A Model for Antigen-Specific T-Cell Anergy: Displacement of CD4-p56 lck Signalosome from the Lipid Rafts by a Soluble, Dimeric Peptide-MHC Class II Chimera

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A Model for Antigen-Specific T-Cell Anergy: Displacement of CD4-p56\textsuperscript{\textit{lck}} Signalosome from the Lipid Rafts by a Soluble, Dimeric Peptide-MHC Class II Chimera\textsuperscript{1}

Sunil Thomas, Rajeev Kumar, Anca Preda-Pais, Sofia Casares, and Teodor-D. Brumeanu\textsuperscript{2}

Soluble, dimeric peptide-MHC chimeras were shown to induce Ag-specific T cell anergy in vitro and in vivo. In this study, we describe a mechanism by which a soluble, dimeric peptide MHC class II chimera (DEF) induces Ag-specific T cell anergy. The anergic cells showed a displacement of the CD4-p56\textsuperscript{\textit{lck}} signaling module from the GM1-rich plasma membrane microdomains (lipid rafts), and subsequently an increase in p56\textsuperscript{\textit{lck}} kinase activity, a dominant expression of p21 inhibitory TCR ζ-chain, and a poor phosphorylation and recruitment of ζ-associated protein of 70 kDa kinase to the TCR’s immunoreceptor tyrosine-based activation motifs. The Th1 and Th2 transcription was suppressed and the cells were arrested in the Th0 stage of differentiation. Recovery from DEF anergy occurred late and spontaneously at the expense of low thresholds for activation-induced cell death. In contrast to DEF, a combination of TCR and CD4 mAbs did not induce such alterations or anergy, indicating that the ligand-mediated topology of TCR and CD4 coengagement can differentially affect the T cell function. Our results argue for a model of anergy in which the defective partitioning of signaling molecules in lipid rafts is an early, negative signaling event in T cells. Physiological ligands like DEF chimeras may provide new tools for silencing the autoimmune processes, and may also help in deciphering new mechanisms of negative regulation in T cells.

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\textsuperscript{1}Abbreviations used in this paper: ZAP-70, \textit{zeta} associated protein of 70 kDa (ZAP-70); p56\textsuperscript{\textit{lck}}, \textit{zeta} associated protein of 70 kDa-associated protein of 70 kDA (ZAP-70); 3) dephosphorylation of p56\textsuperscript{\textit{lck}} kinase by phosphatases (14); and 4) Cbl-mediated ubiquitination and degradation of ZAP-70 protein (15). In this study, we describe a mechanism by which poor phosphorylation and recruitment of ZAP-70 to the TCR occurs in the context of p56\textsuperscript{\textit{lck}} sequestration in the nonlipid rafts of plasma membrane in T cells.

It has been demonstrated that the GM1 glycosphingolipid-rich microdomains of plasma membrane (lipid rafts) are foci of signal transduction (16). Various receptors and signaling molecules are differentially distributed in the lipid rafts of resting T cells. For example, the CD4 coreceptor and its associated p56\textsuperscript{\textit{lck}} kinase are mostly located in the lipid rafts (17, 18), whereas the CD3 ε-chain and TCR reside mostly in the nonlipid rafts (19). In contrast, the TCR ζ-chain is recruited in the lipid rafts of resting T cells (20). During the T cell activation subsequent to the formation of immunological synapse as mediated by TCR interaction with the MHC-peptide complexes on APCs (21), various protein receptors like CD2, CD3, CD28, and TCR (22), and protein tyrosine kinases like p56\textsuperscript{\textit{lck}}, p59\textsuperscript{\textit{src}}, and phosphatidylinositol 3-kinase (23–25), as well as protein commuters like linker for activation of T cells and Grb2 (25, 26), are concentrated in the lipid rafts. Meanwhile, protein phosphatases with a negative regulatory effect on TCR signaling like Src homology protein-1 are recruited into the lipid rafts, while others like CD45 extracellular phosphatase are excluded (16). In this study, we found that the sole interaction of a soluble, dimeric peptide-MHC-II ligand (DEF) with the TCR and CD4 coreceptor on resting CD4 T cells at high receptor occupancy drastically altered the topology of the CD4-p56\textsuperscript{\textit{lck}} signalosome in the plasma membrane microdomains leading to T cell anergy.

Soluble, dimeric peptide-MHC chimeras built on an Ig scaffold were shown to induce Ag-specific T cell anergy in vitro and in vivo (Refs. 7, 8, 27, and 28). These chimeric molecules are physiological ligands genetically engineered to recognize the peptide-specific TCR and CD4 coreceptor on T cells. We have engineered the first soluble, dimeric (HA110–120)-I-E\textsuperscript{d} chimera (DEF) on an
IgG2a scaffold (29), and showed that at low TCR and CD4 occupancy, DEF induces hemagglutinin (HA)-specific Th2 cell differentiation by a negative regulation of STAT4 phosphorylation (30), while at high receptor occupancy induces HA-specific T cell unresponsiveness by a blockade of CD3/TCR signaling (28). In this study, we found that the signaling blockade occurs due to the displacement of the CD-p56^c^k signalosome from the GM1-rich microdomains of the T cell plasma membrane, followed by poor activation of ZAP-70 kinase with subsequent suppression of Th1 and Th2 transcription.

**Materials and Methods**

**Cells**

Naive CD4 T cells were negatively sorted on mouse CD4 column kits (R&D Systems, Minneapolis, MN) from the spleen of nonimmunized HA-specific transgenic mice (29). Specific CD4 T cells express the 13.4d TCR that recognizes the HA110-120 immunodominant epitope of PR8/A/34 influenza virus in context of I-E^b^ class II molecules (31).

**TCR and CD4 ligands**

The soluble dimeric peptide-MHC-II chimera (DER) is a physiological ligand for the TCR and CD4 coreceptor, which consists of the I-E^b^ and I-E^b^ extracellular domains stabilized through a murine Fcgamma fragment at the C terminus of I-E^b^ chain, and expresses the HA110-120 immunodominant CD4 epitope of HA of PR8 influenza virus covalently linked to the N terminus of I-E^b^ chains (29). Negatively sorted, CD4 TCR-CD4 T cells were incubated with DEF for 4 h at 37°C at high TCR and CD4 receptor occupancy (50 ng/ml/10^6^ cells). The CD4 (GK1.5) mAb is a rat IgG2a/k and the TCR V88 (F23) mAb is a mouse IgG1/k obtained by affinity chromatography from hybridoma-secreting cells. The removal of DEF and Ab ligands bound to the cells before carrying out the assays was achieved by washing the cells for 10 min with 0.1 M sodium acetate buffer, pH 4.5, on ice followed by washing with PBS.

**[^3H]Thymidine incorporation ([^3H]Tdr) and cytokine assays**

Spleen cells (10^6^) from TCR-CD4 Tg mice were exposed to the ligands at high TCR and CD4 occupancy (20,000 ligand/ml/10^6^ cells) for 4 h, the ligands were removed, and the HA110-120 peptide (10 pg/ml) was added to the cultures for 3 days, in the presence or absence of rIL-2 (20 IU/ml/10^6^ cells). The tritiated thymidine ([^3H]Tdr) was added for the last 24 h, cells were harvested on Skatron filter paper (Sterling, VA), and the cpm was measured in a beta-sciillation chamber. The production of IL-2, IFN-γ, and IL-4 by the supernatants was measured in the second and third day of proliferation by ELISA kits according to the manufacturer’s instructions (BioSource International, Camarillo, CA).

**Flow fluorescent analyses (FACS)**

To determine the number of cell divisions, negatively sorted CD4 TCR-CD4 T cells were first incubated with 50 µM CFSE (Sigma-Aldrich, St. Louis, MO) for 2 h in RPMI 1640 medium supplemented with 10% FCS, cells were washed, and then treated with ligands as described. Some 2 × 10^6^ cells were rested for 8 and 24 h in ligand-free medium, and the CFSE fluorescence intensity among 20,000 cells was acquired among gated HA-specific T cells (stained with 6.5.2 mAb-PE conjugate) by an Epics Profile Analyzer II cytofluorometer (Corixa, Hialeah, FL). To determine the annexin V expression on cells treated or not with ligands, the cells (2 × 10^5^) were made free of ligands as described, resuspended in 1% BSA/PBS/0.05% NaN_3, and doubly stained for 30 min on ice with annexin V mAb-PE (BD PharMingen, San Diego, CA) and 6.5.2 clonotypic mAb-FITC conjugates. Cells were washed with 1% BSA/PBS/0.05% NaN_3, fixed in 1% paraformaldehyde, and 20,000 events were acquired by a FACS analyzer. To determine the expression level of HA-specific TCR and CD4 on T cells treated or not with ligands, cells were rested for 2 or 18 h in ligand-free medium, washed, and incubated or not with 50 µM cycloheximide, and then doubly stained with 6.5.2 mAb-FITC and GK1.5 mAb-PE conjugates (BD PharMingen). The signal-to-noise background was subtracted from each sample stained with FITC- and PE-labeled isotype control Abs.

**Western blot analyses**

Negatively sorted, splenic CD4 TCR-CD4 Tg T cells (20 × 10^6^) incubated with ligands and then rested in ligand-free medium for 2, 12, or 24 h, were washed and lysed on ice in native conditions in the presence of a mixture of protease inhibitors (Roche Diagnostic, Indianapolis, IN). The total cell lysates were cleared by centrifugation for 10 min at 300 × g and the cell membranes were isolated by ultracentrifugation for 120 min at 200,000 × g. The membrane pellets were solubilized in native lysis buffer and the protein extracts were cleared for 2 h at room temperature with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) bound to isotype control Abs. Some 50 µg of the membrane protein extracts were precipitated for 24 h at 4°C with 10 µg of specific Abs (GK1.5 and F23 mAbs). Primary Abs used in Western blot analyses for the detection of p56^c^k, p59^c^k, and ZAP-70 were from Upstate Biotechnology (Lake Placid, NY). The immunoprecipitates were captured on protein A/G-agarose gel washed two times with lysis buffer and once with PBS, and boiled for 5 min in electrophoresis buffer containing 0.1% SDS and 5% 2-ME. To detect STAT5, the lysates were precipitated with a rabbit STAT5 Ab (Santa Cruz Biotechnology) and separated by SDS-PAGE in 12% homogenous PhastGels (Amersham Pharmacia Biotech, Piscataway, NJ). To isolate the proteins bound to the TCR chain, the protein extracts from the plasma membrane were precipitated with a TCR chain mAb covalently coupled to agarose (Santa Cruz Biotechnology), the Ab precipitate was separated by SDS-PAGE in 8–25% gradient PhastGels, and electrophoresed onto polyvinylidene difluoride (PVDF) membranes in semidry conditions. The PVDF membranes were blocked with 3% BSA/PBS, and developed with RCL/p42, p44 phosphotyrosine-agarose (BD Transduction Laboratories, Lexington, KY). To confirm the identity of phosphorylated proteins, the PVDF membranes were stripped off for 20 min with 0.1 M glycine/ HCl, pH 2.8, blocked again with 3% BSA/PBS, and reprobed with specific Abs followed by incubation for 2 h with protein A-agarose conjugate (1/20,000 dilution; Sigma-Aldrich). The HRP chemiluminescent activity was measured on PVDF membranes by a Lumilight Western Blot Substrate kit according to the manufacturer’s instructions (Roche Diagnostic Systems). For the detection of GM1 glycosphingolipid, individual sucrase gradient fractions (5 µl per fraction) from negatively sorted CD4 TCR-CD4 T cells treated or not with ligands were diluted four times in Laemmli sample buffer and boiled for 5 min. Proteins were separated in 8–16% gradient polyacrylamide gels (Bio-Rad, Hercules, CA) at a 30 mA constant current. The gels were transferred to PVDF membranes (0.45 µm) at 1.5 mA/cm^2^ for 2 h and blocked overnight at 4°C with PBS-3% BSA. The PVDF membranes were incubated for 1 h at 37°C with cholera toxin subunit B (CTB)-HRP conjugate (1 µg/ml in PBS-1% BSA; Sigma-Aldrich), washed with PBS/0.05% Tween 20, incubated with HRP chemiluminescent substrate, and exposed onto X-OMAT Kodak films (Rochester, NY).

**RT-PCR**

Total RNA was isolated from 3 × 10^6^ negatively sorted CD4 TCR-CD4 T cells treated or not with ligands, and rested in ligand-free medium for 2, 12, and 24 h, using a QiAamp RNA Blood Mini Kit (Qiagen, Valencia, CA) as recommended by the manufacturer. One microgram of total RNA was used to prepare first-strand cDNA using a Qiagen One Step RT-PCR kit following the manufacturer’s protocol. The specific primers for each cDNA construct are shown in Table I. A 1/10 dilution of the RT-PCR product was subjected to electrophoresis in 1.5% agarose gel and the percent of transcripts was calculated by Scion Image software analysis (FREDERICK, MD), based on the integration of the percent of pixels and band intensity after normalization against the corresponding β-actin band in each sample.

**Isolation of T cell plasma membrane microdomains**

The plasma membrane microdomains from negatively sorted CD4 TCR-CD4 T cells treated or not with ligands, and rested in ligand-free medium for 2, 12, and 24 h were isolated following a modified protocol described by Cottin et al. (32). Cells (200 × 10^6^) were washed, lysed by sonication on ice (five times of 10 s per cycle at 100 W), and centrifuged for 5 min at 800 × g. The supernatant was incubated for 1 h at 4°C with 1% Brij 58 detergent in buffer A (25 mM Tris, 150 mM NaCl, 5 mM EDTA supplemented with a mixture of protease inhibitors (Complete Roche Diagnostics). The Brij 58 detergent preserves the association of TCR with the lipid rafts, while solubilizing the lipoglycoproteins from the plasma membrane microdomains. Some 1 ml of each cell lysates was mixed with 1 ml of Brij 58-containing buffer, placed at the bottom of ultracentrifuge tube, and then layered with a step gradient of 80% sucrose made in buffer A, and centrifuged for 18 h and 4°C at 38,000 rpm in a SW41 rotor (Beckman Instruments, Fullerton, CA). Nine fractions of 1 ml each were collected from the top and dialyzed in 0.0015 M saline using Seporator dialysis bags (molecular mass cut-off of 1,000 kDa; Sigma-Aldrich). Fractions were concentrated by speed vacuum to 300 µl and kept frozen at −80°C until used.
and then incubated overnight at 4 °C/H9262/NaCl, 0.1 mM EDTA, 10% glycerol, 1.9 mM MgCl2, 0.5 mM DTT, 0.01% Triton X-100, 25 mM poly(dI-dC) containing 1 ng of 32P-labeled RF-PCR

Table 1. Primers used for RT-PCR

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<th>Gene</th>
<th>Primer Sequence (5′-3′)</th>
<th>Amplification Size (bp)</th>
<th>Ref.</th>
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<td>5′-ATGAGGCGCAAGGCCCTAGG</td>
<td>482 ± 38</td>
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<tr>
<td>Bad</td>
<td>5′-AGACGAGGGCTTCTTGTCAAG</td>
<td>257 ± 38</td>
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<tr>
<td>Bcl-2</td>
<td>5′-GAAGCCGCTTGGCCTTGGAGG</td>
<td>394 ± 38</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5′-GGCCCGGCAAAGGAAGGAAG</td>
<td>220 ± 38</td>
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<td>cMaf</td>
<td>5′-TGGACTTCGACGCCTTGGG</td>
<td>388 ± 67</td>
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<td>c-Myb</td>
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<td>463 ± 68</td>
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<tr>
<td>GATA-3</td>
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<tr>
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<td>β-Actin</td>
<td>5′-CCTGGGCGGCCGCTTGGCAAC</td>
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ELISA

To measure the content of GM1 glycosphingolipid in the sucrose gradient fractions from negatively sorted, CD4 TCR-HA T cells treated or not with ligands and rested in ligand-free medium for 2, 12, and 24 h, some 200 μl were coated overnight at 4 °C. Plates were blocked for 2 h at 37 °C with PBS/1% BSA and incubated for 1 h at 37 °C with CTB-HRP 1 μg/ml in PBS/1% BSA, then washed with PBS/0.05% Tween 20, and incubated for 15 min with 200 μl of 1% HEPES buffer, pH 7.4. GM1high and GM1low pooled fractions were precipitated in PBS/1% BSA, then washed, and resuspended in 1% BSA/PBS containing 0.05% sodium azide, stained for 30 min at 4°C with 10 μg/ml CTB-FITC conjugate, washed again, and permeabilized in CytoPerm solution (BD PharMingen). Permeabilized cells were stained for 30 min on ice with a rabbit anti-p56fyn (Molecular Probes, Eugene, OR), washed, and bound p56fyn 1 Abs were revealed after a 30-min incubation on ice with an anti-rabbit IgG-Alexa 594 conjugate (Molecular Probes). Cells were washed, fixed in 1% paraformaldehyde, centrifuged for 5 min on glass covers at 1200 rpm, mounted in Vectashield medium (Vector Laboratories, Burlingame, CA), and sealed with Permount solution (Fisher Scientific, Springfield, NJ). Several 0.5 μm cross-sections were analyzed by four-color CLSM in an inverted Leica microscope (Deerfield, IL) equipped with fluorescence filters for double excitation at λ = 488 and 594 nm.

Results

Cross-linking of TCR and CD4 coreceptor by soluble DEF dimer at high receptor occupancy leads to Ag-specific T cell anergy

We had previously shown that the cross-linking of TCR and CD4 on resting, HA-specific CD4 T cells by a soluble dimeric HA110–120/MHC-II chimera (DEF) at low receptor occupancy (0.5–5 μg/ml/107 cells) leads to type 2 cell differentiation by a negative regulation of STAT4 signaling (30). In contrast, the incubation of resting HA-specific CD4 T cells for 4 h with this soluble DEF chimera at high receptor occupancy (50 μg/ml/107 cells) induced T cell unresponsiveness. The unresponsive cells did not proliferate and did not produce cytokines upon restimulation with HA-pulsed APCs or with plastic-immobilized 2C11 mAb (2 μg/ml) plus anti-CD28 (7D4 mAb, 0.5 μg/ml). Under the same conditions, a soluble anti-TCR Vβ8 (F23 mAb), or anti-CD4 (GK1.5 mAb), or their equimolar combination did not induce T cell unresponsiveness (Fig. 1A). This indicated that the topology of TCR and CD4 engagement is an important parameter that can differentially affect T cell function.

FACS analysis of CFSE-labeled DEF unresponsive cells showed a lack of cell division 8 and 24 h after restimulating the cells in ligand-free medium, while a small population among the cells pre-exposed to TCR/CD4 mAb ligands entered a single cycle of cell division. In contrast, resting CD4 TCR-HA T cells stimulated with plastic-immobilized CD3e mAbs plus anti-CD28 mAbs showed two cycles of division within 24 h (Fig. 1B). DEF unresponsiveness was restored upon the addition of rIL-2 (100 IU/ml/107 cells) to the culture, suggesting that the state of unresponsiveness was the result of anergy. A complete state of anergy could not be induced by incubation of cells for 30 min with DEF at high receptor occupancy, while incubation for 60 min reduced the index of HA...
responsiveness only by 50%, indicating that DEF anergy occurred by a cumulative process of negative signaling events (Fig. 1C).

DEF anergy was Ag specific because restimulation of naive CD4 T cells from the spleen of BALB/c mice (previously exposed to DEF or to TCR and CD4 mAb ligands at high receptor occupancy) with plastic-immobilized CD3 mAb plus CD28 mAb showed indexes of proliferation (52,450 ±1,100 cpm) and cytokine production (IL-2, 1,150 ± 53 pg/ml; for IFN-γ, 275 ± 7.8 pg/ml; and for IL-4, 14.8 pg/ml) similar to the control cells (53,240 ± 1,870 cpm; IL-2, 1,014 ±17.5; IL-4, 258 ± 12.5; IFN-γ, 865 ± 5.6 pg/ml).

DEF anergy does not affect the IL-2R-mediated STAT5 signaling pathway

Because the addition of IL-2 reversed the DEF anergy, we investigated the status of IL-2R-mediated STAT5 signaling. Phosphorylation-mediated activation of STAT5 through Janus kinase 3 subsequent to ligation of IL-2/β by IL-2 leads to STAT5 recruitment to the IL-2R followed by STAT5 dimerization and translocation of STAT5 dimers into the nucleus where they bind to and activate several gene promoters, among which is the IL-2 promoter (36). DEF anergic T cells and those pre-exposed or not to TCR/CD4 mAb ligands at high receptor occupancy were sorted on 6.5.2-mAb beads, stimulated or not on beads with rIL-2, then lysed on beads, and precipitated with STAT5 Ab. STAT5 phosphorylation was determined by Western blot using RC 20 anti-pTyr-HRP conjugate (upper panels). The identity of STAT5 was confirmed on stripped-off membranes reprobed with rabbit STAT5 Ab (lower panels). The results of one of the two experiments conducted are shown.

FIGURE 1. Induction of T cell anergy by soluble, dimeric DEF ligand. A, Proliferation and cytokine production of CD4 TCR-HA T cells exposed to DEF, or to TCR and CD4 mAb ligands at high receptor occupancy. After removal of ligands, the cells were restimulated for 3 days with HA110–120 peptide in the absence or presence of exogenous IL-2. Cell proliferation was measured by [3H]TdR in triplicate wells. Cytokine production was measured by ELISA in the cell culture supernatants after 2 days of proliferation. The mean values (picograms per milliliter) were calculated in duplicate wells (±SD for IL-2, 10.5 ± 1.2 pg/ml; for IL-4, 14.8 ± 0.7 pg/ml; and for IFN-γ, 28 ± 2.8 pg/ml). B, Lack of cell division of DEF anergic T cells. CFSE-labeled cells were exposed to the ligands as described, and 8 and 24 h after resting in ligand-free medium, the cells were stained with 6.5.2 clonotypic mAb-PE conjugate. The CFSE fluorescence of gated 6.5.2-positive cells was determined by FACS. Top right panel, The sequential halving CFSE concentrations corresponding to the number of cell cycle divisions. The positive control was CFSE-labeled cells stimulated with plastic-immobilized 2C11 mAb plus soluble CD28 mAb (right panels). C, Induction of complete anergy by DEF is time-dependent. The CD4 TCR-HA T cells were exposed for various intervals of time to DEF at high receptor occupancy. DEF was removed, the cells were restimulated with HA-pulsed APCs for 3 days, and the thymidine incorporation was determined as described. D, The Con A proliferative response in aliquot samples. D, DEF anergy does not affect STAT5 signaling. DEF anergic T cells and those pre-exposed or not to TCR/CD4 mAb ligands at high receptor occupancy were sorted on 6.5.2-mAb beads, stimulated or not on beads with rIL-2, then lysed on beads, and precipitated with STAT5 Ab. STAT5 phosphorylation was determined by Western blot using RC 20 anti-pTyr-HRP conjugate (upper panels). The identity of STAT5 was confirmed on stripped-off membranes reprobed with rabbit STAT5 Ab (lower panels). The results of one of the two experiments conducted are shown.
DEF anergic cells as well in those pre-exposed to mAb ligands, indicating that IL-2R-mediated STAT5 signaling was not affected by DEF anergy (Fig. 1D).

**DEF anergic T cells do not enter apoptosis**

Strong stimulation of resting CD4 T cells through the TCR alone leads to activation-induced apoptosis in vitro (37). Apoptosis did not occur in DEF anergic T cells, because annexin V, an early marker for the expression of phosphatidylserine on the cell surface (long before DNA fragmentation) was present in <8% of these cells as compared with those pre-exposed for 4 h to TCR/CD4 mAb ligands at high receptor occupancy (12.36%; Fig. 2A). Lack of apoptosis was consistent with the results showing reversal of anergy by restimulation with Ag in the presence of IL-2. It had been previously shown that Bcl-2, and in particular Bcl-xI, mRNA expression is increased in long-term surviving cells such as Th1 memory cells (38). Semiquantitative RT-PCR showed higher Bcl-2 and Bcl-xI mRNA levels in DEF anergic cells than in those pre-exposed to TCR/CD4 mAbs or in control cells, whereas the mRNA levels of Bad and Bax antagonists were lower in DEF anergic cells (Fig. 2B). Also, 12 h after cessation of DEF anergic stimulus, the c-Myb mRNA and its protein expression were increased (Fig. 2C, upper left panel). Western blot analysis detected two elevated c-Myb isoforms in DEF anergic cells (81 and 78 kDa), and only one isoform of 78 kDa in cells pre-exposed to TCR/CD4 mAbs. Both c-Myb isoforms bound to their oligonucleotide consensus motif, as indicated by EMSA (Fig. 2C, upper right panel). The results showed that the Bcl-2 and Bcl-xI antiapoptotic signals were up-regulated by the sole interaction of the TCR and CD4 coreceptor with the DEF ligand, but not with TCR/CD4 mAb ligands. Also, the c-Myb antiapoptotic protein was differentially up-regulated, depending on the nature of ligand-mediated TCR and CD4 coligation, i.e., DEF vs TCR/CD4 mAb ligands.

**TCR and CD4 down-modulation does not play a role in induction of anergy by DEF**

TCR or CD4 down-modulation triggered by strong and continuous cross-linking, leads to receptor unavailability on the cell surface for a subsequent challenge with Ag. The extent to which TCR-HA and CD4 down-regulation may have played in induction of DEF anergy was measured in rested cells for 2 h in ligand-free medium by FACS using 6.5.2 elotypic mAb-FITC and GK1.5 mAb-PE conjugates. There was no significant difference in TCR-HA and CD4 expression on DEF anergic T cells (16.5 and 84% fluorescence intensity, respectively) as compared with control cells (17.2 and 88.5% fluorescence intensity, respectively) as determined 12 h after resting the cells in ligand-free medium (Fig. 2D).

To determine a possible compensatory role of de novo synthesis for the internalization of the TCR and CD4 coreceptor, we measured, by FACS, TCR and CD4 expression 16 h after resting the cells in ligand-free medium containing cycloheximide. Although DEF anergic cells showed a decrease in TCR and CD4 expression by 37.5 and 9.5%, respectively, as compared with control cells, those pre-exposed to TCR/CD4 mAbs showed a decrease by 71.8 and 43%, respectively (Fig. 2D). The results indicated that TCR or CD4 down-regulation in DEF anergic cells was not as high as in cells pre-exposed to TCR/CD4 mAbs (not rendered anergic) and, thereby, it was not crucial for induction of anergy by DEF.

**DEF anergic T cells display unique CD4 and p56	xi{lk} partitioning in the plasma membrane microdomains**

GM1 is the major glycosphingolipid of detergent-resistant microdomains (lipid rafts) in the murine T cell plasma membrane and it is a marker for identification of lipid rafts in plasma membrane (24). We have isolated by sucrose gradient centrifugation nine fractions containing detergent-insoluble and detergent-soluble plasma microdomains from DEF anergic T cells, and from cells pre-exposed for 4 h to TCR/CD4 mAb ligands at high receptor occupancy 2, 12, and 24 h after resting the cells in ligand-free medium.

The sucrose gradient fractions were analyzed for the amount and distribution of TCR, CD4 coreceptor, p56	xi{lk}, and p59	xi{fn} kinases in relation to the GM1 glycosphingolipid. Western blot analysis showed GM1 accumulation mostly in detergent-insoluble fractions 1 and 2 from DEF anergic T cells. A small amount of GM1 was also detected in detergent-soluble fractions 5–8 of DEF anergic T cells, but not in those pre-exposed to TCR/CD4 mAb ligands (Fig. 3A). In contrast, GM1 was distributed exclusively in detergent-insoluble fractions 1–4 from control cells. According to these results, we have designated fractions 1–4 as GM1\textsuperscript{high} (detergent-insoluble microdomains, lipid rafts), and fractions 5–8 as GM1\textsuperscript{low} (detergent-soluble microdomains).

Resting CD4 T cells contain >90% of the TCR and CD3e chain in the nonlipid rafts due to their lack of palmitylation, whereas the CD4 coreceptor is palmitylated and resides mostly in the lipid rafts. ELISA showed that the amount of TCR in GM1\textsuperscript{high} fractions was slightly increased in DEF anergic T cells, whereas in cells pre-exposed to TCR/CD4 mAb ligands was highly increased as compared with control cells (Fig. 3B). In contrast to the cells pre-exposed to TCR/CD4 mAb ligands, the DEF anergic cells showed significantly higher amount of CD4 in GM1\textsuperscript{low} fractions. These alterations were detected 12 h after resting the anergic T cells in DEF-free medium, but they were not found in cells pre-exposed to TCR/CD4 mAbs, indicating that: 1) TCR and CD4 partitioning in the plasma membrane microdomains can be differentially altered depending on the nature of coligation, and 2) DEF-mediated displacement of CD4 from the lipid rafts correlated with the state of T cell anergy.

**Displacement of the CD4-p56	xi{lk} signalosome from the lipid rafts correlates with alteration of PTK activity and early TCR signaling**

The p56	xi{lk} and p59	xi{fn} Src kinases are critical for T cell activation. The p56	xi{lk} mediates phosphorylation and recruitment of ZAP-70 kinase to the TCR’s ITAM motifs (13), while the increase in p59	xi{fn} kinase activity was associated with the anergic status of CD4 T cells (39, 40). The p56	xi{lk} kinase, as well as CD4, is palmitylated in resting CD4 T cells (41) and thereby recruited to the lipid rafts where it becomes associated with the CD4 coreceptor. The p59	xi{fn} kinase is also palmitylated and is almost 70% recruited in the lipid rafts of resting CD4 T cells (42).

Two, 12, and 24 h after resting the cells in ligand-free medium, we investigated the extent to which the displacement of the CD4 coreceptor from the plasma membrane lipid rafts of DEF anergic cells could affect the distribution and activity of p56\textsuperscript{lk}, p59\textsuperscript{fn}, and ZAP-70 kinases. Like the CD4 coreceptor, p56\textsuperscript{lk} kinase was displaced from the GM1\textsuperscript{high} to the GM1\textsuperscript{low} moieties in DEF anergic T cells, while the partitioning of p59\textsuperscript{fn} kinase remained unchanged for at least 12 h, as compared with control cells (Fig. 3C). CLSM analysis showed an accumulation of p56\textsuperscript{lk} that did not colocalize with the GM1 microdomains in the plasma membrane of DEF anergic cells as compared with those pre-exposed to TCR/CD4 mAbs (Fig. 3D). This indicated that p56\textsuperscript{lk} redistribution in the nonlipid rafts followed the pattern of CD4 redistribution in DEF anergic T cells. These alterations persisted only for 12 h after resting the cells in ligand-free medium.

We next compared the PTK activity in the CD4 and TCR immunoprecipitates prepared from the pool of sucrose gradient fractions 1–4 (GM1\textsuperscript{high}) and 5–8 (GM1\textsuperscript{low}). The results of Western
blot analysis in TCR and CD4 recovered from immunoprecipitates correlated with the ELISA data on the distribution of these two receptors in the GM1<sup>high</sup> and GM1<sup>low</sup> moieties. Thus, the amount of CD4 immunoprecipitated in GM1<sup>high</sup> fractions from DEF anergic cells was 4.5 times less than in control cells, whereas in GM1<sup>low</sup> fractions was 3 times higher than in control cells, as determined by Western blot with specific Abs and computer integration by Scion Image software. In contrast, the amount of TCR immunoprecipitated in GM1<sup>high</sup> fractions from cells treated with CD4 and TCR Abs was twice as high as in control cells and almost the same in GM1<sup>low</sup> fractions, whereas the amount of TCR immunoprecipitated in GM1<sup>high</sup> fractions was 2.5 times higher than in control cells, and in GM1<sup>low</sup> fractions was 2 times lower than in control cells (data not shown). For all the experiments of PTK-receptor associated PTK activity, as well as for those on protein tyrosine phosphatase (PTP) activity, samples were normalized with respect to the protein content.

The PTK activity in CD4 immunoprecipitates from DEF anergic cells was significantly higher in the GM1<sup>low</sup> pool, whereas in cells pre-exposed to TCR/CD4 mAbs was higher in the GM1<sup>high</sup> than in the GM1<sup>low</sup> pool. The control cells showed poor PTK activity in the GM1<sup>high</sup> pool, and lack of activity in the GM1<sup>low</sup> pool (Fig.
A phosphorylated band of 56 kDa identified by Western blot as p56\textsuperscript{Lck} was always associated with the kinase activity in the GM1 low pool from DEF anergic cells, but not in those pre-exposed to TCR/CD4 mAbs or in control cells. The presence of tyrosine phosphorylated p56\textsuperscript{Lck} in this in vitro kinase assay was consistent with the findings reported by Bernadetta et al. (43), and it was attributed to the ability of p56\textsuperscript{Lck} to autophosphorylate. In addition, DEF anergic cells showed an extra band of 60 kDa in the GM1 high pool identified by Western blot as p56\textsuperscript{Lck}, which was not tyrosine-phosphorylated as determined by Western blot (Fig. 4 B), but was most likely serine-phosphorylated as previously described by Feder and Bishop (35). We also found a lack of p59\textsuperscript{Fyn} phosphorylation and p59\textsuperscript{Fyn}-related PTK activity in the CD4 immunoprecipitates of GM1\textsuperscript{high} and GM1\textsuperscript{low} pools from either DEF anergic cells, cells pre-exposed to TCR/CD4 mAbs, or control cells. These results indicated first that the PTK activity on the CD4 coreceptor was mostly attributed to the p56\textsuperscript{Lck} kinase. Secondly, higher p56\textsuperscript{Lck} activity in GM1\textsuperscript{high} than in GM1\textsuperscript{low} fractions correlated with the displacement of the CD4 coreceptor from the GM1 high to GM1 low fractions in DEF anergic cells. This was consistent with the ELISA and CLSM results showing accumulation of p56\textsuperscript{Lck} in the nonlipid rafts in these cells. These alterations persisted only for 12 h after resting the cells in ligand-free medium, which coincided with the duration of p56\textsuperscript{Lck} sequestration in the nonlipid rafts.

The PTK activity in TCR immunoprecipitates was hardly detected in control cells (Fig. 4 C). However, DEF anergic cells, but not those pre-exposed to TCR/CD4 mAbs, showed the highest PTK activity in GM1\textsuperscript{high} moieties and it was mostly associated with p59\textsuperscript{Fyn} kinase as confirmed by Western blot (Fig. 4D). The p59\textsuperscript{Fyn} kinase can also catalyze the enolase substrate (35).
pattern of PTK activity persisted up to 12 h after the cessation of DEF stimulus.

Analysis of total PTP activity in the GM1 high and GM1 low pools against a monopeptide substrate (Glu and Tyr phosphorylated by natural kinases) at 2 and 12 h after resting the cells in ligand-free medium, using a Universal Tyrosine Phosphatase Assay kit (Takara Bio, Shiga, Japan), showed no differences between the DEF anergic T cells and those pre-exposed to the TCR/CD4 mAb ligands, i.e., 0.1 IU/H9262/l/min and 0.25 IU/H9262/l/min, respectively, as compared with control cells (0.18 IU/H9262/l/min).

Shortly after productive stimulation of T cells through the TCR, the ZAP-70 kinase is recruited to the TCR’s ITAMs where it is phosphorylated by p56\(^{\text{lck}}\). Western blot analysis showed that ZAP-70 was inefficiently recruited to, and poorly if any at all phosphorylated on the TCR-H9256-chain, in both GM1 high and GM1 low pooled fractions from DEF anergic cells (Fig. 4D). In addition, Western blot analysis conducted in immunoprecipitates from GM1\(^{\text{high}}\) and GM1\(^{\text{low}}\) pools from DEF anergic cells (after normalization with respect of protein content) showed an increase in p59\(^{\text{fyn}}\) phosphorylation on the CD3/TCR complex in the lipid rafts (GM1\(^{\text{high}}\) pooled fractions) of DEF anergic cells (Fig. 4D), and a dominant expression of the p21 inhibitory isoform of the TCR-H9256-chain (Fig. 4E). There was no p59\(^{\text{fyn}}\) association with the CD4 coreceptor in either GM1 high or GM1 low moieties from DEF anergic cells (data not shown), but only with the TCR, as found by Western blot analysis. The ZAP-70, TCR-H9256-chain, and p59\(^{\text{fyn}}\) alterations were detected no later than 12 h after resting the cells in DEF-free medium (data not shown), and they were not detected in cells pre-exposed to TCR/CD4 mAbs (Fig. 4, D and E).

In assembly, the results showed that the p56\(^{\text{lck}}\) kinase was responsible for most of the CD4-associated PTK activity and it was displaced together with the CD4 coreceptor from the lipid rafts in
nonlipid raft microdomains of DEF anergic T cells. In contrast, most of the PTK activity associated with the TCR came from p59fyn kinase and it was distributed mostly in the lipid rafts. In contrast to the PTK activity, there was no significant alteration in the PTP activity in DEF anergic cells as compared with control cells, or to those treated with TCR and CD4 Abs. The altered pattern of PTK and TCR signaling persisted up to 12 h after cessation of DEF anergic stimulus, which correlated with the duration of CD4-p56lck displacement from the lipid rafts.

**TH1 and TH2 transcription is down-regulated in DEF anergic T cells**

Several transcription factors are instrumental for differentiation of CD4 T cells (44). AP-1 assists the IL-2 gene activation, whereas STAT4 and the recently discovered Th1 transcription factor T-box expressed in T cells (T-bet) induces secretion of IFN-γ. Among the Th2 transcription factors, STAT6, cMaf, and GATA-3 are critical for IL-4 gene activation. Although NF-ATc appears to down-regulate late IL-4 production, the NF-ATc is an early positive regulator of IL-4. We have analyzed the gene expression and activity of these Th1 and Th2 transcription factors in cells rendered anergic by DEF, as compared with cells pre-exposed to TCR/CD4 mAb ligands at high receptor occupancy. Negatively sorted CD4 splenic T cells from CD4 TCR-HA transgenic mice, pre-exposed to the ligands as described, were rested for 2 and 72 h in ligand-free medium, and then restimulated for 4 h on plastic-immobilized DEF (5 μg/ml). We and others had previously shown that DEF-like chimeras induce potent activation of Ag-specific T cells when immobilized on the plastic surface (29, 45). Stimulation of T cells with plastic-immobilized DEF leaves the CD4 T cells untouched other than CD4-specific T cells for the HA110–120 peptide. The binding of AP-1, NF-AT, and cMaf, but not of GATA, to their oligonucleotide consensus motifs was drastically reduced in DEF anergic T cells up to 72 h after resting the cells in DEF-free medium. In contrast, cells pre-exposed to TCR/CD4 mAbs showed no alterations in AP-1, cMaf, and NF-AT activity, as demonstrated by EMSA (Fig. 5A). Semiquantitative RT-PCR also showed reduction of cMaf, NF-ATc, STAT4, STAT6, and T-bet, but not of GATA-3 and NF-ATp messages (Fig. 5B). In contrast, the NF-ATp mRNA level was up-regulated in DEF anergic cells. Consistent with the data on cell proliferation and cell division, these results indicate that the DEF anergic T cells were arrested in a Th0 state of differentiation (Fig. 5C).
DEF anergic T cells recover late and spontaneously from anergy, at the expense of low thresholds for activation-induced cell death (AICD)

We have analyzed the fate of DEF anergic T cells after spontaneous recovery from anergy, 4 days after resting the cells in DEF-free medium. These cells proliferated and secreted cytokines upon restimulation with HA-pulsed APCs in the absence of exogenous IL-2. However, the pattern of proliferation and cytokine production was quite different from that observed in cells pre-exposed to TCR/CD4 mAb ligands (Fig. 6). DEF anergic cells recovering from anergy reached the peak of proliferation 24 h after restimulation, secreted mostly IFN-γ, and ceased to proliferate 48 h later. At 48 and 72 h after restimulation, the cell viability was decreased to 35 and 10%, respectively, as determined by trypan blue exclusion method. In contrast, cells pre-exposed to TCR/CD4 mAbs reached the peak of proliferation 72 h after restimulation, and secreted mainly IL-2. The viability of these cells was reduced by 58%, only 4 days after restimulation. The results demonstrated that in contrast to CD4 T cells treated with mAb ligands (that were not rendered anergic), DEF anergic T cells recovered late and spontaneously from anergy, and displayed high sensitivity to AICD upon Ag restimulation in the presence of APC-derived costimulation.

Discussion

Soluble, dimeric peptide-MHC chimeras were shown to induce Ag-specific T cell anergy in vitro and in vivo (7, 8, 27, 28). In this study, we deciphered a mechanism underlying the Ag-specific anergy of CD4 T cells as induced by a soluble, dimeric peptide-MHC-II chimera (DEF). The anergy occurred shortly after high occupancy of the TCR and CD4 coreceptor by DEF, but not by a combination of TCR and CD4 Ab ligands, indicating that the nature of ligand-mediated TCR and CD4 cross-linking plays an important role for T cell function. We had previously ruled out the possibility of TCR masking by DEF, and thereby DEF competition for the binding of MHC-II-peptide complexes on APCs by TCR (7).

Van Rensen et al. (46) showed that induction of T cell anergy in vitro by liposomes incorporating peptide-MHC-II complexes occurred in the context of TCR down-modulation. This could be mostly related to a bystander effect due to the internalization of liposomes in T cells. We found that TCR and CD4 down-modulation was not critical for the induction of anergy by the soluble DEF dimer. A low extent of TCR and CD4 down-modulation in DEF anergic T cells was most likely due to its inability to provide a strong interaction with the receptors but rather, it provided a tickling of the receptors. The nuclear magnetic resonance data showed indeed that the soluble peptide-MHC-II molecules exert low binding avidity for the TCR as compared with Abs (47).

DEF anergy did not affect the IL-2R-mediated STAT5 signaling pathway, which argues for a lack of cross-talking between the TCR/CD4 and IL-2R signaling pathways in this anergy system. The anergic cells showed protection against apoptosis for a period of 4 days in the context of Bcl-2, Bcl-xL, and c-Myb up-regulation. The resistance to apoptosis by a mechanism of c-Myb-dependent up-regulation of Bcl-2 has been described in mouse T cell lines (48). Up-regulation of c-Myb itself requires endogenous IL-2 (49). We had previously shown that the cross-linking of the TCR and CD4 coreceptor with the soluble DEF dimer in the absence of APC-derived costimulation induces initially small amounts of endogenous IL-2 (30). This explains why c-Myb could be up-regulated in cells exposed to DEF.

Our data argue for a model of anergy, which relies on defective partitioning of signaling molecules in the lipid rafts leading to early negative signaling events in T cells. In support of this theory are previous data showing that the anergy induced by a suboptimal dose of soluble CD3 (2C11) mAb correlated with the lack of translocation of the CD3/TCR complex into the lipid rafts, followed by
its accumulation in the nonlipid raft microdomains (50). According to the model of DEF anergy, low affinity interactions with the CD4 and TCR at the interface of lipid rafts were unable to induce receptor internalization during a 4-h interval, but were sufficient to induce allosteric changes in the plasma membrane microdomains, leading to the displacement of the CD4-p56\({\kappa}\)-binding signaling module from the lipid rafts, and its accumulation in the nonlipid raft microdomains. Sequestration of p56\({\kappa}\) on ligation with the HIV gp120 protein on the cytoskeleton leading to TCR desensitization has been previously described (51), although a p56\({\kappa}\)-independent CD4-mediated TCR desensitization was observed in this system (52).

The TCR content in the lipid rafts of DEF anergic cells was also slightly increased. It has been shown that the lipid raft microdomains are heterogeneously organized in terms of protein content, and that the ligand-mediated cross-linking of GM1 moieties in these microdomains facilitates an exchange of proteins (53). At a high order of cross-linking by soluble DEF dimer, a lateral diffusion of TCR residing in the marginal zone of the nonlipid rafts toward the lipid raft microdomains may have been promoted by a pseudo-cross-linking of TCR and CD4. Thus, one arm of DEF toward the lipid raft microdomains may have been promoted by a high order of cross-linking by soluble DEF dimer, a lateral diffusion-mediated TCR desensitization was observed in this system (52).

In conclusion, the results of this study showed first that DEF-mediated topology of TCR and CD4 coengagement could differentially affect T cell function. That is because, first, the cross-linking of the TCR and CD4 by DEF at low receptor occupancy polarized the cells toward a type 2 cell differentiation (30), and, second, at high receptor occupancy, only cells exposed to DEF, but not those exposed to TCR/CD4 mAb ligands, were rendered anergic. Second, an early event associated with DEF anergy was the displacement of the CD4-p56\({\kappa}\) signalingosome from the lipid rafts followed by alteration of TCR signaling, and the arrest of Th1 and Th2 transcription. Such alterations were not detected in T cells treated with TCR/CD4 mAb ligands. Third, although the DEF anergic cells recovered late and spontaneously from anergy, they were highly sensitive to AICD. According to these data, one may assume that Ag-specific ligands like DEF chimeras may provide new tools for silencing an Ag-driven autoimmune process, and may also help in searching for new mechanisms of negative regulation in T cells.

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