Rudimentary TCR Signaling Triggers Default IL-10 Secretion by Human Th1 Cells


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Rudimentary TCR Signaling Triggers Default IL-10 Secretion by Human Th1 Cells


Understanding the process of inducing T cell activation has been hampered by the complex interactions between APC and inflammatory Th1 cells. To dissociate Ag-specific signaling through the TCR from costimulatory signaling, rTCR ligands (RTL) containing the α1 and β1 domains of HLA-DR2b (DRA*0101:DRB1*1501) covalently linked with either the myelin basic protein peptide 85–99 (RTL303) or CABL-b3a2 (RTL311) peptides were constructed to provide a minimal ligand for peptide-specific TCRs. When incubated with peptide-specific Th1 cell clones in the absence of APC or costimulatory molecules, only the cognate RTL induced partial activation through the TCR. This partial activation included rapid TCR z-chain phosphorylation, calcium mobilization, and reduced extracellular signal-related kinase activity, as well as IL-10 production, but not proliferation or other obvious phenotypic changes. On restimulation with APC/peptide, theRTL-pretreated Th1 clones had reduced proliferation and secreted less IFN-γ; IL-10 production persisted. These findings reveal for the first time the rudimentary signaling pattern delivered by initial engagement of the external TCR interface, which is further supplemented by coactivation molecules. Activation with RTLs provides a novel strategy for generating autoantigen-specific bystander suppression useful for treatment of complex autoimmune diseases. * The Journal of Immunology, 2001, 167: 4386–4395.

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cells and T cell clones in the absence of costimulatory interactions that complicate dissection of the information cascade initiated by MHC/peptide binding to the TCRα and TCRβ chains. A minimum TCR ligand conceptually consists of the surface of an MHC molecule that interacts with the TCR and the three to five amino acid residues within a peptide bound in the groove of the MHC molecule that are exposed to solvent, facing outward for interaction with the TCR.

In previous studies, we described the design, biochemistry, and biophysical characterization of recombinant TCR ligands (RTLs) derived from MHC class II (12–14). MHC class II molecules are membrane-anchored heterodimers on the surface of APC that bind to the TCR, initiating a cascade of interactions that result in Ag-specific activation and differentiation of clonal populations of T cells. Each polypeptide chain consists of four extracellular domains (α1/2, β1/2), a transmembrane region, and a short cytoplasmic tail. We have used rational drug design and protein molecular engineering to express the α1 and β1 domains of HLA-DR2 as a single exon of ~200 aa residues with various N-terminal extensions containing antigenic peptides. These HLA-DR2-derived RTLs fold to form the peptide-binding/T cell recognition domain of the native MHC class II molecule (14).

Rat MHC class II RT1.B-derived RTLs have been used successfully to protect and treat actively induced and passively induced experimental autoimmune encephalomyelitis (12–16). Here we address a relatively straightforward hypothesis: can the phenotype of autoreactive CD4+ human T cells be modulated therapeutically by treatment with human RTLs? Inflammatory Th1, CD4+ T cells are activated in a multistep process that is initiated by coligation of the TCR and CD4 with MHC-peptide complex present on APC. This primary, Ag-specific signal must be presented in the proper context, which is provided by costimulation through interactions of additional T cell surface molecules such as CD28 with their respective ligands on APC. Stimulation through the TCR in the absence of costimulation, rather than being a neutral event, can induce a range of cellular responses from full activation to anergy or cell death (17). In this report, we describe experiments that document the ability of Ag-specific RTLs to induce a variety of human T cell signal transduction processes as well as modulate effector functions, including cytokine profiles and proliferative potential.

Materials and Methods

RTLs

General methods for cloning, expression, purification, in vitro folding, and biochemical analysis of these molecules have been described previously (12–16). DNA constructs encoding the RTLs on PET-21d plasmid were transformed into BL21(DE3)-competent cells for protein expression. Positive clones were chosen for expression of the HLA-DR2 β2m1-derived RTL303 and RTL311 covalent peptide-coupled molecules. Bacteria were grown in 1-liter cultures to midlogarithmic phase (OD600 0.6–0.8) in Luria-Bertani broth (BD Biosciences, Sparks, MD) containing carbenicillin (50 μg/ml) at 37°C. Recombinant protein production was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside. After incubation for 3 h, the cells were centrifuged and stored at −80°C before processing. All subsequent manipulations of the cells were at 4°C. The cell pellets were resuspended in ice-cold PBS, pH 7.4, and sonicated for 4 × 20 s with the cell suspension cooled in a salt-ice-water bath. The cell suspension was then centrifuged, the supernatant fraction was poured off, and the cell pellet was resuspended and washed three times in PBS and then resuspended in 20 mM ethanolamine, 6 M urea, pH 10, for 4 h. After centrifugation, the supernatant containing the solubilized recombinant protein of interest was collected and stored at 4°C until purification. RTL constructs were purified and concentrated by FPLC ion exchange chromatography using Source 30Q anion exchange chromatography (Amersham Pharmacia Biotech, Piscataway, NJ) in an XK26/20 column (Amersham Pharmacia Biotech), using a step gradient with 20 mM ethanolamine, 6 M urea, 1 M NaCl, pH 10. Homogeneous peaks of the appropriate size were collected, dialyzed extensively against PBS at 4°C, and concentrated by centrifugal ultrafiltration with Centrifloc-10 membranes (Amicon, Beverly, MA). For purification to homogeneity, a finish step used size exclusion chromatography on Superose 6 (Pharmacia Biotech) in an HR16/50 column (Amersham Pharmacia Biotech). The final yield of purified protein varied between 5 and 10 mg/L of bacterial culture, and the molecules used in these studies form well-defined aggregates that are highly soluble in aqueous buffers.

Synthetic peptides

MBP85–99 peptide (ENPVVVFKNIVTPR) and BCR-ABL b3a2 peptide (ATMIFQKSSKALQRPVAS, CABL) (18) were prepared on Applied Biosystems 432A (Foster City, CA) peptide synthesizer using solid phase synthesis. The myelin basic protein (MBP) peptide was numbered according to the bovine MBP sequence (19). Peptides were prepared with C-terminal amide groups and cleaved using thioanisole-1,2-ethanedithiol-dih2O in trifluoroacetic acid for 1.5 h at room temperature with gentle shaking. Cleaved peptides were precipitated with six washes in 100% cold methanol, ethanol, and methanol and stored at −20°C under nitrogen. The purity of peptides was verified by reverse phase HPLC on an analytical VyDAC C18 column.

T cell clones

Peptide-specific T cell clones were selected from PBMC of a multiple sclerosis (MS) patient homozygous for HLA-DRB1*1501 and a healthy donor homozygous for HLA-DRB1*07, as determined by standard serological methods and further confirmed by PCR amplification with sequence-specific primers (20). Frequencies of T cells specific for human MBP85–99 and CABL were determined by limiting dilution assay. BCM were prepared by Ficol1 (American Pharmacdia Biotech, Uppsala, Sweden) gradient centrifugation and cultured with 10 μg/ml of either MBP85–99 or CABL peptide at 50,000 BCM/well of a 96-well U-bottom plate plus 150,000 irradiated (2500 rad) PBMC/well as APC in 0.2 ml medium (RPMI 1640 with 1% human pooled AB serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin G, and 100 μg/ml streptomycin) for 5 days, followed by adding 5 ng/ml IL-2 (R&D Systems, Minneapolis, MN) twice per week. After 3 wk, the culture plates were examined for “clump formation” by visual microscopy and the cells from the “best” 20–30 clump-forming wells among a total of 200 wells per each peptide in each replicate were 5-fold diluted in 5 ml for another 5–6 wk. These cells were evaluated for peptide specificity by the proliferation assay, in which 50,000 T cells/well (washed three times) were incubated in triplicate with 150,000 freshly isolated and irradiated APC/well plus either medium alone, 10 μg/ml MBP85–99 or 10 μg/ml CABL peptide for 3 days, with [3H]thyminidine added for the last 18 h. Stimulation index (SI) was calculated by dividing the mean cpm of peptide-added wells by the mean cpm of the medium alone control wells. T cell isolates with the highest SI for a particular peptide Ag were selected and expanded in medium containing 5 ng/ml IL-2, with survival of 1–6 mo, depending on the clone, without further stimulations.

Subcloning and expansion of T cell number

Selected peptide-specific T cell isolates were subcloned by limiting dilution at 0.5 T cells/well plus 100,000 APC/well in 0.2 ml medium containing 5 ng/ml anti-CD3ε (145-2C11, BioSource, Camarillo, CA) and 5 ng/ml anti-CD28 (93, BD Biosciences) followed by addition of 5 ng/ml IL-2 twice per wk for 1–3 wk. All wells with growing T cells were screened for peptide-specific response by the proliferation assay, and the well with the highest SI was selected and continuously cultured in medium plus IL-2. The clonality of cells was determined by RT-PCR, with a clone defined as a T cell population utilizing a single TCR V gene. T cell clones were expanded by stimulation with 10 ng/ml anti-CD3 in the presence of 5 × 105 irradiated (2500 rad) EBV-transformed B cell lines and 25 × 105 irradiated (2500 rad) autologous APC per 25-cm2 flask in 10% AB pooled serum (BioWhittaker, Walkersville, MD) for 5 days, followed by washing and resuspending the cells in medium containing 5 ng/ml IL-2, with fresh IL-2 additions twice a week. Either autologous or heterologous B cell lines and PBMC were usable, but heterologous cell lines were determined empirically to be even better in supporting T cell expansion. As professional APC, the transformed B cells were enriched with costimulatory molecules and related B cell-derived cytokines which were essentially required for T cell expansion using anti-

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*Abbreviations used in this paper: RTL, recombinant TCR ligand; MS, multiple sclerosis; MBP, myelin basic protein; CABL, BCR-ABL b3a2 peptide (ATMIFQKSSKALQRPVAS, CABL); ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; EP, electrostatic potential; LP, lipophilic potential; P-ERK, phosphorylated ERK; T-ERK, total cellular ERK; MAPK, mitogen-activated protein kinase.
CD3 stimulation. Otherwise, T cells stimulated with anti-CD3 alone or with inadequate costimulation would be turned to an anergic status. In this article, we report a method using an existing EBV-transformed heterologous B cell line and freshly isolated autologous PBMC, similar to a protocol reported by us previously (21). Expanded T cells were evaluated for peptide-specific proliferation and the selected, expanded T cell clone with the highest proliferation SI was used for experimental procedures.

**AV and BV gene expression by RT-PCR**

The clonality of the Th1 cells used in this study was determined by RT-PCR (22), using the AV and BV gene-specific primers in Table 1. Briefly, total RNA was isolated from fresh or frozen pellets cells using the Stratagene RNA Isolation Kit (Stratagene, La Jolla, CA). cDNA was synthesized in a 20-μl volume using Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and an oligo(dT)12-18 primer (Life Technologies), following the manufacturer’s recommendations. For amplification of TCRBV cDNA, a panel of 26 BV and a single BC primer was used. A portion of the BC primer was labeled (either 2–3% was end labeled at the 5’ end of TCRBV cDNA, a panel of 26 BV and a single BC primer was used. A portion of the BC primer was labeled (either 2–3% was end labeled with [32P]ATP, or 50% was end labeled at the 5’ end of the fluorochrome, Cy3 (Amersham Pharmacia Biotech). As a positive control for the reaction, two BC primers (forward and reverse) were used, and the reverse primer was labeled as above. As a positive control for the reaction, two BC primers (forward and reverse) were used, one labeled as above. PCR conditions were as described above. After amplification, 10 μl of each reaction were loaded on a 6% polyacrylamide gel and run at 250 V for 22 min. If the DNA was radioactively labeled, the gel was dried for 1 h, exposed to a phosphor screen for 30 min–1 h, and analyzed by phosphorimaging (Bio-Rad Molecular Imager FX; Bio-Rad, Hercules, CA). If the DNA was fluorescently labeled, the gel was directly imaged on a fluorescent imager (Bio-Rad Molecular Imager FX). In either case, the PCR products of the correct size were quantitated by measuring phosphor or fluorescent signal intensity, and the background was subtracted using an adjacent region below the bands.

**Cytokine detection by ELISA**

Cell culture supernatants were recovered at 72 h and frozen at −80°C until use. Cytokine measurement was performed by ELISA as previously described (23), using cytokine-specific capture and detection Abs for IL-2, IFN-γ, IL-4, and IL-10 (BD PharMingen). Standard curves for each assay were generated using recombinant cytokines (BD PharMingen), and the cytokine concentration in the cell supernatants was determined by interpolation.

**Flow cytometry**

Two-color immunofluorescent analysis was performed on a FACSscan instrument (BD Biosciences, Mountain View, CA) using CellQuest software. Quadrants were defined using isotype-matched control Abs.

**3-Phosphorylation assay**

T cells were harvested from culture by centrifuging at 400 × g for 10 min, washed, and resuspended in fresh RPMI. Cells were treated with RTLs at 20 μM final concentration for various amounts of time at 37°C. Treatment

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Table 1. **AV and BV gene-specific primers**

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Table 2. **TCR BV Primer Sequences**

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*Sources for primer sequences: AV1–8, AV9–29, Ref. 48; (AC/HCA23), AV8.1, and AV8.2, designed by our laboratory based on the sequences published in Ref. 49; BV1–11, BV14–16, BV18–20, BV22–24, BC(5’), BC(3’), AC(5’), and AC(3’), Ref. 50; BV12(B), BV13.1(B), and BV13.2(B), Ref. 51; BV17(B), Ref. 52; BV21, Ref. 53.*
was stopped by addition of ice-cold RPMI, and cells were collected by centrifugation. The supernatant was decanted, and lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl)benzenesulfonfonyl fluoride-HCl) containing 0.8 μM aprotinin, 50 μM bestatin, 20 μM leupeptin, 10 μM pepstatin A, 1 mM activated sodium orthovanadate, 50 mM NaF, 0.25 mM potassium bisperoxo(10-phenanthroline) oxovanadate, 50 μM phenylarsine oxide) was added immediately. After a mixing at 4˚C for 15 min to dissolve the cells, the samples were centrifuged for 15 min, and cell lysate was added. The lysate was mixed and boiled for 5 min, and then aliquots were separated by 15% SDS-PAGE. Protein was transferred to a polyvinylidene difluoride membrane for Western blot analysis. Western blot block buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20, 1% BSA) was added. Primary Ab was anti-phosphotyrosine, clone 4G10 (Upstate Biotechnology, Lake Placid, NY). Secondary and tertiary Ab was determined from an ECF Western blotting kit (Amersham). The dried blot was scanned using a Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA), and chemiluminescence was quantified using ImageQuant version 5.01 (Molecular Dynamics).

ERK activation assay

T cells were harvested and treated with RTLs as for ζ-phosphorylation assay. Western blot analysis was performed using anti-phospho-extra-cellular signal-related kinase (ERK) (Promega) at 1/5000 dilution or anti-ERK kinase (New England Biolabs, Beverly, MA) at 1/1500 dilution and visualized using an ECF Western blotting kit. Bands of interest were quantified as described for ζ-phosphorylation assay.

Ca\(^{2+}\) imaging

Human T cells were plated on polystyrene-coated 35-mm glass bottom dishes and cultured for 12–24 h in medium containing IL-2. Fura-2-ace-toxymethyl ester (5 mM; Molecular Probes, Eugene, OR) dissolved in the culture medium was loaded on the cells for 30 min in CO\(_2\) incubator. After a rinse of fura-2 and an additional 15-min incubation in the culture medium, the cells were used for calcium measurement. Fluorescent images were obtained by an upright microscope (Axioskop FS; Zeiss, Oberkochen, Germany) with a water immersion objective (UplanFL 60×/0.8; Olympus, Melville, NY). Two wavelengths of the excitation UV light (340 or 380 nm) switched by a monochromator (Polychrome 2; T.I.L.L. Photonics, Martinarine, Germany) were exposed for 73 ms at 6-s interval. The intensity of 380 nm UV light was attenuated by a balancing filter (Omega Optical, Brattleboro, VT). The excitation UV light was reflected by a dichroic mirror (FT 395 nm; Zeiss), and the fluorescent image was band-passed (BP500–530; Zeiss), amplified by an image intensifier (C7039-02; Hamamatsu Photonics, Middlesex, NJ), and exposed to a multiple format-cooled CCD camera (C4880; Hamamatsu Photonics). The UV light exposure, CCD control, image sampling, and acquisition were done with a digital imaging system (ARGUS HiSCA; Hamamatsu Photonics). The background fluorescence was subtracted by the imaging system. During the recording, cells were kept in a culture medium maintained at 30°C by a stage heater (DTC-200; Dia Medical Systems, Tokyo, Japan). The volume and timing of drug application were regulated by a trigger-driven superfusion system (DAD-12; ALA Scientific Instruments, Westbury, NY).

Results

This study was initiated to analyze the mechanism by which human RTLs affect human T cell clones in vitro. Two different MHC class II DR2-derived RTLs (HLA-DR2b: DRA*0101, DRB1*1501) were used in this study (Fig. 1). RTL303 (β1α1/MBP\(_{53–99}\)) and RTL311 (β1α1/CABL) differ only in the Ag genetically encoded at the N terminus of the single exon RTL. The

FIGURE 1. Structure-based design of the human HLA-DR2-derived RTLs. A, Schematic scale model of an MHC class II molecule on the surface of an APC. The polypeptide backbone extracellular domain is based on the crystallographic coordinates of HLA-DR1 (Protein Data Bank accession code 1AQD) (24, 25). The transmembrane domains are shown schematically as 0.5-nm cylinders, roughly the diameter of a polyglycine α helix. Color scheme: α-chain, red; β-chain, blue. Residues conserved between human HLA-DR, HLA-DP, HLA-DQ, murine I-A and I-E, and rat RT1.B class II molecules are magenta. Bound peptide is green. The C termini of the MHC class II heterodimers are labeled.

Middle, The HLA-DR2-β1α1-derived RTL303 molecule containing covalently coupled CABL peptide. The backgrond fluorescence was subtracted by the imaging system. During the recording, cells were kept in a culture medium maintained at 30°C by a stage heater (DTC-200; Dia Medical Systems, Tokyo, Japan). The volume and timing of drug application were regulated by a trigger-driven superfusion system (DAD-12; ALA Scientific Instruments, Westbury, NY).
MBP₈₅₋₉₉ peptide represents the immunodominant MBP determinant in DR2 patients (24), and the CABL peptide (18) contains the appropriate motif for binding DR2. The human T cell clones used in this study were selected from a DR2-homozygous patient and a DR7-homozygous healthy control.

Structure-based design of the human HLA-DR2-derived recombinant TCR ligands (RTLs)

Sequence alignment of MHC class II molecules from human, rat, and mouse species provided a starting point for our studies, as previously described (13). Structure-based homology modeling was performed using the refined crystallographic coordinates of human DR2 (25) as well as DR1 (26, 27), murine I-Ek molecules (28), and scorpion toxins (29). Because a number of amino acid residues in human DR2 (Protein Data Bank accession number 1BX2) were missing/not located in the crystallographic data (25), the correct side chains based on the sequence of DR2 were substituted in the sequence, and the peptide backbone was modeled as a rigid body during structural refinement using local energy minimization. These relatively small (~200-aa) RTLs were produced in Escherichia coli in large quantities and refolded from inclusion bodies, with a final yield of purified protein between 15 and 30 mg/L of bacterial culture (14). Fig. 1 shows a schematic scale model of an MHC class II molecule on the surface of an APC (Fig. 1A). The HLA-DR2 β1α1-derived RTL303 molecule containing covalently linked MBP₈₅₋₉₉ peptide (Fig. 1B, left) and the HLA-DR2 β1α1-derived RTL311 molecule containing covalently coupled CABL peptide (Fig. 1C, left), are shown with the color scheme used in Fig. 1A with the primary TCR contact residues colored yellow for clarity. The P2 histidyl, P3 phenylalanyl, and P5 lysyl residues derived from the MBP peptide are prominent, solvent-exposed residues. These residues are important for TCR recognition of the MBP peptide. The corresponding residues in the CABL peptide (P2 threonine, P3 glycine, P5 lysine) are also shown. Immediately striking is the percentage of surface area that is homologous across species. When colored according to electrostatic potential (EP) (Ref. 30; Fig. 1, B and C, middle), or according to lipophilic potential (LP) (Ref. 31; Fig. 1, B and C, right), subtleties between the molecules are resolved that likely play a specific role in allowing TCR recognition of Ag in the context of the DR2-derived RTL surface.

The design of the constructs allows for substitution of sequences encoding different antigenic peptides using restriction enzyme digestion and ligation of the constructs. Structural characterization using circular dichroism demonstrated that these molecules retained the anti-parallel β sheet platform and antiparallel α helices observed in the native class II heterodimer, and the molecules exhibited a cooperative two-state thermal unfolding transition (14).

The RTLs with the covalently linked Ag-peptide showed increased stability to thermal unfolding relative to “empty” RTLs, similar to that observed for rat RT1.B RTLs (13).

Human T cell clones

DR2 and DR7 homozygous donor-derived Ag-specific T cell clones expressing a single TCR BV gene were used to evaluate the ability of Ag-specific RTLs to directly modify the behavior of T cells. Clonality was verified by TCR BV gene expression (data not shown), and each of the clones proliferated only when stimulated by specific peptide presented by autologous APC. DR2 homozygous T cell clone MR#3-1 was specific for the MBP₈₅₋₉₉ peptide, and DR2-homozygous clone MR#2-87 was specific for the CABL peptide. The DR7 homozygous T cell clone CP#1-15 was specific for the MBP₈₅₋₉₉ peptide (Fig. 2).

RTL treatment induced early signal transduction events

We examined ζ-chain phosphorylation in the DR2-homozygous T cell clones MR#3-1 and MR#2-87. MR#3-1 is specific for the MBP₈₅₋₉₉ peptide carried by RTL303, and MR#2-87 is specific for the CABL peptide carried by RTL311. The antigenic peptide on the N-terminal end of the RTLs are the only difference between the two molecules. The TCR-ζ chain is constitutively phosphorylated in resting T cells, and changes in levels of ζ-chain phosphorylation are one of the earliest indicators of information processing through the TCR. In resting clones, ζ was phosphorylated as a pair of

FIGURE 3. TCR ζ chain phosphorylation induced by RTL treatment is Ag specific. DR2-restricted T cell clones MR#3-1 specific for MBP₈₅₋₉₉ peptide or MR#2-87 specific for CABL-b3a2 peptide were incubated at 37°C with medium alone (control) or with 20 μM RTL303 or RTL311. A, Western blot analysis of phosphorylated ζ shows a pair of phosphoprotein species of 21 and 23 kDa, termed p21 and p23, respectively. B, Quantification of these bands showed a distinct change in the p21/p23 ratio that peaked at 10 min. Each experiment shown is representative of at least three independent experiments. Points represent mean ± SEM.
phosphoprotein species of 21 and 23 kDa, termed p21 and p23, respectively. Treatment of clone MR#3-1 with 20 μM RTL303 showed a distinct change in the p23:p21 ratio that reached a minimum at 10 min (Fig. 3). This same distinct change in the p23:p21 ratio was observed for clone MR#2-87 when treated with 20 mM RTL311 (Fig. 3). OnlyRTLs containing the peptide for which the clones were specific induced this type of ζ-phosphorylation, previously observed after T cell activation by antagonist ligands (32, 33).

We monitored calcium levels in the DR2-homozygous T cell clone MR#3-1 specific for the MBP85–99 peptide using single-cell analysis. Although there is a general agreement that calcium mobilization is a specific consequence of T cell activation, the pattern of response and dosage required for full activation remain controversial (34). Four general patterns of intracellular calcium mobilization have been observed, but only the most robust has been correlated with full T cell proliferation. RTL303 treatment induced a sustained high calcium signal, whereas RTL301 (identical with RTL303 except a single point mutation that altered folding properties, F150L) showed no increase in calcium signal over the same time period (Fig. 4).

We further analyzed the effects of RTL on levels of the ERKs key components within the Ras signaling pathway involved in the control of T cell growth and differentiation (35). The activated form of ERKs are themselves phosphorylated (36); thus, a straightforward measure of ERK activity was to compare the fraction of ERKs that were phosphorylated (P-ERK) relative to the total cellular ERKs (T-ERK) present. Within 15 min after treatment with RTLs, the level of P-ERK was drastically reduced in an Ag-specific fashion. 20 μM RTL303 reduced P-ERK by 80% in clone #3-1, and 20 mM RTL311 reduced P-ERK by 90% in clone #2-87 (Fig. 5).

The early signal transduction events that were altered by Ag-specific RTL treatment on the cognate T cell clones led us to investigate the effect of RTL treatment on cell surface markers, proliferation, and cytokines. Cell surface expression levels of CD25, CD69, and CD134 (OX40) were analyzed by multicolor flow cytometry at 24 and 48 h after treatment with RTLs and compared with APC-peptide- or Con A-stimulated cells. CD69 (37) was already very high (~80% positive) in these clones. APC-peptide induced Ag-specific increases in CD25 (38) and CD134 (39) that peaked between 48 and 72 h (data not shown), whereas RTL treatment had no effect on these cell surface markers. Anti-CD3 treatment down-modulated TCR off the surface of the clones, whereas RTL treatment had no effect on TCR cell surface expression levels (data not shown). RTL treatment induced only subtle increases in apoptotic changes as quantified using annexin V staining, and these were not Ag-specific (data not shown). Treatment of T cell clones with RTLs did not induce proliferation when added in solution, when immobilized onto plastic microtiter plates, or in combination with the addition of anti-CD28 (data not shown).

On activation with APC plus Ag, clone MR#3-1 (MBP85–99 specific) and MR#2-87 (CABL-specific) showed classic Th1 cytokine profiles that included IL-2 production, high IFN-γ, and little or no detectable IL-4 or IL-10. As is shown in Fig. 6A, activation through the CD3-ε chain with anti-CD3 Ab induced an initial burst of strong proliferation and production of IL-2, IFN-γ, and surprisingly IL-4, but no IL-10. In contrast, on treatment with RTL303, clone MR#3-1 continued production of IFN-γ but in addition substantially increased its production of IL-10 (Fig. 6A). IL-10 appeared within 24 h after addition of RTL303, and its production continued for >72 h, to 3 orders of magnitude above the untreated or RTL311-treated control. In contrast, IL-2 and IL-4 levels did not show RTL-induced changes (Fig. 6A). Similarly, after treatment with RTL311, clone MR#2-87 (CABL specific) also showed a dramatic increase in production of IL-10 within 24 h that continued for >72 h above the untreated or RTL303-treated control (Fig. 6B). Again, IL-2 and IL-4 levels did not show detectable RTL-induced changes, and IFN-γ production remained relatively constant (Fig. 6B). The switch to IL-10 production was exquisitely Ag specific, with the clones responding only to the cognate RTL carrying peptide Ag for which the clones were specific. The DR7 homozygous T cell clone CP#1-15 specific for MBP85–99 showed no response to DR2-derived RTLs (data not shown), indicating that RTL induction of IL-10 was also MHC restricted.

To assess the effects of RTL pretreatment on subsequent response to Ag, T cell clones pretreated with anti-CD3 or RTLs were restimulated with APC-peptide, and cell surface markers, proliferation, and cytokine production were monitored. RTL pretreatment had no effect on the cell surface expression levels of CD25, CD69, or CD134 (OX40) induced by restimulation with APC-peptide compared with T cells stimulated with APC-peptide that
had never seen RTLs, and there were no apoptotic changes observed during a 72-h period using annexin V staining (data not shown).

As anticipated, anti-CD3-pretreated T cells were strongly inhibited, exhibiting a 71% decrease in proliferation and a 95% inhibition of cytokine production, with continued IL-2R (CD25) expression (Table II; Fig. 7), a pattern consistent with classical anergy (39). Clone MR#3-1 showed a 42% inhibition of proliferation when pretreated with 20 μM RTL303, and clone MR#2-87 showed a 57% inhibition of proliferation when pretreated with 20 μM RTL311 (Table II; Fig. 7). Inhibition of proliferation was also MHC class II specific, as clone CP#1-15 (HLA-DR7-homozygous donor; MBP 85–99 specific) showed little change in proliferation after pretreatment with RTL303 or RTL311 (Table II). Clone MR#3-1 pretreated with RTL303 followed by restimulation with APC-Ag showed a 25% reduction in IL-2, a 23% reduction in IFN-γ, and no significant changes in IL-4 production (Fig. 7). Similarly, clone MR#2-87 showed a 33% reduction in IL-2, a 62% reduction in IFN-γ production, and no significant change in IL-4 production. It was of critical importance, however, that both RTL-pretreated T cell clones continued to produce IL-10 on restimulation with APC-peptide (Fig. 7).

Discussion

The physical design and protein engineering involved in creating the human recombinant TCR ligands (RTLs) were based on our previous work engineering rat RT1.B-derived RTLs (12, 13). The logic for extending this work to human DR2 has recently been described (14). The molecules used in these studies form well-defined aggregates that are highly soluble in aqueous buffers. These aggregates may allow for the “cooperative effect of multipoint binding” (41) necessary for signaling through the TCR. In this article, we demonstrate at the signal transduction level the Ag specificity of these novel human RTLs. The oligomeric state of the RTLs may have allowed us to observe their biological activity and to make the unexpected finding that IL-10 production occurred after Ag-specific RTL treatment on cognate T cells.

The results presented above demonstrate clearly that the rudimentary TCR ligand embodied in the RTLs delivered signals to Th1 cells and support the hypothesis of specific engagement of RTLs with the αβ-TCR signaling complex. Signals delivered by RTLs have different physiological consequences than those that occur after anti-CD3 Ab treatment.

In our system, anti-CD3 induced strong initial proliferation and secretion of IL-2, IFN-γ, and IL-4 (Fig. 6). Anti-CD3-pretreated T cells that were restimulated with APC-Ag had markedly reduced levels of proliferation and cytokine secretion, including IL-2, but retained expression of IL-2R, thus recapitulating the classical anergy pathway (Fig. 7). In contrast, direct treatment with RTLs did...
autologous PBMC (150,000/well) plus peptide-Ag (MBP85 or RTL311 for 48 h, followed by washing with RPMI and restimulation with medium, anti-CD3, or 20 μg/ml RTLs, and IL-10 production was maintained even after stimulation with anti-CD3 or RTL treatment. Pretreatment does not (data not shown), another clear difference between anti-CD3 and RTL treatment. Thus, it is apparent that the focused activation of T cells through Ab cross-linking of the CD3ε chain had vastly different consequences than activation by RTLs, presumably through the exposed TCR surface. It is probable that interaction of the TCR with MHC-Ag involves more elements and a more complex set of signals than activation by cross-linking CD3ε chains, and our results indicate that signal transduction induced by anti-CD3 Ab may not accurately portray ligand-induced activation through the TCR, suggesting that CD3 activation alone likely does not comprise a normal physiological pathway.

The signal transduction cascade downstream from the TCR is very complex. Unlike receptor tyrosine kinases, the cytoplasmic portion of the TCR lacks intrinsic catalytic activity. Instead, the induction of tyrosine phosphorylation following engagement of the TCR requires the expression of nonreceptor kinases. Both the Src (Lck and Fyn) family and the Syk/Zap-70 family of tyrosine kinases are required for normal TCR signal transduction (40). The transmembrane CD4 coreceptor interacts with the MHC class II β2 domain (10). This domain has been engineered out of the RTLs. The cytoplasmic domain of CD4 interacts strongly with the cytoplasmic tyrosine kinase Lck, which enables the CD4 molecule to participate in signal transduction. Lck contains an SH3 domain that is able to mediate protein-protein interactions (42) and that has been proposed to stabilize the formation of Lck homodimers, potentiating TCR signaling after coligation of the TCR and coreceptor CD4 (43). Previous work indicated that deletion of the Lck SH3 domain interfered with the ability of an oncogenic form of Lck to enhance IL-2 production, supporting a role for Lck in regulating cytokine gene transcription (44, 45). T cells lacking functional Lck fail to induce Zap-70 recruitment and activation, which has been implicated in downstream signaling events involving the mitogen-activated protein kinases (MAPK) ERK1 and ERK2 (46).

Although the complete molecular signal transduction circuitry remains undefined, RTLs induce rapid antagonistic effects on ζ-chain phosphorylation and ERK activation. The intensity of the

### Table II. Ag-specific inhibition of T cell clones by preculturing with RTLs

<table>
<thead>
<tr>
<th>Donor 1</th>
<th>Precultured with RTL303</th>
<th>Precultured with RTL311</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>20 μM</td>
</tr>
<tr>
<td>Donor 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone 3-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ APC</td>
<td>439 ± 221</td>
<td>549 ± 70</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>-42.3 (p &lt; 0.01)</td>
<td>-5.6</td>
</tr>
<tr>
<td>Clone 2-87</td>
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<td></td>
</tr>
<tr>
<td>+ APC</td>
<td>1,166 ± 24</td>
<td>554 ± 188</td>
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<tr>
<td>Inhibition (%)</td>
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<table>
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<tr>
<th>Donor 2</th>
<th>Precultured with RTL303</th>
<th>Precultured with RTL311</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>20 μM</td>
</tr>
<tr>
<td>Donor 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone 1-15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ APC</td>
<td>258 ± 48</td>
<td>124 ± 7</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>-5.1</td>
<td></td>
</tr>
<tr>
<td>+ APC + MBP85 (10 μg/ml)</td>
<td>7,840 ± 1,258</td>
<td>7,299 ± 1,074</td>
</tr>
</tbody>
</table>

* Soluble RTL303 or RTL311 were cocultured with T cell clones at 200,000 T cells/200 μl medium for 48 h followed by two washings with RPMI 1640 before the assay. The p values were based on comparison with “untreated” control.

* Irradiated (2500 rad) autologous PBMC (2 × 10^5) were added at an APC:T ratio of 4:1 for 3 days with [3H]thymidine incorporation for the last 18 h.
p21 and p23 forms of $\zeta$ increased together in a nonpeptide-Ag-specific fashion (Fig. 3A), whereas the p23:p21 ratio varied in a peptide-Ag-specific manner (Fig. 3B), due to a biased decrease in the level of the p23 moiety. The antagonistic effect on ERK phosphorylation also varied in a peptide-Ag-specific manner (Fig. 5A). RTL treatment also induced marked calcium mobilization (Fig. 4). The fact that all three of these pathways were affected in an Ag-specific manner strongly implies that the RTLs are causing these effects through direct interaction with the TCR. Experiments are currently under way to quantify the association and dissociation constants of the RTLs for soluble single-chain TCR. These studies will allow us to tailor the affinity of DR2-derived RTLs for engagement with cognate $\alpha$-$\beta$-TCR.

How do we reconcile the fact that the RTLs induce calcium (Fig. 4) but reduced MAPK activation? A sustained calcium flux implies activated ras, which in turn should activate MAP kinases. This sequence is thought to occur during T cell activation. The fact that it is altered with RTL treatment and/or coupled to antagonistic $\zeta$-chain phosphorylation may identify a breakpoint or keypoint of regulation in this signal transduction pathway-feedback loop. Signal transduction studies are under way to investigate the role of MAPK phosphatases in our model system to determine whether they are being specifically up-regulated by RTL treatment. A plausible hypothesis would be that MAPK phosphatases are up-regulated in the absence of coreceptor and/or costimulatory input, shifting the balance toward a lower level of activated MAPK.

The most important new finding presented above is the Ag-specific induction by RTLs of IL-10 secretion. This result was unexpected, given the lack of IL-10 production by the Th1 clones when stimulated by APC-Ag or by anti-CD3 Ab. Moreover, the continued secretion of IL-10 on restimulation of the RTL-pretreated clones with APC-Ag indicates that this pathway was not substantially attenuated during reactivation. This result suggests that TCR interaction with the RTL results in default IL-10 production that persists even on re-exposure to specific Ag. The elevated level of IL-10 induced in Th1 cells by RTLs has important regulatory implications for autoimmune diseases such as MS because of the known anti-inflammatory effects of this cytokine on Th1 cell and macrophage activation.

It is likely that the pathogenesis of MS involves autoimmune Th1 cells directed at one or more immunodominant myelin peptides, including MBP$_{85-99}$. Conceivably, RTLs such as RTL303 could induce IL-10 production by these T cells, thus neutralizing their pathogenic potential. Moreover, local production of IL-10 after Ag stimulation in the CNS could result in the inhibition of activation of bystander Th1 cells that may be of the same or different Ag specificity, as well as macrophages that participate in demyelination. Thus, this important new finding implies a regulatory potential that extends beyond the RTL-ligated neuroantigen-specific T cell. RTL induction of IL-10 in specific T cell populations that recognize CNS Ags could potentially be used to regulate the immune system while preserving the T cell repertoire and may represent a novel strategy for therapeutic intervention of complex T cell-mediated autoimmune diseases such as MS.

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References


