Cutting Edge: MHC Class II-Restricted Peptides Containing the Inflammation-Associated Marker 3-Nitrotyrosine Evade Central Tolerance and Elicit a Robust Cell-Mediated Immune Response

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Nitrotyrosine is widely recognized as a surrogate marker of up-regulated inducible NO synthase expression at sites of inflammation. However, the potential immunogenicity of autologous proteins containing nitrotyrosine has not previously been investigated. Herein, we used the I-Ek-restricted T cell epitope of pigeon/moth cytochrome c (PCC/MCC88–103) to assess the ability of T cells to recognize ligands containing nitrotyrosine. Substitution of the single tyrosine (Y97) in PCC/MCC88–103 with nitrotyrosine abrogates recognition by the MCC88–103-specific T cell hybridoma 2B4. CBA (H2k) mice immunized with MCC88–103 or nitrated MCC88–103 peptides produce T cell responses that are mutually exclusive. Transgenic mice that constitutively express PCC under the control of an MHC class I promoter are tolerant toward immunization with MCC88–103 but exhibited a robust immune response against nitrated MCC88–103. Analysis of T cell hybridomas specific for nitrated-MCC88–103 indicated that subtle differences in TCR VDJ gene usage are sufficient to allow nitrotyrosine-specific T cells to escape the processes of central tolerance. The Journal of Immunology, 2003, 171: 528–532.

Protein-associated nitrotyrosine is widely recognized as a hallmark of inflammation and is often used as a surrogate marker for the up-regulation of inducible NO synthase (iNOS). Accumulation of protein-associated nitrotyrosine has been documented in inflammatory conditions of diverse origin such as atherosclerosis (1), respiratory disease (2), transplant rejection (3), multiple sclerosis (4), Alzheimer’s disease (5), celiac disease (6), arthritis (7–9), ischemia-reperfusion injury (10), autoimmune diabetes (11), autoimmune uveitis (12), and infectious diseases (13). Although the precise molecular mechanism(s) responsible for in vivo formation of nitrotyrosine is still not firmly established, peroxynitrite and other reactive nitrogen species generated during periods of inflammation are thought to be primary intermediates (14, 15). The conversion of tyrosine to nitrotyrosine has been shown to have considerable biological ramifications, including alteration of enzymatic activity (16). However, the effect of this modification on immunological reactivity has received only limited attention. A number of polyclonal sera (17) and mAbs (17, 18) specific for protein-associated nitrotyrosine have been generated (usually using nitrotyrosine-containing keyhole limpet hemocyanin as an immunogen). This clearly indicates that the nitrotyrosine moiety of modified proteins can contribute to an Ab epitope. These Abs have proven to be sensitive and specific tools for the detection of nitrotyrosine in vivo and in vitro. However, to the best of our knowledge, there is no prior evidence as to whether the conversion of tyrosine to nitrotyrosine can be detected by components of the cellular immune system. If conversion of tyrosine to nitrotyrosine in autologous proteins can render these proteins recognizable as immunogenic autoantigens, this could have important implications for autoimmune diseases. Recent studies have indicated that a host of post-translational modifications, including glycosylation (19, 20), phosphorylation (21, 22), and cysteinylation (23, 24), can all affect T cell immunoreactivity. Indeed, it has been proposed that these modifications may play a role in the initiation and/or maintenance of autoimmune disease by contributing to the breakdown of immunological tolerance (25, 26).

In the present study we have used an in vivo model system to determine whether the conversion of tyrosine to nitrotyrosine can affect Ag recognition by components of the cellular immune system. For this purpose we have used the well-established I-Ek-restricted peptide from the model Ag moth/pigeon cytochrome c.
Materials and Methods

Experimental animals

Wild-type CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). PCC-transgenic mice (28) bred onto a B10.A background (line 119) (30) were a gift from Dr. R. Schwartz (National Institutes of Health, Bethesda, MD). All animals were used at 6–12 wk of age.

Reagents

Human IL-2 was provided by Dr. C. Reynolds (National Cancer Institute, Bethesda, MD). IL-2 production was assessed using the IL-2-dependent cell line CTL-L2 (31) obtained from American Type Culture Collection (Manassas, VA). For ELISA experiments, IFN-γ capture Ab (R4-6A2) and biotinylated anti-mouse IFN-γ (XMG1.2) were purchased from BD PharMingen (San Diego, CA). The T cell hybridoma 2B4 and the IEk-expressing CHO cells have been described previously (29) and were gifts from Dr. M. Davis (Stanford, CA). The MCC88103 peptide was synthesized by the Peptide Synthesis Facility at Queen’s University (Kingston, Canada), and the nitrated MCC88–103 (nMCC88–103) peptide was synthesized by Sigma-Genosys (Woodlands, TX).

Cell preparation and culture

Mice (CBA/J and PCC-transgenic) were immunized in the rear footpad with 25 μg of peptide (MCC88–103 or nMCC88–103) mixed with IFA. Ten days after immunization, mice were euthanized, and the draining popliteal lymph node cells were recovered. Mononuclear cells were prepared from lymph pools and cells (2 × 10⁶ cells/well in 96-well plates) were cultured for 3 days in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Sigma-Genosys), 25 mM HEPES buffer, 50 μM 2-ME, penicillin-streptomycin, and 1-glutamine (Life Technologies) in the presence or the absence of the indicated peptides. On day 3 of culture half the culture supernatant was removed and the indicated amount of IFN-γ-secreting ELISA, and the remaining cells were pulsed overnight with [³H]thyminidine (1 μCi/well) to measure peptide-induced proliferation. Lymph node cells from mice (CBA/J and PCC-transgenic) immunized with nMCC88–103 peptide were also stimulated with nMCC88–103 peptide in bulk (1 × 10⁶ cells/well in six-well plates). After 10-day stimulation cells were fused with BW5147 thymoma cells (obtained from American Type Culture Collection) using previously described protocols (32) to generate T cell hybridomas. Hybridomas were cloned in 96-well plates by limiting dilution and were assessed for CD3 surface expression by flow cytometry and for IL-2 production in response to IEk-expressing CHO cells in the presence or the absence of MCC88–103 or nMCC88–103 peptide.

TCR β-chain gene usage

119IF5 T hybridomas were lysed using TR1zol reagent (Life Technologies), and total RNA was used for analysis of TCR β-chain gene usage employing a degenerate primer PCR approach as previously described (33). Briefly, cDNA was prepared by standard methodology using oligo(dT) and reverse transcriptase. The TCR β-chain sequence was then amplified by PCR using a degenerate TCR β-chain variable region consensus primer 5′-TAAGCGGCGCGATGSLYTGTGATWXXACG-3′ (S = A/G; L = A/G; T = C/G; W = A/G; X = A/G) and a β-chain constant region primer 5′-CAGCT CGACGCCACCTGG-3′. The PCR reaction was performed using three cycles with a low stringency annealing step (1 min at 94°C, 1 min at 37°C, 1 min at 72°C), followed by 27 cycles using a high stringency annealing step (1 min at 94°C, 1 min at 55°C, 1 min at 72°C). The resulting PCR product was cloned into the TA vector (Invitrogen, San Diego, CA) and was subjected to automated nucleotide sequence analysis at the University of Ottawa Biotechnology Research Institute.

Results and Discussion

We first assessed the consequence of tyrosine to nitrotyrosine conversion in terms of recognition by the MCC88–103specific T cell hybridoma 2B4. As previously described (29), 2B4 responds to MCC88–103 peptide presented in the context of IEk by synthesizing IL-2 in a dose-dependent manner (Fig. 1C). In contrast, 2B4 T cells were completely nonresponsive to stimulation with a synthetic peptide (nMCC88–103) containing a nitrotyrosine in place of Y97, even at the highest concentrations tested. Although the loss of recognition of nMCC88–103 by the T cell hybridoma 2B4 constituted a negative result, this observation provided the first clear evidence that modification of tyrosine to nitrotyrosine could have an impact on the process of T cell recognition. Previous studies have indicated that Y97 of MCC88–103 is not involved in MHC binding (29); however, we wanted to eliminate the possibility that conversion of Y97 to nitrotyrosine has profound consequences in terms of immunological reactivity. More importantly, we observed that self Ags containing a tyrosine to nitrotyrosine conversion can evade the process of central tolerance, rendering them potentially important autoantigens.
nitrotyrosine disrupted presentation of the nMCC88–103 Peptide by MHC. To test this, we immunized CBA (H2k) mice with either MCC88–103 or nMCC88–103 peptides and assessed their cellular immune response using in vitro recall assays. Draining lymph node cells from mice immunized with MCC88–103 peptide secreted IFN-γ in a dose-dependent manner in response to in vitro stimulation with MCC88–103 peptide (Fig. 1D). These same cells also secreted IFN-γ in response to stimulation with nMCC88–103 peptide; however, the response to the latter was significantly weaker than the response to MCC88–103 peptide. Strikingly, mice immunized with nMCC88–103 peptide had the opposite pattern of recognition. Cells from nMCC88–103-immunized animals secreted IFN-γ in response to in vitro stimulation with nMCC88–103 peptide, but were completely unresponsive to MCC88–103 peptide. The presence of a robust nMCC88–103-specific immune response provided strong evidence that conversion of the single tyrosine residue of MCC88–103 to nitrotyrosine does not significantly impact on the ability of this peptide to be presented by IE2. This is consistent with previous results showing that Y97 does not make direct contact with the peptide binding groove of IE2 (27, 29). Furthermore, these results demonstrate unequivocally that conversion of tyrosine to nitrotyrosine has potentially profound consequences in terms of T cell recognition.

We next examined whether nitration of an autologous protein might be capable of rendering it immunogenic and potentially recognizable as an autoantigen. To address this question, we used transgenic mice that constitutively express PCC under the control of the MHC class I promoter (28). These mice are unresponsive to immunization with MCC88–103 due to the process of central tolerance whereby potentially autoreactive T cells are eliminated during maturation in the thymus via the process of negative selection. In agreement with earlier findings (28), we could not detect an MCC88–103-specific cellular immune response in PCC transgenic mice after immunization with MCC88–103 peptide (Fig. 2). However, immunization of PCC transgenic mice with nMCC88–103 peptide elicited a robust cellular immune response against nMCC88–103 as measured by Ag-specific in vitro proliferation and IFN-γ production. Together these results indicate that autologous proteins containing a tyrosine to nitrotyrosine conversion may not be subject to the constraints of immunological tolerance. Accordingly, we propose that the high

![FIGURE 2](image-url)
levels of tissue nitrotyrosine observed in various types of inflammation may actually be provoking a chronic inflammatory condition by acting as potential autoantigens.

To further characterize the response of PCC transgenic mice against nMCC88–103 peptide we generated a panel of nMCC88–103-specific T cell hybridomas from mice that had been immunized with nMCC88–103 peptide (three from wild-type CBA mice and nine from PCC transgenic mice). Although the T cell hybridomas varied in terms of the absolute amount of IL-2 produced in response to Ag stimulation, all 12 hybridomas showed exquisite sensitivity and specificity for nMCC88–103 compared with MCC88–103 peptide; only two of the hybridomas (119-4C9 and CBA-4C8) exhibited weak responsiveness to nonmodified MCC88–103 peptide (Fig. 3A). Once again, the reactivity of T cell hybridomas in response to stimulation with nMCC88–103 peptide plus IEβ-transfected CHO cells confirms that nitration of Y97 does not abrogate the ability of the nMCC88–103 peptide to bind MHC, but, rather, selectively activates a distinct repertoire of IEβ-restricted T cells. To gain a better understanding of this repertoire, TCR β-chain usage was evaluated for one of the hybridomas derived from PCC transgenic mice (119-1F5). The sequence of the TCR β-chain of 119-1F5 was very similar to that of a previously described MCC88–103-specific T cell clone (6.9R.D6) (34). Both clones use the combination of Vβ1 and Jβ1.2; however, they differ in the area of the D region (Fig. 3B).

An additional description of the v structure of MCC88–103 bound to IEβ indicates that the aromatic ring of Y97 is in close contact with the neighboring residue K99, and that Y97 plays a role in proper positioning of K99, which is a critical TCR contact residue (27). By introducing a negative charge on Y97, it is conceivable that the interaction between Y97 and K99 has been disrupted. Regardless of the mechanism, it is interesting to note that the very subtle alteration of TCR β-chain usage found on nMCC88–103-specific T cells is sufficient to allow their escape from negative selection in PCC transgenic mice. Once again, this finding provides evidence that nitrated peptides are unlikely to participate in the process of thymic negative selection and that T cells bearing TCRs capable of recognizing nitrated self-peptides escape this process and enter the periphery.

Lastly, we began to assess the requirements for the production of nitrated ligands in vivo. We used the nMCC88–103-specific T cell hybridoma 119-1F5 as a tool to detect the conversion of MCC88–103 to nMCC88–103 (Figs. 1 and 2 suggest that 0.1 μM is the limit of detection for this system). When APC (peritoneal exudate cells or splenic mononuclear cells) from IEβ-expressing mice were stimulated with IFN-γ plus LPS, they did not convert MCC88–103 to nMCC88–103 at levels detectable by 119-1F5 T cells. This is consistent with the proposed role of the intermediate peroxynitrite in the formation of nitrotyrosine. Peroxynitrite is formed by the combination of NO plus superoxide, the latter of which is unlikely to be produced in the present system. Peroxynitrite itself is very toxic, and addition of low levels of perynitrin (1–2 μM) directly to the assay resulted in almost complete cell death (data not shown). However, when MCC88–103 peptide was treated with peroxynitrite and then added to the assay, 119-1F5 T cells responded robustly, suggesting that if peroxynitrite is produced at low concentrations in vivo, then it is likely that immunoreactive nitrated ligands could accumulate at sites of chronic inflammation. The results presented herein formally demonstrate that autologous proteins containing nitrotyrosine can elicit a robust anti-self-immune response. This phenomenon may be of broad potential significance in the field of autoimmunity, as nitrotyrosine-containing proteins are often present in tissues that are targeted during autoimmunity (35). Thus, we hypothesize that nitrotyrosine-containing autologous proteins may be recognized by the immune system as foreign and may therefore contribute to the process of autoimmunity. Continuous activation of autoreactive nitrotyrosine-specific T cells would
be expected to result in inflammation and an influx of inflammatory cells, such as neutrophils and macrophages, that produce superoxide and NO, leading to further production of nitrosyrtosine-containing autoantigens. Indeed, treatment with inhibitors of iNOS has been repeatedly shown to have a dramatic beneficial effect in models of autoimmunity (36) or in clinical settings such as transplantation (37, 38). We are currently engaged in the process of identifying nitrated proteins at sites of inflammation associated with autoimmune disease to assess whether these proteins constitute targets of the cellular immune response.

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