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Cutting Edge: T Cells Monitor N-Myristoylation of the Nef Protein in Simian Immunodeficiency Virus-Infected Monkeys

Daisuke Morita,*† Tatsuhiko Igarashi,*‡ Mariko Horiike,*§ Naoki Mori,*§ and Masahiko Sugita*†

The use of the host cellular machinery is essential for pathogenic viruses to replicate in host cells. HIV and SIV borrow the host-derived N-myristoyl-transferase and its substrate, myristoyl-CoA, for coupling a saturated C\textsubscript{14} fatty acid (myristic acid) to the N-terminal glycine residue of the Nef protein. This biochemical reaction, referred to as N-myristoylation, assists its targeting to the plasma membrane, thereby supporting the immunosuppressive activity proposed for the Nef protein. In this study, we show that the host immunity is equipped with CTLs capable of sensing N-myristoylation of the Nef protein. A rhesus macaque CD8+ T cell line was established that specifically recognized N-myristoylated, but not unmodified, peptides of the Nef protein. Furthermore, the population size of N-myristoylated Nef peptide-specific T cells was found to increase significantly in the circulation of SIV-infected monkeys. Thus, these results identify N-myristoylated viral peptides as a novel class of CTL target Ag.

Materials and Methods

**Ags**

Chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated. Peptides were synthesized by a manual Fmoc solid-phase peptide synthesis technique using Wang-resin precoupled with a relevant C-terminal amino acid (EMD Chemicals, Gibbstown, NJ). Acylation was carried out by reacting the N-terminal amino group with acid anhydrides prepared with N,N-diisopropylcarbodiimide, followed by release of the acylated peptides in 95% trifluoroacetic acid. Crude Ags were purified by HPLC with a gradient elution based on water and methanol with 0.1% trifluoroacetic acid. After freeze-drying, the samples were subjected to liquid chromatography mass spectrometry analysis, using a C18 column (GL Sciences) at a flow rate of 0.5 ml/min with a solvent system of water and methanol with 0.1% formic acid.

**Establishment of lipopeptide-specific T cell lines**

PBMCs (1.2 × 10^7/well) obtained from rhesus macaque monkeys were cultured with synthetic lipopeptides at a concentration of 5 μg/ml, and antigenic stimulation was repeated every 2 wk in the presence of irradiated autologous PBMCs. IL-2 was added at 0.3 nM after the second stimulation, and the concentration was gradually increased to 3 nM by the fourth stimulation. The culture medium used was RPMI 1640 (Invitrogen, Carlsbad, CA).

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CA supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 2-ME (Invitrogen), penicillin, and streptomycin.

**T cell assays**

T cells (5 × 10^4/well) were incubated with the indicated concentrations of Ags in the presence of irradiated autologous or allogeneic PBMCs (3 × 10^5/well) using 96-well, flat-bottom microtiter plates. After 24 h, aliquots of the culture supernatants were collected, and the amount of IFN-γ released into the media was measured using a human/monkey IFN-γ ELISA kit (Mabtech, Nacka Strand, Sweden).

**Flow cytometry**

The surface expression of T cell markers on the 2N5.1 T cells was analyzed by flow cytometry as described previously (5). For the cytotoxicity assay, the T cell line was labeled by incubating the cells with 500 nM CFSE (Invitrogen) for 10 min at 37˚C. Allogeneic PBMCs (3 × 10^5/well) were cultured in the presence or absence of the CFSE-labeled 2N5.1 T cells (1 × 10^5/well) and/or the C14nef5 Ag (5 μg/ml). After 4.5 h culture, the whole cells were harvested and stained with PE-labeled mouse mAbs to CD3 (SP34-2), CD14 (M5E2), or CD20 (2H7) for 30 min on ice. The labeled cells were washed, and propidium iodide (PI; BD Biosciences) was added to gate out dead cells. The cell samples were analyzed by flow cytometry using a BD FACScanto II flow cytometer (BD Biosciences). Data were collected at a constant flow rate (120 μl/min).

**Animals and viral infection**

The rhesus macaques (Macaca mulatta) used in this study were treated humanely in accordance with the institutional regulations, and experimental protocols were approved by the Committee for Experimental Use of Non-human Primates at the Institute for Virus Research, Kyoto University. Six healthy monkeys were inoculated with SIVmac239 (6) i.v. at a dose of 2000 50% tissue culture-infective dose, and the titer of plasma viral RNA was determined by quantitative RT-PCR as described previously (7).

**IFN-γ ELISPOT assays**

ELISPOT plates (Millipore, Billerica, MA) were coated with the GZ-4 anti–IFN-γ Ab (10 μg/ml) at 4˚C overnight. After blocking with RPMI 1640

**FIGURE 1.** The response of 2N5.1 to N-myristoylated Nef peptides. A, The N-terminal 5-mer peptide derived from the mac239 strain of SIV was synthesized, and a myristic acid was conjugated with the glycine residue to generate the C14nef5 lipopeptide. B, The synthetic compound was analyzed by liquid chromatography mass spectrometry. A single peak was observed at a retention time of 19 min (left panel), and the ions with a mass-to-charge ratio of 614.4 and 636.4 were detected that corresponded to the proton and sodium adducts of C14nef5 with the exact mass of 613.41 (right panel). C and D, The 2N5.1 T cells (5 × 10^4/well) were stimulated with the indicated Ags (5 μg/ml) in the presence of irradiated autologous PBMCs (3 × 10^5), and the amount of IFN-γ released into the culture media was measured. Assays were performed in triplicate samples, and the mean values with the SD are shown. E and F, The 2N5.1 T cells were stimulated with either C14nef5 or mutant Ags, and the amount of IFN-γ released into the media was measured.

**FIGURE 2.** Expression of CD8 molecules and perforin by the 2N5.1 T cells. A, The 2N5.1 T cells were tested for their expression of T cell markers by flow cytometry. A dotted line in each panel indicates a histogram with a negative control Ab. B, The 2N5.1 T cells (5 × 10^4/well) were cultured with autologous PBMCs (3 × 10^5/well) in either the presence or the absence of the C14nef5 Ag (5 μg/ml), and the amount of perforin released into the culture media was measured.
supplemented with 10% FCS, monkey PBMCs (2.5 × 10^5/well) were incubated with Ags (5 μg/ml) for 20 h. The plates were then washed with PBS, and spots representing IFN-γ–secreting cells were detected by sequential incubation with the biotinylated 7-B6-1 anti–IFN-γ Ab (1 μg/ml) for 2 h at room temperature, followed by HRP-conjugated streptavidin for 1 h and tetramethylbenzidine for 10 min (all from Mabtech). Samples were analyzed in duplicate, and the mean numbers of the spots were calculated.

Results and Discussion
To address whether T cells could specifically recognize N-myristoylated Nef peptides, PBMCs obtained from rhesus macaque (M. mulatta) monkeys were stimulated repeatedly in vitro with an array of synthetic N-myristoylated peptides derived from the SIV Nef protein. As a result of this attempt, a T cell line, termed 2N5.1, was established that proliferated in response to the N-myristoylated Nef 5-mer peptide (C14nef5) (Fig. 1A, 1B). The cells produced IFN-γ in response to C14nef5, but not N-myristoylated 3-mer (C14nef3), 4-mer (C14nef4), and 6-mer (C14nef6) of the Nef peptide (Fig. 1C). Furthermore, the 2N5.1 T cell activation was not observed when myristic acid and the 5-mer peptide were added as a free form (Fig. 1D), suggesting that

FIGURE 3. Elimination of C14nef5-pulsed monocytes by the 2N5.1 T cells. A, Allogeneic PBMCs (3 × 10^5/well) were incubated in either the presence or the absence of the CFSE-labeled 2N5.1 T cells (1 × 10^5/well) and the C14nef5 Ag for 4.5 h. The cells were then stained with PE-labeled Abs to CD3, CD20, or CD14, and PI-unstained live cells were analyzed by flow cytometry. PBMC-derived T cells, B cells, and monocytes that survived were boxed in the top, middle, and bottom panels, respectively. Note that monocytes were specifically eliminated when PBMCs were incubated in the presence of the 2N5.1 T cells and the C14nef5 Ag (indicated with an arrow). B, The absolute number of recovered CD3+ (top panel), CD20+ (middle panel), and CD14+ cells (bottom panel) during 1 min of flow cytometric analysis is shown.

FIGURE 4. T cell responses to myristoylated Nef peptides in SIV-infected monkeys. A, Six healthy rhesus monkeys were inoculated with SIVmac239 i.v. at a dose of 2000 50% tissue culture-infective dose. Before (n = 3, left panel) and 5 wk postinfection (n = 6, right panel), PBMCs were obtained and stimulated with the indicated Ags, followed by detection of IFN-γ–producing cells in ELISPOT assays. The numbers of Ag-specific spots in individual subjects are shown with dots, and the mean values are indicated with bars. B, The numbers of C14nef5-specific spots and the virus load at 7 wk postinfection are plotted for each subject. The linear line was drawn on the basis of the linear least-square method, and the value of correlation coefficient was −0.41 (n = 6). *p < 0.05.
the T cells specifically recognized the 5-mer peptide that was conjugated covalently with myristic acid. Freshly isolated PBMCs responded to Con A, but not C14nef5 (Fig. 1E), ruling out the possibility that C14nef5 functioned as a non-specific T cell mitogen.

An additional series of mutational analyses revealed a fine specificity for the lipopeptide recognition by the T cells. The 5-mer Nef peptide conjugated with a shorter (C10) saturated fatty acid (C10-GGAIS) showed reduced T cell stimulation activity compared with C14nef5, and no T cell response was detected for C6-GGAIS (Fig. 1F), further confirming that the peptide modification with a fatty acid of the C14 chain length (myristic acid) was essential for activation of the 2N5.1 cells. The N-terminal amino acid sequence (GGAIS) of the Nef protein matches with a typical N-myristoylation motif, Gly-X-X-X-(Ser/Thr), in which X is any amino acid (3). Whereas the serine-to-threonine substitution (C14-GGAI→AIS) did not affect the antigenic activity, alanine substitution for either the second glycine residue (C14-GAAS) or the isoleucine residue (C14-GGAAS) located between the conserved flanking amino acid residues totally abrogated the activity (Fig. 1F). Furthermore, an add of an amide linkage to the carboxyl group of the C-terminal serine residue (C14-GGAAS-NH2) resulted in total loss of the antigenic activity (Fig. 1G). These results indicated that the amino acid sequence of the C14nef5 lipopeptide constituted a T cell epitope that was short in length but still stringent in terms of amino acid selection compared with that recognized by classical MHC-restricted, peptide-specific T cells.

As expected from the highly specific recognition of peptide sequences, the 2N5.1 T cell line expressed clonotypic TCR-α- and β-chains with random N-additions (data not shown). The 2N5.1 T cells were found to be CD4 negative and positive for CD8α- and CD8β-chains (Fig. 2A). Upon antigenic stimulation, the cells could secrete perforin (Fig. 2B), suggesting that the 2N5.1 T cells were both phenotypically and functionally defined as CTLs. To directly assess the Ag-dependent cytolytic activity exerted by the T cells, freshly isolated PBMCs were used as target cells for CFSE-labeled 2N5.1 T cells in an in vitro culture in either the presence or absence of the C14nef5 Ag. After 4.5 h, all of the cells were harvested and labeled with PE-conjugated Abs to CD3, CD20, or CD14. Whereas PI-stained dead cells were gated out, PBMC-derived T cells (CD3+, CSFE- cells), B cells (CD20+ cells), and monocytes (CD14+ cells) as well as the 2N5.1 effector cells (CSFE+ cells) were monitored by flow cytometry (Fig. 3A). The cell populations representing the recovered target T cells (Fig. 3A, boxed in the top panels) and B cells (Fig. 3A, boxed in the middle panels) were unchanged throughout the panels, and the absolute numbers of the cells were virtually the same regardless of the presence or absence of the T cells and the C14nef5 Ag (Fig. 3B, top and middle panels). In sharp contrast, the number of recovered CD14+ monocytes was markedly reduced after culture in the presence of both the 2N5.1 T cells and the C14nef5 Ag (indicated with an arrow in Fig. 3A and shown in the bottom panel of Fig. 3B), demonstrating Ag-dependent killing of monocytes by the T cells. Consistent with this, peripheral blood monocytes purified by an MACS-based procedure could fully stimulate the 2N5.1 cells to produce IFN-γ (data not shown). On the basis of these observations, we concluded that the C14nef5-specific T cells were CTLs with monocytes as a major target cell type.

The results described above underscored the capacity for T cells to recognize the myristoylated Nef peptide, but the relevance of this to SIV infection in vivo remained to be established. We therefore wished to determine if such T cell responses directed specifically against myristoylated viral peptides might be elicited in SIV-infected monkeys. PBMCs were obtained from monkeys before and 5 wk postinfection with the mac239 strain of SIV and tested for their reactivity to C14nef3, C14nef4, C14nef5, C14nef6, and three N-terminally myristoylated SIV Gag peptides, C14-GVRN (C14gag4), C14-GVRSNS (C14gag5), and C14-GVRSNSV (C14gag6), in IFN-γ ELISPOT assays. Whereas no Ag-specific T cell responses were detected before infection, the number of T cells that recognized C14nef5 was significantly increased after SIV infection (Fig. 4A), suggesting that the immune recognition of C14nef5 indeed occurred in response to SIV infection. In addition, a significant fraction of T cells recognized C14nef6 in infected monkeys, which had not been expected from the study with the 2N5.1 T cell line. Finally, the plasma viral load at 7 wk postinfection in each SIV-infected monkey appeared to correlate reciprocally with the number of C14nef5-specific T cells (Fig. 4B).

It has been a challenge over the past two decades to develop effective vaccines against human infection with HIV. Unfortunately, the development of HIV vaccines designed for activating classical peptide-specific, MHC class I-restricted CTLs has had only limited success so far (8). The vaccine potential of the myristoylated Nef peptides will be tested directly, but one can predict that this new class of lipopeptide vaccine candidates may have a couple of important advantages over classical protein/peptide vaccines. Introducing amino acid mutations in the target proteins is an efficient strategy that HIV has evolved to escape from CTL attack, but the short stretch of the N-terminal amino acid residues of the Nef protein that contains N-myristoylation signal is hard to mutate without affecting the function of the protein (4). Although our preliminary studies indicated that the lipopeptide presentation could occur independently of CD1 function, the C14nef5-specific 2N5.1 T cell response was elicited by using PBMCs from any donor rhesus macaque monkeys as APCs, suggesting that the response could potentially be mediated by nonpolymorphic elements shared in all of the individuals. HIV uses the host cellular machinery for N-myristoylation of the Nef protein to favor its replication in the host, but the current study indicates that this results in unintended expansion of the Ag repertoire recognized by the host immune system. Lipid modifications are critical for a significant number of pathogenic viral proteins to function, but the host immunity appears to have evolved the ability to sense this post-translational event.

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Disclosures
The authors have no financial conflicts of interest.
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