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Accelerated Turnover of MHC Class II Molecules in Nonobese Diabetic Mice Is Developmentally and Environmentally Regulated In Vivo and Dispensable for Autoimmunity

Alessandra De Riva,* Mark C. Varley,*+ Leslie J. Bluck,‡ Anne Cooke,§ Michael J. Deery,‡ and Robert Busch*

The H2-A\(^{\beta}\) (A\(^{\beta}\)) MHC class II (MHCII) allele is required for type 1 diabetes (T1D) in NOD mice. A\(^{\beta}\) not only has a unique peptide-binding profile, it was reported to exhibit biochemical defects, including accelerated protein turnover. Such defects were proposed to impair Ag presentation and, thus, self-tolerance. Here, we report measurements of MHCII protein synthesis and turnover in vivo. NOD mice and BALB/c controls were labeled continuously with heavy water, and splenic B cells and dendritic cells were isolated. MHCII molecules were immunoprecipitated and digested with trypsin. Digests were analyzed by liquid chromatography/mass spectrometry to quantify the fraction of newly synthesized MHCII molecules and, thus, turnover. MHCII turnover was faster in dendritic cells than in B cells, varying slightly between mouse strains. Some A\(^{\beta}\) molecules exhibited accelerated turnover in B cells from young, but not older, prediabetic female NOD mice. This acceleration was not detected in a second NOD colony with a high incidence of T1D. Turnover rates of A\(^{\beta}\) and H2-A\(^{\alpha}\) were indistinguishable in (NOD × BALB/c) F1 mice. In conclusion, accelerated MHCII turnover may occur in NOD mice, but it reflects environmental and developmental influences, rather than a structural deficit of the A\(^{\beta}\) allele. Moreover, this phenotype wanes before the onset of overt T1D and is dispensable for the development of autoimmune diabetes. Our observations highlight the importance of in vivo studies in understanding the role of protein turnover in genotype/phenotype relationships and offer a novel approach for addressing this fundamental research challenge. The Journal of Immunology, 2013, 190: 5961–5971.

N onobese diabetic mice have been studied extensively as a paradigm of complex autoimmune disease, which develops under the control of genetic, hormonal, and environmental influences. Lymphocyte infiltration of pancreatic islets develops at a young age, leading to progressive β cell destruction and, starting around 12 wk of age, to overt type 1 diabetes (T1D), with a female sex bias (1). Genetic control of T1D in NOD mice includes critical contributions from both H2-A and H2-E MHC class II (MHCII) loci, alongside multiple non-MHCII genes, most of which affect T cell function (2, 3). NOD mice lack E\(\alpha\) transcription; in addition, the unique H2-A\(^{\beta}\) (A\(^{\beta}\)) β-chain differs by 17 aa from the protective A\(\beta\) allele (4). T1D development is reduced or abrogated in NOD mice transgenic for E\(\alpha\) or other H2-A alleles (3). MHCII glycoproteins normally present peptides to CD4+ T lymphocytes, and NOD mice harbor pathogenetic CD4+ effector T cells that recognize islet autoantigens presented by A\(^{\beta}\) (reviewed in Ref. 5). A\(\beta\) shares key polymorphisms, in particular a non-Asp residue at position 57, and peptide-binding preferences with human HLA-DQB1 alleles that confer risk for T1D (6–8). Nonetheless, no unifying explanation for the link between these alleles and islet autoimmunity has emerged from considerations of peptide-binding specificity (5). Moreover, the potential for A\(^{\beta}\)-restricted T cell autoreactivity extends to self-Ags that are systemically expressed, both in the “autoproliferation” phenotype of NOD mice (9, 10) and in the K/B\(\times\)N model of autoimmune arthritis (11, 12). These considerations prompted suggestions that A\(^{\beta}\) confers a broader tendency toward autoreactivity, perhaps mediated by unique biochemical properties other than their peptide-binding specificity.

Indeed, A\(^{\beta}\) exhibits several unusual biochemical properties compared with other MHCII alleles (5). Many MHCII alleles remain stable as αβ heterodimers in SDS at room temperature when loaded stably with peptides, but A\(^{\beta}\) dimers readily dissociate under these conditions (13). Genetic studies indicate that this relative instability represents an intrinsic structural property of the A\(^{\beta}\) heterodimer. SDS instability also correlates with the T1D risk or protection associated with human DQ alleles (14). In [\(^{35}\)S]-pulse/chase experiments performed in vitro, A\(^{\beta}\) exhibited faster turnover in cultured NOD splenocytes than did other H2-A alleles in splenocytes from other mouse strains (13). Based on these observations, it was proposed that a biochemical stability defect of
A^β7, leading to accelerated turnover, might impair T cell tolerance (9, 10).

This view has been controversial, however. SDS instability is shared by other H2-A alleles, which are not associated with spontaneous autoimmunity; A^β7 may be at the lower end of normal allelic variation in this regard (5, 15). Initial evidence that SDS instability was attributable to poor peptide binding by A^β7 (13) was not borne out by subsequent peptide-binding and extraction studies (5). Moreover, several HLA-DQ alleles that confer risk for T1D in humans retain peptides well, despite interacting poorly with HLA-DM during endosomal peptide loading (5). Structural studies in which A^β7 was expressed and crystallized did not indicate any marked defects in stability or folding (16).

More recent studies showed that MHCII protein life span is affected by extrinsic variables. Wild-type APCs of different lineages vary in their MHCII-internalization rates, correlating with the length of ubiquitin chains attached to MHCII β-chains (17). Ubiquitination is modulated during activation of dendritic cells (DCs) by proinflammatory stimuli in vitro (18), correlating with large differences in MHCII protein life span (19). Genetic deficiency in the peptide-exchange cofactor, DM, reduces the surface expression of A^β7 and other MHCII alleles with low affinity for invariant chain peptides and increases their turnover (20, 21). Similar effects on MHCII life span could result from negative regulation of DM activity by HLA-DO/H2-DO (22), which is regulation of DM activity by HLA-DO/H2-DO (22), which is involved in certain disease conditions (23, 24). In NOD mice, MHCII associations with invariant chain may be abnormal, and the development and frequencies of B cells and DCs were reported to be altered, with unknown consequences for the regulation of MHCII protein life span (5).

Moreover, T1D development is subject to environmental control. In NOD colonies worldwide, T1D incidence varies between from ~20 to ≥80% in females at 30 wk of age (26), suggesting that diabetes development is influenced by environmental and epigenetic factors. Environmental factors that contribute to this variation include multiple viral, bacterial, and eukaryotic pathogens; the resident intestinal microflora; as well as experimentally administered proinflammatory stimuli, all of which modify host immune responsiveness (27). In addition, hormonal regulation is suggested by the much lower disease incidence in males; this, too, is influenced by the microbiome (28). Whether disease-relevant environmental factors affect MHCII protein life span in vivo remains unclear.

A definitive assessment of the determinants of MHCII protein instability and its role in autoimmune pathogenesis requires quantification of turnover rates in vivo in APCs of living animals. Methodologies for addressing this problem have been lacking until recently. Radiolabeling, a standard approach for measuring protein life span in vitro (29), is difficult to use in vivo. Stable isotope labeling of amino acids in cell culture (SILAC) has been applied in vivo, but complete labeling of proteins with essential amino acids takes several generations in mice (30), and the precursor/product relationships are not well understood in vivo. Stable isotope approaches have been used for proteome-wide surveys of steady-state protein expression levels and for kinetic proteomics; in contrast, little work has focused on assessing the effects of structural polymorphism on the turnover rates of specific proteins of interest.

We (31, 32) and, independently, other investigators (33, 34) developed an alternative approach to address this problem: stable isotope labeling of nonessential amino acids with heavy water (SINEW). We showed that this approach can be used to quantify the biosynthetic dynamics of different MHC allotypes (31). Deuterated water (heavy water, H2O) is used as a nonradioactive metabolic tracer, which does not interfere detectably with normal physiology at the concentrations used for labeling (35). Following H2O administration, H2 equilibrates rapidly across body water pools and is incorporated biosynthetically into nonessential amino acids and, hence, into proteins of interest (31, 32), with minimal and transient interference from dietary amino acids (32, 36). The approach is now well-validated for quantification of protein synthesis on time scales of a few hours or longer. In this study, we applied SINEW to characterize MHCII turnover in vivo. Surprisingly, our findings indicate that environmental and developmental variables, rather than structural polymorphism, are key determinants of accelerated turnover of A^β7 in NOD APCs and that this phenotype is dispensable for development of T1D.

Materials and Methods

Mice and cell lines

Animal studies were performed according to institutional and national guidelines under UK Home Office Project Licenses 80/2156 and 80/2440. For most studies, NOD mice (named "NOD^ad" where necessary to avoid ambiguity) were bred under specific pathogen-free conditions at the Centre for Biomedical Services, University of Cambridge, from founder stock kindly provided by Prof. Linda Wicker. NOD genotype (100%) was authenticated in a breeding trio using an array of 384 single nucleotide polymorphisms (Charles River Laboratories), spaced ~7 MB apart across the entire genome, which distinguish common inbred mouse strains. BALB/c mice were purchased from Charles River Laboratories and used after 10 d of acclimatization in the same facility. Female BALB/c mice were bred with NOD males to generate F1 mice. Animals were maintained in individually ventilated cages and received standard rodent chow and water ad libitum.

A second colony of NOD mice (named "NOD^high") was bred in the animal facility of the Department of Pathology, University of Cambridge, and maintained under barrier conditions. These NOD mice were also derived from founder stock kindly provided by Prof. Linda Wicker in 2002. T1D incidence increased over time in the facility in the Department of Pathology and subsequently remained high (≥80%) by 30 wk of age in females.

Both colonies were maintained by breeding the first litter in each generation and were kept on the same diet. For incidence studies, animals were aged to 30 wk and observed regularly for signs of diabetes; incidence was confirmed by measurement of glucosuria, using Diastix (Bayer Diagnostics). Routine veterinary microbiological screening of sentinel mice in both colonies was carried out to a standard exceeding current recommendations of the Federation of European Laboratory Animal Sciences Associations (B&K Diagnostics).

M12 NOD is a derivative of the M12 murine B lymphoma cell line transfected with A^β7 genes. These cells, as well as murine A20 B lymphoma cells and the EBV-transformed B cell line, Priess, were cultured as previously described (31).

Labeling experiments

Mice were labeled to 4–5 atom percent enrichment (a.p.e.) of 2H2O in body water by initial i.p. bolus injection of 35 ml/kg 99% a.p.e.2H2O (CK Gas) containing 0.9% w/v NaCl. This material was depleted of LPS using Detoxi-Gel Columns (Thermo Scientific) and sterile filtered. The initial body water enrichment was maintained by continuous administration of 8% a.p.e.2H2O in drinking water ad libitum. Control animals received injections of LPS-depleted 0.9% (w/v) NaCl in natural-abundance water and were maintained concurrently. At various time points following i.p. bolus, animals were exanguinated under terminal general anesthesia, and spleens and femurs were removed following cervical dislocation.

Abs

MK-D6 (IgG2a anti-H2-As) (37) and 14.4.A.5 (IgG2a anti-H2-E) (38) hybridomas were kind gifts from Dr. B. Stockinger. ME-1 (IgG1 anti-HLA-B27) (39) and W6/32 (IgG2a anti-HLA-A/B/C) (40) were kindly provided by Prof. J.S.H. Gaston and used as isotype controls. The mAbs were used as culture supernatants or purified over protein A or protein G affinity columns (GE Biosciences). For flow cytometry, MK-D6 was FITC labeled using Lightning-Link Fluorescein (Innova Biosciences). Other Abs (developed in mice, unless mentioned otherwise) were obtained commercially: IgG1-FITC isotype control, clone MOPC21, from BD Biosciences; IgG2a-FITC isotype control, cat no. MG2A01, from Invitrogen; and CD19-PE/Cy5, clone eBio1D3, and rat anti-mouse CD86-PE, clone P03.1.
from eBioscience. The following Abs were from BioLegend: CD11c–allophycocyanin, clone N418; OX-6 anti–RT1.B, cross-reactive with H-2Aβ7, unconjugated and FITC–conjugated; anti–H-2Aβ76 (Aβ6)–Alexa Fluor 647, clone 39-10-8; rat IgG2b-PE, clone RTK 530; and IgG3–Alexa Fluor 647 isotype control, clone MG3-35.

**Cell isolation and flow cytometry**

Spleens were disrupted mechanically after enzymatic dissociation with Liberase CI (Roche), following the manufacturer’s instructions. After incubation in mouse FeR blocking Reagent (Miltenyi Biotec), CD11c+ and B220+ cells were sequentially isolated by immunomagnetic labeling with CD11c+ and CD55B220+) MACS MicroBeads (Miltenyi Biotec) and double-positive selection using an AutoMACS magnetic cell sorter (Miltenyi Biotec), following the manufacturer’s instructions. Cells isolation were monitored by staining aliquots with fluorochrome–Ab conjugates against CD11c, CD19, and MHCII or isotype controls. Analysis was performed using a FACS Canto II flow cytometer (BD Biosciences) and FlowJo software (TreeStar). B cells and DCs were enumerated as CD19+MHCII+ and CD11c+MHCII+, respectively, after gating on intact splenocytes by forward and side scatter. MHCII and CD646 levels (median fluorescence intensities) were quantified after gating on cells expressing the appropriate lineage Ag within the scatter gate. Purified cell samples were washed in PBS, counted, and stored as pellets at −20°C.

**Protein isolation**

Cell pellets were extracted in ice-cold TBS (pH 7.4) containing 1% IGEPAL CA-630 (Sigma) and protease inhibitors (Complete; Roche; plus 10 m M iodoacetamide). Extracts were centrifuged at 10,000 × g to pellet nuclei and debris (31). The calculations were adjusted for variations in the precursor pool enrichment and adjusting the number of biosynthetic mode, modeled by MIDA, using the measured body water enrichment to represent except where mentioned. Summary estimates of fractional protein synthesis and SD of data pooled from at least two independent labeling experiments were derived by averaging data from all available peptides (at least two), with an overall SD < 6%. Data points with greater analytical errors, in most cases due to low peptide ion abundance, were excluded.

**DNA processing and calculation of fractional cell turnover**

DNA processing and calculation of fractional cell turnover was done as described (35), with minor modifications. Briefly, DNA-containing pellets from extracted cell samples (see above) were boiled in deionized water, undissolved material was removed by centrifugation, and the supernatant was subjected to DNA hydrolysis, mild acid release of deoxyribose from nucleotides, and chemical modification with pentfluorobenzyl hydroxylamine and acetic anhydride to generate the pentfluorobenzyl triacetyl derivative. The derivative was extracted with dichloromethane, dried overnight in vacuo, suspended in ethyl acetate, and injected in duplicate into a benchtop gas chromatograph/mass spectrometer (Hewlett-Packard). Measurements were performed in negative chemical ionization mode, with selected ion monitoring of the ion formed by loss of HF from the derivative, with m/z 435 (m1) or 436 (m2). The fractional molar abundance of the m1 mass isomer [m1/(m1+m2)] was calculated for all samples by integration of the major peak, representing the trans isomer of the derivative, in extracted ion chromatograms, using a previously described algorithm (44) in a MatLab implementation (M.C. Varley, unpublished observations). Unlabeled deoxyribose standards spanning the abundance range of samples were run, and abundance-corrected baseline values (35) were subtracted to generate excess m1 (Em1) values. Bone marrow harvest was performed from femurs after >10 d of 2H2O labeling of mice was used as a fully labeled control. Em1 values for unlabeled and fully labeled deoxyribose conformed closely to those observed in previous work (35), and duplicate measurements of Em1 were routinely within 0.2% of the mean. Fractional DNA synthesis was calculated as 

\[
\text{f}_{\text{cell}} = \frac{\text{Em}_{\text{1sample}} - \text{Em}_{\text{1BM}}}{\text{Em}_{\text{1sample}}} \times 100%
\]

where [BM] refers to the average of Em1 values for fully labeled bone marrow.

**Analysis of 2H2O enrichment**

Sera and media were diluted gravimetrically, and 2H2O enrichment was quantified by isotope ratio MS, as previously described (31).

**Statistics**

Statistical analysis was performed using GraphPad Prism software. Means and SD of data pooled from at least two independent labeling experiments were used as descriptive statistics. Strain differences in immunophenotypes were evaluated by unpaired, two-tailed Student t test. Variation between peptides from the same protein sample was analyzed by one-way ANOVA. Two-way ANOVA was used to analyze the effects of cell lineage (B cell or DC) and either molecules/strains (Ag7 in NOD versus H2-Ad or H2-Aβ7 in BALB/c versus H2-Ed [Eβ7] in BALB/c) or sex of NOD mice. The time course of f was modeled for each molecule or cell population as either a single-exponential rise to maximum ([f](t) = 1 - e−kt), with rate constant k and fmax, ln(2) or 45%, or the sum of two single-exponential terms, with rate constants k1 and k2, weighted in relative proportions w1 and w2 ([f](t) = w1(1 - e−k1t) + w2(1 - e−k2t)), by nonlinear least-squares fit to the data. Best-fit half-lives were reported with 95% confidence intervals. Curve fits using alternative models were compared using the F test. In addition, the F test was used to compare single-exponential half-lives between different molecules/strains or sexes. Kaplan–Meier analysis of cumulative disease incidence was performed, and incidence curves were compared by the log-rank test.

**Results**

**Use of SINEW for measurement of MHCII protein turnover in vivo**

We used the recently developed SINEW approach to address the role of MHCII turnover in NOD autoimmunity in vivo (31). SINEW uses MS to quantify the relative abundances of peptide mass variants (“mass isomeropes”, differing by ≥1 Da as the result of the presence of stable isotopes of any element at any position in the peptide). The resultant mass isomerope distributions change upon 2H incorporation into proteins during continuous biosynthetic labeling with heavy water (2H2O), in proportion to the cumulative fraction of old molecules that has been replaced by new ones during the labeling interval (31). This measure, also known as fractional protein synthesis, equals protein turnover, provided that cell turnover is

\[
\text{protein synthesis} = \text{protein turnover} = k = \frac{1}{t_{1/2}}
\]

where t1/2 is the half-life for the average molecule in the cell population. The results were expressed as mean ± SD of data pooled from at least two independent labeling experiments.
negligible and levels of proteins of interest remain at steady state (Fig. 1A); these assumptions are tested further below.

The experimental design is summarized in Fig. 1B; steps a–d represent the measurement of fractional protein synthesis. Six-week-old female, prediabetic NOD and control mice (BALB/c; H2-A^d and -E^d) were labeled in vivo with 2H2O for several weeks (Fig. 1B, step a). 3H enrichments in body water were kept at ∼4–5% throughout the labeling period, as verified by MS analysis of sera (Fig. 1C, data not shown).

Animals were sacrificed after various times; splenocytes were dissociated enzymatically and used for sequential MACS isolation of DCs (CD11c^+) and B cells (B220^+; Fig. 1B, step b). Flow cytometric enumeration of APCs before MACS enrichment revealed strain differences in APC composition (Fig. 2A–C). Spleens of young NOD females contained significantly greater proportions and absolute numbers of DCs, smaller proportions of B cells, and, thus, a higher ratio of DCs/B cells than were found in age- and sex-matched BALB/c mice. This was consistent with previous reports of lymphopenia and abnormal B cell development in NOD mice (45, 46). Despite these differences, DCs and B cells from both strains were successfully enriched to >90% purity by MACS (Fig. 2D, data not shown). Indeed, >95% of MHCII^+ cells were of the desired lineage after enrichment (Figs. 2D, data not shown).

Next, MHCII molecules were immunoprecipitated from detergent extracts of APCs, using allele-specific mAbs (Fig. 1B, step c). MHCII α and β-chains were separated by nonreducing SDS-PAGE and excised following silver staining (Fig. 3A). Tryptic digests were used for peptide mapping by tandem MS, which confirmed the presence of the MHCII molecules of interest (Fig. 3B). Mass isotopomer distributions of selected peptides were then quantified by LC/MS (31). In this example, the presence of MHCII molecules turned over much more actively in DCs than in B cells (Fig. 4C). Strikingly, all three MHCII molecules in inbred mice were selected for each of the MHCII molecules of interest, based on their total ion abundance, absence of peak contamination, agreement with MIDA predictions, and the magnitude of the shifts in the mass isotopomer distributions following 2H2O labeling. The peptides are shown in bold type in Fig. 3B; their mass isotopomer distributions and labeling statistics are summarized in the supplemental material (Supplemental Fig. 1, Supplemental Table I). Informative peptides were selected for each of the MHCII molecules of interest, based on their total ion abundance, absence of peak contamination, agreement with MIDA predictions, and the magnitude of the shifts in the mass isotopomer distributions following 2H2O labeling. The peptides are shown in bold type in Fig. 3B; their mass isotopomer distributions and labeling statistics are summarized in the supplemental material (Supplemental Fig. 1, Supplemental Table I).

Previous work showed that, in partially labeled mass isotopomer distributions of peptides, the abundance of each individual mass variant is intermediate between unlabeled and fully labeled distributions, in proportion to the fractional synthesis of the protein of origin (31). On this basis, fractional synthesis values were calculated from multiple MHCII-derived peptides. Fig. 4B shows an analysis of B cells from individual mice labeled for 4 h. Within error, analysis of different peptides from the same MHCII molecule gave indistinguishable estimates of fractional protein synthesis (Fig. 4B). Subsequently, at least two informative peptides in each sample were analyzed, and fractional protein synthesis values were averaged (SD ∼ 6%).

**Effect of cell lineage on the turnover of different MHCII molecules in inbred mice**

Fig. 4C summarizes the fractional synthesis of E^d, A^d, and A^e7 at 4 h for B cells, as well as DCs. Strikingly, all three MHCII molecules turned over much more actively in DCs than in B cells (Fig. 4C); these differences were more pronounced than those between MHCII molecules. Moreover, the allelic differences also depended on the APC type: in DCs, A^e7 had higher turnover than E^d or A^d.

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**FIGURE 1.** Measurement of MHCII turnover in vivo by SINEW. (A) Fractional protein synthesis, measured by SINEW, includes potential contributions from protein turnover, net protein synthesis, and cell proliferation. The latter contributions can be corrected for by quantifying steady-state protein levels and fractional DNA synthesis or neglected if they are small. (B) Work flow for measurements of fractional MHCII protein and DNA synthesis in APC populations of 2H2O-labeled living mice. See text for details. (C) Equivalent 2H2O labeling of NOD and BALB/c mice. 2H2O a.p.e. was measured by isotope ratio MS in serum from 6-wk-old female BALB/c (n = 15) and NOD (n = 16) mice after up to 24 h of 2H2O labeling. There was no significant difference in average 2H2O labeling between strains (p > 0.05, Student t test). Serum 2H2O enrichments after 24 h (data not shown) were similar, on average, but less variable than at the earlier time points.
but in B cells, $E^d$ turnover was similar to $A^d$ and faster than $A^e$. We concluded that structural polymorphism is not the sole determinant of MHCII turnover in vivo, whereas the cell lineage is a major determinant.

When tracked over time, MHCII protein fractional synthesis in B cells increased continuously, approaching 100% replacement of old molecules within a week (Fig. 5A, 5B; statistics are summarized in Fig. 5C). Single-exponential curve fits indicated average fractional synthesis $t_{1/2}$ $\sim$10–12.5 h. Consistent with data obtained at 4 h, the half-lives for $A^e$ and $E^d$ were slightly, but significantly, shorter than for $A^d$ (Fig. 5C; compared by F test). Interestingly, however, data for $A^e$ turnover deviated significantly from a single-exponential model with a uniform half-life (by Runs test) and were significantly better modeled by a mixture of fast- and slow-turnover subpopulations (Fig. 5B, 5C). The fast phase explained the difference between $A^d$ and $A^e$ at 4 h, which disappeared later in the experiment. Thus, fast turnover appears to be characteristic of only a fraction of $A^e$ molecules.

In contrast, turnover of $A^e$ in M12 B lymphoma transfectants labeled in vitro was uniformly slow, without evidence of a fast phase (Supplemental Fig. 2) (31). Thus, the presence of kinetic heterogeneity seemed to depend on the cellular context.

**Fractional synthesis is a measure of MHCII protein turnover in vivo**

In addition to the need to replace turned-over protein, another reason for the synthesis of new cell-associated molecules could be the requirement to maintain steady-state protein levels as cells divide (Fig. 1A). To assess whether cell division contributed substantially to the need for MHCII protein synthesis, fractional DNA synthesis was quantified by $^3$H$_2$O labeling, using previously established, well-validated methods (Fig. 1B, steps e and f) (35). As expected, B cell proliferation occurred on much slower time scales ($t_{1/2}$ $\sim$2 wk) than did MHCII fractional synthesis (Fig. 5A, 5B). DC renewal was faster (data not shown), but it did not contribute substantially to protein synthesis at early time points (5–6 wk). Thus, proliferative cell renewal did not contribute substantially to the observed rates of MHCII fractional synthesis in vivo. In contrast, $A^e$ fractional protein synthesis in the M12 B lymphoma line was entirely attributable to cell growth, with no detectable additional contribution from protein turnover.
The fraction of new MHCII molecules is shown after 4 h of $^{2}$H$_2$O labeling (cells from eight (Ed) or nine (Ad) individual BALB/c mice and five NOD isotopomer distributions of a typical peptide from Ed quantified in B cells isolated after 4 h of $^{2}$H$_2$O labeling in vivo. Data are from the same MHCII protein. Fractional MHCII protein synthesis was mates of fractional synthesis obtained by analysis of different peptides aged across all informative mass isotopomers. (interpolation between the unlabeled and fully labeled distribution, averaged across all informative mass isotopomers. (B) Indistinguishable estimates of fractional synthesis obtained by analysis of different peptides from the same MHCII protein. Fractional MHCII protein synthesis was quantified in B cells isolated after 4 h of $^{2}$H$_2$O labeling in vivo. Data are shown for different peptides (identified by the first three amino acids in each sequence; compare with Fig. 3B) from individual animals. Data were pooled from at least two independent labeling experiments for each strain. Fractional synthesis estimates from different tryptic fragments of the same protein were statistically indistinguishable ($p > 0.05$, one-way ANOVA). (C) APC lineage and mouse strain affect MHCII turnover at 4 h in vivo. The fraction of new MHCII molecules is shown after 4 h of $^{2}$H$_2$O labeling of female, 4–6-wk-old mice. Mean ± SD are shown for splenic B220$^+$ B cells from eight (E$^d$) or nine (A$^d$) individual BALB/c mice and five NOD mice, as well as for CD11c$^+$ DCs from five BALB/c mice and two independently labeled pools of three NOD mice. Fractional synthesis was averaged from two or three mass isotopomers of two to seven MHCII-derived peptides/animal. Data from at least two independent labeling experiments/group were pooled. Effects of APC lineage ($p < 0.0001$) and molecules/strains ($p < 0.0001$) on turnover, as well as their mutual interaction (i.e., the hierarchy of molecules depends on the cell type: $p = 0.0175$), were significant (two-way ANOVA). The $p$ values in the Figure show significant differences between molecules within each cell lineage (Bonferroni posttest). n.s., Not significant.

(Supplemental Fig. 2). We estimated that the half-life for A$^{d}$ turnover was $\sim 42$ h in this cell line.

In addition, we considered the possibility that a proportion of splenic B cells might be activated in NOD B cells and that this might contribute to heterogeneous A$^{d}$ turnover (47). As a positive control, upregulation of MHCII, CD86, and other activation markers was readily detected on B cells stimulated in vitro with LPS over a wide dose range (data not shown). However, flow cytometric analysis of MHCII (Fig. 6A, 6B) and CD86 (Fig. 6C, 6D) expression. In addition, these experiments showed no evidence of inadvertent activation of APCs in vivo due to the i.p. bolus injection of $^{3}$H$_2$O used to initiate labeling. Pilot experiments revealed the presence of low levels of LPS in some batches of $^{2}$H$_2$O, which were sufficient to cause slight activation of APCs in vitro (A. De Riva, unpublished observations). To avoid the possibility that LPS activation could confound the turnover measurements, LPS was routinely removed from $^{2}$H$_2$O preparations used for i.p. bolus injection. Lastly, there was no evidence for changes in MHCII levels over the course of the labeling experiments (Fig. 6A, 6B, data not shown), arguing against the possibility that some of the measured new synthesis might be attributable to net accumulation of new MHCII molecules (Fig. 1A). Taken together, these additional controls showed that changes in MHCII levels and cell proliferation did not contribute markedly to fractional MHCII protein synthesis, implying that fractional synthesis is equivalent to protein turnover in this system (Fig. 1A).

Biological variables affecting the accelerated turnover of A$^{d}$ molecules in NOD mice

Further experiments were undertaken to identify determinants of the difference in turnover between H2-A$^{d}$ in BALB/c B cells and...
A\textsuperscript{A\textgamma} in NOD B cells at early time points. If this difference reflects an inherent structural defect of A\textsuperscript{A\textgamma}, then it should be detectable in (NOD × BALB/c) F\textsubscript{1} mice. However, A\textsuperscript{A\textgamma} and A\textsuperscript{A\textgamma} isolated from F\textsubscript{1} B cells showed equal turnover at 4 h (Fig. 7A). The two molecules were not mutually contaminated, as judged by analysis of tryptic digests (Supplemental Table II); F\textsubscript{1} B cells were highly pure and not activated, as judged by analysis of MHCII and CD86 expression. Thus, the turnover difference between A\textsuperscript{A} and A\textsuperscript{A\textgamma} in the parental strains is not determined by the allelic difference but by the context in which the H\textsubscript{2}-A molecules is expressed.

In contrast, developmental variables affected A\textsuperscript{A\textgamma} turnover in NOD B cells. Turnover was indistinguishable between males and females, both at 4 h and in B cells over time (Fig. 8A, 8B), but it declined by 12 wk of age at rates similar to A\textsuperscript{A} turnover in young BALB/c mice (Fig. 8C).

To address the role of environmental variables in the regulation of MHCII turnover, A\textsuperscript{A\textgamma} turnover at 4 h in splenic B cells from young female mice was compared between two NOD colonies housed in different animal facilities (Fig. 9). In the colony used for the previous experiments, ∼22% of females developed T1D by 30 wk of age, which is at the low end of the range of disease penetrance observed worldwide (“NOD\textsuperscript{low},” Fig. 9A) (26). In contrast, as many as 91% of the females in the second (“NOD\textsuperscript{high}) colony developed diabetes. Unlike the NOD\textsuperscript{low} colony, A\textsuperscript{A\textgamma} in B cells from young female NOD\textsuperscript{high} animals showed no evidence of accelerated turnover at the informative 4-h time point (Fig. 9B). The purity of MACS-enriched NOD\textsuperscript{high} B cells (Fig. 9C), their nonactivated phenotype (Fig. 9D, 9E), and B cell subset composition (data not shown) were similar to NOD\textsuperscript{low} animals (Fig. 6). Together, these data show that environmental differences affect A\textsuperscript{A\textgamma} turnover in apparently nonactivated B cells. Interestingly, A\textsuperscript{A\textgamma} turnover was not accelerated in the high-incidence colony (Fig. 9A, 9B), indicating that accelerated turnover was dispensable for the development of T1D.

Discussion
To our knowledge, this study reports the first measurements of MHCII protein turnover in living mice. Moreover, this study provides evidence against the hypothesis that an intrinsic stability defect in A\textsuperscript{A\textgamma} contributes to the pathogenesis of autoimmune diabetes.

The SINEW approach, previously validated to measure MHC protein synthesis in cultured cell lines (31), was used in this study in vivo to compare the turnover rates of different MHCII molecules in NOD and control mice. The studies were carefully controlled to avoid perturbation of the system by LPS contamination during \textsupers\textsuperscript{2}H\textsubscript{2}O label administration. There was no evidence for perturbation of APCs by \textsuperscript{2}H\textsubscript{2}O itself. Measurements of the proliferative renewal of APCs and of MHCII levels showed that protein turnover was the only significant contributor to MHCII fractional synthesis. In principle, cell turnover could be a confounding variable in measuring the turnover rates of long-lived, cell-associated proteins, yet this is rarely considered in kinetic proteomics experiments. The ability of \textsuperscript{2}H\textsubscript{2}O to label newly synthesized proteins and DNA (the latter representing cell turnover) over) represents a distinct advantage of SINEW over SILAC. The SINEW measurements were analytically precise and accurate, as judged by the close agreement of data with MIDA predictions. The measurements were validated by analysis of multiple peptides from each MHCII molecule. SINEW relies on quantitative shifts in peptide mass isotopomer distributions following \textsuperscript{2}H\textsubscript{2}O labeling; these shifts are subtler than the larger mass shifts created in SILAC by use of all-\textsuperscript{13}C-labeled amino acids. Nonetheless, the analytical characteristics of the SINEW approach permitted small differences in MHCII protein turnover to be detected.

Key determinants of MHCII protein turnover in vivo were identified. APC type had a substantial effect. In DCs, the ∼40% replacement of old by new molecules after 4 h of labeling corresponds to an average t\textsubscript{1/2} of ∼5–6 h, about twice as fast as that observed in B cells (t\textsubscript{1/2} ∼10–12 h). These findings correlate with recent studies, showing that murine DCs have higher rates of...
ubiquitin-dependent MHCII internalization than do B cells in vitro (17); this is thought to regulate lysosomal degradation of MHCII molecules. MHCII turnover was much slower still ($t_{1/2} \approx 42$ h) in transfected M12 B lymphoma cells. Thus, some immortalized cell lines, which are useful in studying other aspects of Ag presentation, are poor models for studying the physiological regulation of MHCII protein turnover.

In B cells, the early kinetics of H2-A turnover measured by SINEW in vivo was comparable to the kinetics observed previously in vitro by pulse-chase analysis of unseparated splenocytes for up to 8 h (13). This was expected, given that B cells dominated quantitatively among splenic APCs in both mouse strains. However, possible confounding effects of APC composition must be considered when interpreting turnover measurements in whole splenocytes. We found that NOD spleens contain a higher ratio of DO, a regulator of DM-mediated peptide exchange (23, 25). This could affect peptide loading, with possible implications for MHC protein stability (22). However, $A^d$- and $E^d$-labeling kinetics in BALB/c B cells conformed closely to a single-exponential curve fit, suggesting that the turnover rates of these molecules in the major B cell subsets are fairly uniform. Nonetheless, rare subsets could exhibit distinct rates of turnover.

In vitro, activation of APCs by proinflammatory stimuli is a major regulator of MHCII protein turnover. This is clearly evident in LPS- and cytokine-stimulated human and murine DCs, which, following an initial burst of MHCII synthesis, shut down ubiquitin-dependent MHCII internalization and degradation to enable persistent Ag presentation (19). The resultant shutdown in MHCII turnover is readily observed in vitro in LPS-stimulated human DCs by our SINEW approach (C. Prevosto, R. Busch, unpublished observations). In our studies, there was no evidence for activation of B cells or DCs in the spleens of $^{3}H_{2}O$-labeled mice when analyzed ex vivo; as a positive control, MHCII, CD86, and other activation markers were upregulated in splenic B cells stimulated with LPS in vitro (data not shown). We concluded that the MHCII turnover rates observed in vivo represent nonactivated conditions; the impact of APC activation will be an interesting direction of further research.

Compared with the large differences in MHCII turnover between APC types, the differences between MHCII molecules were less pronounced. In B cells and in DCs from inbred strains, initial measurements revealed slightly faster turnover rates for $A^{d}$ than for $A^{r}$, reproducing the direction of trends observed previously.
in vitro (13). Albeit statistically significant and consistent within and between experiments, the difference was ~20% or less, whether it was measured at a single informative time point or in terms of average half-lives calculated from single-exponential curve fits. Moreover, in B cells, the turnover of protective H2-E molecules was indistinguishable from that of the A\(^{\beta}\) susceptibility allele. Thus, differences in overall turnover rates were minor and did not correlate with the contributions of different MHCII molecules to T1D risk. This is consistent with our serological studies (described above, and A. De Riva, unpublished observations), which provide no support for the notion that A\(^{\beta}\) is defective in surface expression compared with other MHCII alleles.

Several observations indicated that the fast-turnover A\(^{\beta}\) subpopulation did not arise from an intrinsic stability defect of the A\(^{\beta}\) allele. First, an intrinsic defect would predict a uniformly fast rate of A\(^{\beta}\) turnover, but a single-exponential labeling curve with a short half-life was inconsistent with our data. Second, A\(^{d}\) and A\(^{\beta}\) turnover were indistinguishable in B cells from H2\(^d\times 8\) F1 mice, showing that, in one and the same cellular environment, the 17-aa differences between these alleles have no detectable effect on turnover. Third, the turnover rate of E\(^d\) was similar to that of A\(^d\) in DCs, but it was similar to A\(^{\beta}\) in B cells, indicating that the cellular context influences relative turnover rates of different MHCII molecules. An overriding effect of the cellular microenvironment was indicated by the fact that the A\(^{\beta}\) molecules in the M12 B lymphoma transfected were uniformly long-lived. Male and female NOD mice had similar A\(^{\beta}\) turnover; however, the accelerated turnover at 4 h was not observed in older NOD mice, and it was not present in B cells from a second colony of NOD mice housed under different environmental conditions. This responsiveness to developmental and environmental variables argued that A\(^{\beta}\) turnover is not determined by any intrinsic stability defect.

The environmental regulation of MHCII turnover contrasts with the marked influence of structural polymorphism on the SDS stability of different H2-A alleles, noted previously by other investigators (13). Our results imply that MHCII turnover rates and SDS stability may be discordantly regulated, so that SDS instability does not necessarily predict a shortened MHCII life span.

We considered the possibility that cellular heterogeneity could contribute to the kinetic heterogeneity of A\(^{\beta}\) molecules in NOD B cells. Preliminary experiments showed no difference in A\(^{\beta}\) turnover at 4 h between MACS-isolated total B220\(^+\) B cells and FACS-sorted follicular B cells (data not shown). Moreover, the difference in A\(^{\beta}\) turnover between the two colonies of NOD mice was not explained by differences in phenotypic activation of splenic B cells (which was undetectable in both colonies) or in their subset composition (data not shown). These experiments do not rule out cellular heterogeneity as a contributing variable. Alternatively, heterogeneous A\(^{\beta}\) turnover could arise from heterogeneous editing of the bound peptide repertoire. This possibility is difficult to address directly with our current methodology, which is not able to measure the turnover rates of specific peptide/MHCII protein complexes. Regardless of the underlying mechanisms, our data indicate that environmental and developmental factors modify the handling of MHCII molecules in splenic B cells, even in the absence of overt phenotypic activation.

Collectively, our studies argue strongly against a disease-promoting role for accelerated turnover in the mechanisms linking the A\(^{\beta}\) allele to T1D pathogenesis, which was postulated earlier (9, 13). As argued above, the complex dynamics of A\(^{\beta}\) turnover in NOD spleens was determined by environmental and developmental variables, rather than by MHCII polymorphism; thus, it did not offer a link between MHCII polymorphism and disease. It had only a small effect on average molecular life spans and no discernible effect on the steady-state expression of A\(^{\beta}\), limiting its potential impact on T cell tolerance. Most importantly, accelerated turnover of some A\(^{\beta}\) molecules in splenic B cells was observed only in a NOD colony with low T1D incidence and not...
in a second colony with high disease incidence. Thus, the dynamic abnormality was not required for T1D development in this high-incidence NOD colony. The simplest explanation is that this phenotype represents an epiphenomenon in T1D pathogenesis.

Although these data argue strongly against a causal role for a generalized, inherent defect in $A^b$ turnover, abnormalities in specialized niches could prove to be relevant to pathogenesis. This could include, for example, activated APCs in lesional tissue, which contribute to T1D pathogenesis from an early stage, and thymic APCs, which maintain central tolerance. The sample requirements of SINEW experiments have not permitted analyses of these less abundant populations. However, with regard to thymic APCs, we note recent work that suggests that central tolerance in NOD mice is not compromised by an Ag-presentation defect, but rather is overwhelmed by a surfeit of $\alpha\beta$ over $\gamma\delta$ T cell precursors during thymic development (48).

Independently of the question of whether $A^b$ turnover contributes to T1D pathogenesis, the differences between the two NOD colonies raised important questions for future research. A key priority will be the isolation of specific environmental variables that determine the large differences in disease incidence. We excluded genetic contamination of the low-incidence colony. Differences in microbiological status, identified by veterinary and in-house screening (data not shown), did not involve species known to influence T1D incidence in NOD mice (27), and broader differences in microbiota and minor differences in husbandry practices could also contribute. A related question is how these environmental variables could influence the dynamics of $A^b$ turnover in splenic B cells without causing overt activation or differences in B cell subset composition. We speculate that the impaired intestinal barrier function of NOD mice (49) could allow splenic B cells to be exposed to chronic, low-level stimulation by intestinal microbiota or their products or cytokines. Extensive further work will be required to test these possibilities.

In conclusion, these studies begin to define determinants of MHCII protein turnover in vivo, which include APC lineage, age, strain background, and environmental variables, but not, surprisingly, allelic polymorphism. Our findings overturn the long-standing, but controversial, hypothesis that autoimmunity in NOD mice arises from an inherent susceptibility of the disease-associated $A^b$ allele to rapid turnover. Context, rather than structural MHCII polymorphism, determines the abnormal turnover of a proportion of $A^b$ molecules in NOD mice. In B cells, which are essential for T1D, $A^b$ turnover in splenic B cells declines with age before the onset of T1D (50) and harbor the great majority of splenic MHCII molecules, small differences in turnover among $A^b$, $A^{d}$, and $E^d$ do not correlate with their effects on T1D risk. Accelerated $A^b$ turnover in NOD splenic B cells declines with age before the onset of T1D in a low-incidence NOD colony and is not observed in a high-incidence NOD colony, indicating that fast $A^b$ turnover is not required for T1D.

Our findings have broader implications. Any effects of genetic polymorphisms on protein turnover, as well as the role of protein turnover in genotype/phenotype relationships, are best evaluated in vivo, with due consideration of cellular and molecular heterogeneity. SINEW offers a widely applicable approach to this important problem.

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