PIMT-deficient mice (22). Therefore, we investigated the influence of Ag stimulation of CD4$^+$ T cells isolated from AND-transgenic mice on isoAsp accumulation. MRL$^{+/+}$.AND and MRL/*lpr*.AND CD4$^+$ T cells proliferated significantly more than B10.AND CD4$^+$ T cells in response to stimulation with PCC 88–104 in both young (8–10 wk) (Fig. 4*D*) and in older mice (data not shown). Similar to Fig. 4*B*, the intracellular isoAsp content was elevated in PCC 88–104-stimulated lupus-prone transgenic CD4$^+$ T cells compared with unstimulated cells isolated from young (8–10 wk; data not shown) and old mice (18–20 wk; Fig. 4*E*). Together, the intracellular isoAsp content increases significantly in autoimmune-prone CD4$^+$ T cells stimulated with Ag. Conversely, nonautoimmune prone T cells repair isoAsp modifications efficiently such that increased modifications were not observed.

*The increase of isoAsp content occurs in spontaneously activated MRL/*lpr *CD4$^+$ T cells in vivo*

The age-related increase of spontaneous T cell activation is a character in MRL-prone mice (33). We examined whether the increased isoAsp content in cultured activated MRL-prone T cells also occurred in vivo. As shown in Fig. 5 and Table III, naive (CD44^{low}CD62L^{high}), CD4$^+$ T cells were the major population (77.9 ± 3.7%) in 10-wk-old B10.BR mice. In contrast, 10-wk-old MRL/*lpr* mice experience spontaneous activation of CD4$^+$ T cells. This activation is apparent by the analysis of ex vivo-purified CD4$^+$ T cells; naive (23.5 ± 7.8%), activated (28.1 ± 2.3%), and memory (42.7 ± 5.6%) population. isoAsp content was assessed in purified naive, activated, and memory CD4$^+$ T cells. Interestingly, the intracellular isoAsp content was increased in spontaneous activated CD4$^+$ T cells (102.95 ± 14.21 pmol/mg protein) as compared with naive CD4$^+$ T cells (68.75 ± 2.19 pmol/mg protein) in MRL/*lpr* mice. isoAsp content was diminished in memory cells in MRL/*lpr* mice (79.17 ± 0.24 pmol/mg protein), although the levels were still greater than those found in naive CD4$^+$ T cells. The lack of significant numbers of spontaneously activated T cells in wild-type B10.BR mice precluded our ability to measure isoAsp content in these populations. As another approach, we first activated T cells from B10.BR mice through their TCRs with anti-CD3 and anti-CD28. Naive, activated, and memory populations were then purified as above with MRL T cells. As illustrated in Table IV, these three populations do not differ in intracellular isoAsp content from B10.BR mice. As discussed below, this observation demonstrates that the process of T cell activation itself does not trigger an increase in isoAsp content. In contrast, it supports the observation that the isoAsp increase is a primary defect in T cell activation in MRL mice.

Discussion

The mechanisms by which most posttranslational protein modifications control and/or influence autoimmune responses are not clearly understood. However, two areas, in particular, include 1) the role of protein modifications in biochemical processing and presentation of Ags and 2) the influence of intracellular posttranslational modifications in lymphocyte development and proliferation. The present study demonstrates that isoAsp protein modification is significantly increased in the cells and tissues of the lupus-prone MRL strain of mice as compared with nonautoimmune mouse strains. The increase in isoAsp modification is coincident with aberrant T cell hyperproliferation, the appearance of

Table II. *Amount of isoAsp residues and PIMT activity in NZB/W F_1 mice of different ages*

	isoAsp Residue[a] (pmol/mg protein)		PIMT Activity[a] (pmol/min/mg protein)	
Tissue Extract	8- to 10-wk-old	16- to 18-wk-old	8- to 10-wk-old	16- to 18-wk-old
Erythrocytes	6.10 ± 2.70	5.84 ± 1.28	0.33 ± 0.05	0.35 ± 0.05
Brain	317.8 ± 61.13	311.7 ± 27.79	185.14 ± 28.14	193.33 ± 23.47
Kidney	159.2 ± 27.16	135.67 ± 19.54	107.57 ± 12.01	132.83 ± 30.71
T cells	74.34 ± 8.06	66.97 ± 7.5	2.71 ± 0.72	2.46 ± 0.71

[a] $n = 5$.

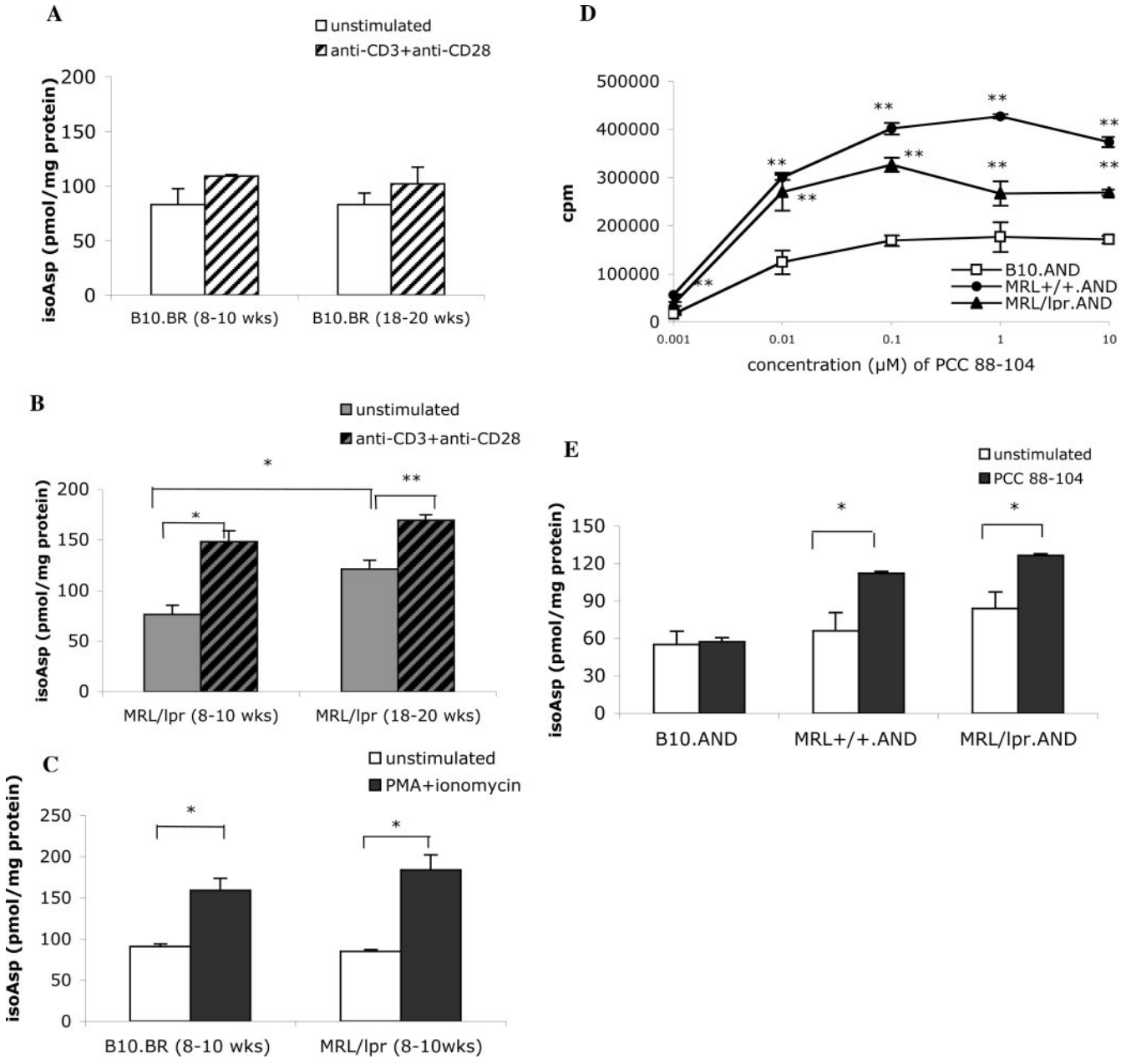

FIGURE 4. Concentration of intracellular isoAsp residues is elevated in T cells of lupus-prone mice after TCR and antigenic stimulation. isoAsp residues were measured in either unstimulated T lymphocyte lysates and in anti-CD3 (10 μg/ml) plus anti-CD28 mAb (1 μg/ml; *A* and *B*) or PMA (50 ng/ml) plus ionomycin (1 μg/ml; *C*)-stimulated T cell lysates from young and older B10.BR (*A*) or MRL/*lpr* (*B*) mice. *D*, $CD4^+$ AND-transgenic T cells from 8- to 10-wk-old mice were stimulated in vitro by CH27 APCs pulsed with varying concentrations of PCC 88–104. Proliferation was measured at 72 h by [^{3}H]thymidine incorporation. **, $p < 0.005$ for comparison of MRL$^{+/+}$.AND or MRL/*lpr*.AND to B10.AND cells. *E*, Control $CD4^+$ T cells purified from 18- to 20-wk-old mice were used as unstimulated $CD4^+$ T cells. As control activated cells, purified $CD4^+$ T cells were stimulated by 10 mM PCC 88–104 in the presence of CH27 cells for 2 days. isoAsp residues were measured in each cell population. The error bars indicate SD of the mean ($n = 3$ for each bar). *, $p < 0.05$; **, $p < 0.005$.

systemic autoimmunity, both autoantibodies and T cell autoimmunity, and with lupus pathology. Our data are consistent with the accelerated production of intracellular isoAsp proteins, in that the repair mechanisms using PIMT are normal in lupus-prone mice.

isoAsp modifications are common inhabitants of cells and arise at physiologic pH and temperature (reviewed in Refs.7, 8, and 20). As extensively reported elsewhere, isoAsp residues are the product of the isomerization of Asp or of the deamidation of Asn within the cell. isoAsp modification occurs most frequently at amino acid sequences bearing Asp-Gly, Asn-Gly, and Asn-Ser and is known to be increased in ageing cells and cells that undergo various forms of stress, such as heat shock.

We found that the amount of intracellular isoAsp proteins did not change significantly in stimulated B10.BR T cells but increased in stimulated MRL-prone T cells by anti-CD3 and anti-CD28 mAb treatment. However, the isoAsp modification was increased in PMA- and ionomycin-treated B10.BR and MRL-prone T cells. Our previous studies (22) in normal mouse strains demonstrated that an inability to repair intracellular isoAsp proteins leads to T cell hyperproliferation through the TCR and lupus-like

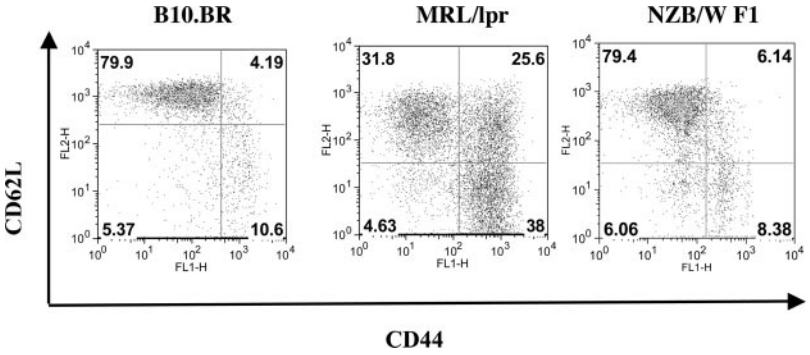

FIGURE 5. Spontaneous $CD4^+$ T cell activation in MRL/*lpr* mice. Pooled spleen and lymph node cells were analyzed via three-color FACS from 10-wk-old B10.BR, MRL/*lpr*, and NZB/W F_1 mice. $CD4^{high}$ gated cells were analyzed for CD44 (FITC) and CD62L (PE) expression.

autoimmunity and $PIMT^{-/-}$ T cells proliferated in the same manner as wild-type T cells stimulated with PMA and ionomycin. In anti-CD3- and anti-CD28 mAb-treated $PIMT^{-/-}$ T cells, the proximal signaling pathways including MEK1/2, ERK1/2, ribosomal S6 kinase 1, p70 S6K, protein kinase Bα, and protein kinase Cα were hyperphosphorylated compared with wild-type T cells (22). Similarly, an altered pattern of phosphorylation in protein tyrosine kinase and ERK has also been described (34, 35) in activated T cells from human lupus patients and MRL mice. Taken together, these observations suggest that proximal signaling defects linked to intracellular isoAsp proteins contribute to T cell hyperproliferation in lupus-prone mice.

We first sought to determine whether aberrant isoAsp modifications inhabit cells and tissues in the MRL mouse. Erythrocytes provide an excellent source of cellular proteins for studying nonenzymatic protein modification because de novo protein biosynthesis does not occur, thereby leading to an inability to replace modified self-proteins (36). We found that the amounts of isoAsp proteins isolated from lupus-prone mice were significantly higher compared with normal mice beginning at 8–10 wk of age. isoAsp-modified proteins accumulated with age in lupus-prone mice. It was possible that the increase of isoAsp-modified proteins might result from either the higher production of the damaged proteins or the lower activity of the isoAsp-repairing enzyme PIMT in the MRL mice. Our data support the former hypothesis in that the PIMT enzyme activity in all cells examined did not differ between normal and lupus-prone mice. PIMT in lupus-prone cells is fully active and can repair isoAsp-modified proteins, but likely does not keep up with the rapid production and accumulation of isoAsp-modified proteins in the cell. In support of this conclusion, other investigators have shown (37, 38) that isoAsp modifications were enhanced in cells under oxidative stress or in UV-irradiated human melanoma M14 cells but PIMT activity remained remarkably stable under these treatments.

PIMT is an active methyltransferase in cells, account for a large portion of AdoMet consumption. Farrar and Clarke (39) have demonstrated that higher levels of AdoMet and lower levels of *S*-adenosylhomocysteine were found in the brains of PIMT-deficient mice. Consistent with this observation, we have also found that the plasma homocysteine concentration was decreased in $PIMT^{-/-}$ mice (data not shown) and in lupus-prone mice. Human SLE is marked by increases in plasma homocysteine, an inhibitor of PIMT and other methyltransferase reactions, which may serve to increase intracellular isoAsp content and lead to alter T cell immune responses (40). This possibility is presently under study in our laboratory. We did not detect any genetic polymorphisms in PIMT between lupus-prone and normal strains of mice that may explain differences in the specific activity of the enzyme, as has been observed among normal human populations (30, 31). Finally, we cannot rule out the possibility that the methyl donor for PIMT activity, AdoMet, is limited or altered within MRL cells, thereby altering the repair process of abnormal isoAsp residues.

In both human and murine models of SLE, T cells exhibit hyperresponsiveness to antigenic stimulation (23, 25, 34). Our laboratory has previously shown that features similar to those seen in murine lupus were found in mice lacking the isoAsp repair enzyme PIMT, notably, T cell hyperproliferation upon TCR stimulation (22). As a natural extension of these studies, we have now found that intracellular isoAsp-modified proteins were elevated with age in MRL mice. In contrast, isoAsp content was not elevated in lymphocytes or tissues of NZB/W F_1 lupus-prone mice. Also, there is no spontaneous activation of NZB/W F_1 T cells as is found in MRL mice (Fig. 5).

The age at which elevated isoAsp proteins are found in MRL T cells is coincident with the onset of lupus and with hyperproliferative T lymphocyte functions. isoAsp protein content is greatly elevated until at least 26 wk of age, at which time peak concentrations are maintained throughout the life of the mouse. MRL B cells do not have abnormal accumulations of isoAsp modifications nor do they have known defects in proliferative responses to Ag. These observations indicate that abnormal isoAsp accumulation is not a trait of all hemopoietic cells in MRL mice.

Table III. *isoAsp content is elevated in spontaneously activated T cells from MRL mice*[a]

	Percentages (±SD)		isoAsp Residue (pmol/mg protein ± SD)	
$CD4^+$ T Cell Subsets	B10.BR mouse strain (10-wk-old)	MRL/*lpr* mouse strain (10-wk-old)	B10.BR mouse strain (10-wk-old)	B10.BR mouse strain (10-wk-old)
Naive	77.9 ± 3.7%	23.5 ± 7.8%	71.75 ± 2.89	68.75 ± 2.19
Activated	3.5 ± 0.1%	28.1 ± 2.3%	ND	102.95 ± 14.21
Memory	10.9 ± 0.9%	42.7 ± 5.6%	ND	79.17 ± 0.24

[a] Cell percentages were obtained via FACS analysis for the naive ($CD44^{low}$ $CD62L^{high}$), activated ($CD44^{high}CD62L^{high}$), and memory ($CD44^{high}CD62L^{low}$) subsets as shown in Fig. 5. After sorting by the expression profile of CD44 and CD62L, cell lysates from naive, activated, and memory $CD4^+$ T cells were measured for intracellular isoAsp content. $n = 3$.

Table IV. *The intracellular isoAsp levels are not changed in naive, activated, and memory CD4$^+$ T cells from B10.BR normal mice*[a]

Mouse Strain	CD4$^+$ T Cell Subsets	isoAsp Residue (pmol/mg protein ± SD)
B10.BR	Naive	70.15 ± 5.16
	Activated	78.05 ± 3.18
	Memory	62.3 ± 11.87

[a] CD4$^+$ T cells were purified from 8- to 10-wk-old B10.BR mice and activated with anti-CD3 and anti-CD28 mAb for 2 days in vitro. Naive, activated, or memory CD4$^+$ T cell populations were purified by FACS using CD44 and CD62L expression patterns and followed by the measurement of intracellular isoAsp content.

The roles of isoAsp-modified proteins are beginning to be appreciated in a number of clinical syndromes. For example, isoAsp-modified amyloid protein is known to be a major component in the brain plaques of patients with Alzheimer's disease (41) and isoAsp-modified plasma proteins are increased in uremic patients (42). As a coincidence, PIMT-deficient mice exhibit severe neurologic disorders that are cured by the repair of isoAsp modifications in the brain (43).

Although there are many known explanations for the aberrant hyperproliferative T cell response in lupus, this study links the presence of increased intracellular isoAsp formation with the spontaneous expression of lupus autoimmunity. As described earlier, our studies in PIMT-deficient mice display virtually identical T cell proliferative disorders as well as lupus-like autoimmunity. We believe that the abnormal accumulation of intracellular isoAsp protein modification may be one contributing factor to the initiation and/or perpetuation of autoimmune disease. Our future studies intend to examine how increasing the repair processes of this modification may ameliorate or reverse the phenotype of lupus-prone autoimmune lymphocytes in mice and/or humans.

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Disclosures

The authors have no financial conflict of interest.

References

1. Doyle, H. A., and M. J. Mamula. 2001. Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol.* 22: 443–449.
2. van Boekel, M. A., and W. J. van Venrooij. 2003. Modifications of arginines and their role in autoimmunity. *Autoimmun. Rev.* 2: 57–62.
3. Corthay, A., J. Backlund, and R. Holmdahl. 2001. Role of glycopeptide-specific T cells in collagen-induced arthritis: an example how post-translational modification of proteins may be involved in autoimmune disease. *Annu. Med.* 33: 456–465.
4. Myers, L. K., J. Myllyharju, M. Nokelainen, D. D. Brand, M. A. Cremer, J. M. Stuart, M. Bodo, K. I. Kivirikko, and A. H. Kang. 2004. Relevance of posttranslational modifications for the arthritogenicity of type II collagen. *J. Immunol.* 172: 2970–2975.
5. Pritzker, L. B., S. Joshi, J. J. Gowan, G. Harauz, and M. A. Moscarello. 2000. Deimination of myelin basic protein, 1: effect of deimination of arginyl residues of myelin basic protein on its structure and susceptibility to digestion by cathepsin D. *Biochemistry* 39: 5374–5381.
6. Mamula, M. J., R. J. Gee, J. I. Elliott, A. Sette, S. Southwood, P. J. Jones, and P. R. Blier. 1999. Isoaspartyl post-translational modification triggers autoimmune responses to self-proteins. *J. Biol. Chem.* 274: 22321–22327.
7. Geiger, T., and S. Clarke. 1987. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides: succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* 262: 785–794.
8. Tashima, G., Hancock, W. S., and Canova-Davis, E. 1995. Effect of deamidation and isoaspartate formation on the activity of proteins. In *Deamidation and Isoaspartate Formation in Peptides and Proteins*. D. W. Aswad, ed. CRC, Boca Raton, FL, pp. 167–192.
9. Aswad, D. W., M. V. Paranandi, and B. T. Schurter. 2000. Isoaspartate in peptides and proteins: formation, significance, and analysis. *J. Pharm. Biomed. Anal.* 21: 1129–1136-1136.
10. Lindner, H., and W. Helliger. 2001. Age-dependent deamidation of asparagine residues in proteins. *Exp. Gerontol.* 36: 1551–1563.
11. Clarke, S. 2003. Aging as war between chemical and biochemical processes: protein methylation and the recognition of age-damaged proteins for repair. *Ageing Res. Rev.* 2: 263–285.
12. George-Nascimento, C., J. Lowenson, M. Borissenko, M. Calderon, A. Medina-Selby, J. Kuo, S. Clarke, and A. Randolph. 1990. Replacement of a labile aspartyl residue increases the stability of human epidermal growth factor. *Biochemistry* 29: 9584–9591.
13. Potter, S. M., W. J. Henzel, and D. W. Aswad. 1993. In vitro aging of calmodulin generates isoaspartate at multiple Asn-Gly and Asp-Gly sites in calcium-binding domains II, III, and IV. *Protein Sci.* 2: 1648–1663.
14. Brennan, T. V., J. W. Anderson, Z. Jia, E. B. Waygood, and S. Clarke. 1994. Repair of spontaneously deamidated HPr phosphocarrier protein catalyzed by the L-isoaspartate-(D-aspartate) *O*-methyltransferase. *J. Biol. Chem.* 269: 24586–24595.
15. Shimizu, T., H. Fukuda, S. Murayama, N. Izumiyama, and T. Shirasawa. 2002. Isoaspartate formation at position 23 of amyloid β peptide enhanced fibril formation and deposited onto senile plaques and vascular amyloids in Alzheimer's disease. *J. Neurosci. Res.* 70: 451–461.
16. Sandmeier, E., P. Hunziker, B. Kunz, R. Sack, and P. Christen. 1999. Spontaneous deamidation and isomerization of Asn108 in prion peptide 106–126 and in full-length prion protein. *Biochem. Biophys. Res. Commun.* 261: 578–583.
17. Paranandi, M. V., A. W. Guzzetta, W. S. Hancock, and D. W. Aswad. 1994. Deamidation and isoaspartate formation during in vitro aging of recombinant tissue plasminogen activator. *J. Biol. Chem.* 269: 243–253.
18. Chazin, W. J., J. Kordel, E. Thulin, T. Hofmann, T. Drakenberg, and S. Forsen. 1989. Identification of an isoaspartyl linkage formed upon deamidation of bovine calbindin D9k and structural characterization by 2D 1H NMR. *Biochemistry* 28: 8646–8653.
19. Lanthier, J., A. Bouthillier, M. Lapointe, M. Demeule, R. Beliveau, and R. R. Desrosiers. 2002. Down-regulation of protein L-isoaspartyl methyltransferase in human epileptic hippocampus contributes to generation of damaged tubulin. *J. Neurochem.* 83: 581–591.
20. Clarke, S. 1985. Protein carboxyl methyltransferases: two distinct classes of enzymes. *Annu. Rev. Biochem.* 54: 479–506.
21. Lowenson, J. D., E. Kim, S. G. Young, and S. Clarke. 2001. Limited accumulation of damaged proteins in L-isoaspartyl-(D-aspartyl) *O*-methyltransferase-deficient mice. *J. Biol. Chem.* 276: 20695–20702.
22. Doyle, H. A., R. J. Gee, and M. J. Mamula. 2003. A failure to repair self-proteins leads to T cell hyperproliferation and autoantibody production. *J. Immunol.* 171: 2840–2847.
23. Stekman, I. L., A. M. Blasini, M. Leon-Ponte, M. L. Baroja, I. Abadi, and M. A. Rodriguez. 1991. Enhanced CD3-mediated T lymphocyte proliferation in patients with systemic lupus erythematosus. *Arthritis Rheum.* 34: 459–467.
24. Kong, P. L., J. M. Odegard, F. Bouzahzah, J. Y. Choi, L. D. Eardley, C. E. Zielinski, and J. E. Craft. 2003. Intrinsic T cell defects in systemic autoimmunity. *Ann. NY Acad. Sci.* 987: 60–67.
25. Vratsanos, G. S., S. Jung, Y. M. Park, and J. Craft. 2001. CD4$^+$ T cells from lupus-prone mice are hyperresponsive to T cell receptor engagement with low and high affinity peptide antigens: a model to explain spontaneous T cell activation in lupus. *J. Exp. Med.* 193: 329–337.
26. Zielinski, C. E., S. N. Jacob, F. Bouzahzah, B. E. Ehrlich, and J. Craft. 2005. Naive CD4$^+$ T cells from lupus-prone Fas-intact MRL mice display TCR-mediated hyperproliferation due to intrinsic threshold defects in activation. *J. Immunol.* 174: 5100–5109.
27. Peng, S. L., S. Fatenejad, and J. Craft. 1996. Induction of nonpathologic, humoral autoimmunity in lupus-prone mice by a class II-restricted, transgenic $\alpha\beta$ T cell: separation of autoantigen-specific and -nonspecific help. *J. Immunol.* 157: 5225–5230.
28. Ladino, C. A., and C. M. O'Connor. 1992. Methylation of atypical protein aspartyl residues during the stress response of HeLa cells. *J. Cell. Physiol.* 153: 297–304.
29. Perna, A. F., D. Ingrosso, C. Lombardi, F. Acanfora, E. Satta, C. M. Cesare, E. Violetti, M. M. Romano, and N. G. De Santo. 2003. Possible mechanisms of homocysteine toxicity. *Kidney Int. Suppl.* 84: S137–S140.
30. Tsai, W., and S. Clarke. 1994. Amino acid polymorphisms of the human L-isoaspartyl/D-aspartyl methyltransferase involved in protein repair. *Biochem. Biophys. Res. Commun.* 203: 491–497.
31. DeVry, C. G., and S. Clarke. 1999. Polymorphic forms of the protein L-isoaspartate-(D-aspartate) *O*-methyltransferase involved in the repair of age-damaged proteins. *J. Hum. Genet.* 44: 275–288.
32. Kagan, R. M., H. J. McFadden, P. N. McFadden, C. O'Connor, and S. Clarke. 1997. Molecular phylogenetics of a protein repair methyltransferase. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 117: 379–385.
33. Giese, T., and W. F. Davidson. 1992. Evidence for early onset, polyclonal activation of T cell subsets in mice homozygous for *lpr*. *J. Immunol.* 149: 3097–3106.

34. Liossis, S. N., X. Z. Ding, G. J. Dennis, and G. C. Tsokos. 1998. Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus: deficient expression of the T cell receptor ζ chain. *J. Clin. Invest.* 101: 1448–1457.
35. Yi, Y., M. McNerney, and S. K. Datta. 2000. Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. *J. Immunol.* 165: 6627–6634.
36. Galletti, P., D. Ingrosso, C. Manna, G. Clemente, and V. Zappia. 1995. Protein damage and methylation-mediated repair in the erythrocyte. *Biochem. J.* 306: 313–325.
37. Ingrosso, D., S. D'Angelo, E. di Carlo, A. F. Perna, V. Zappia, and P. Galletti. 2000. Increased methyl esterification of altered aspartyl residues in erythrocyte membrane proteins in response to oxidative stress. *Eur. J. Biochem.* 267: 4397–4405.
38. D'Angelo, S., D. Ingrosso, B. Perfetto, A. Baroni, M. Zappia, L. L. Lobianco, M. A. Tufano, and P. Galletti. 2001. UVA irradiation induces L-isoaspartyl formation in melanoma cell proteins. *Free Radical Biol. Med.* 31: 1–9.
39. Farrar, C., and S. Clarke. 2002. Altered levels of *S*-adenosylmethionine and *S*-adenosylhomocysteine in the brains of L-isoaspartyl (D-aspartyl) *O*-methyltransferase-deficient mice. *J. Biol. Chem.* 277: 27856–27863.
40. Asanuma, Y., A. Oeser, A. K. Shintani, E. Turner, N. Olsen, S. Fazio, M. F. Linton, P. Raggi, and C. M. Stein. 2003. Premature coronary-artery atherosclerosis in systemic lupus erythematosus. *N. Engl. J. Med.* 349: 2407–2415.
41. Roher, A. E., J. D. Lowenson, S. Clarke, C. Wolkow, R. Wang, R. J. Cotter, I. M. Reardon, H. A. Zurcher-Neely, R. L. Heinrikson, M. J. Ball, et al. 1993. Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem.* 268: 3072–3083.
42. Perna, A. F., P. Castaldo, N. G. De Santo, E. di Carlo, A. Cimmino, P. Galletti, V. Zappia, and D. Ingrosso. 2001. Plasma proteins containing damaged L-isoaspartyl residues are increased in uremia: implications for mechanism. *Kidney Int.* 59: 2299–2308.
43. Kim, E., J. D. Lowenson, D. C. MacLaren, S. Clarke, and S. G. Young. 1997. Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. *Proc. Natl. Acad. Sci. USA* 94: 6132–6137.