Cutting Edge: In Vivo Identification of TCR Redistribution and Polarized IL-2 Production by Naive CD4 T Cells

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Within minutes of conjugate formation, the TCR and several signal transducers cluster on the T cell membrane at the point of contact with an APC bearing the relevant peptide–MHC molecule (1, 2). Clustering may be explained by the coalescence of lipid microdomains, in which the TCR and many signal transducers are concentrated (3), to the site of T cell–APC contact. Recent experiments have shown that the movement of lipid microdomains (4) and several surface proteins (5) to the site of T cell–APC interface is greatly enhanced by signals through CD28, whereas TCR redistribution did not. These results show that Ag-stimulated CD4 T cells produce IL-2 in a polarized fashion and undergo CD28-independent TCR redistribution in vivo. The Journal of Immunology, 2001, 166: 4278–4281.

Methods and Materials

Mice

BALB/c mice were purchased from Sasco (Omaha, NE) or The Jackson Laboratory (Bar Harbor, ME). DO11.10 TCR-transgenic BALB/c mice (9) were bred in a specific pathogen-free facility according to National Institutes of Health guidelines. CD28-deficient DO11.10 BALB/c mice were produced by crossing DO11.10 BALB/c mice with CD28-deficient BALB/c mice (The Jackson Laboratory) for two generations. Peripheral blood cells from progeny of the second cross were screened for expression of the DO11.10 TCR as described previously (10) and for CD28 by staining with fluorescent-labeled anti-CD28 mAb (BD PharMingen, San Diego, CA). DO11.10 BALB/c SCID mice were produced as previously described (11).

Cell transfer

Single-cell suspensions of spleen and lymph node cells were prepared from DO11.10 mice. A small sample was stained with fluorescent-labeled anti-CD4 mAb and the KJ1-26 mAb, which uniquely recognizes the DO11.10 TCR (12), and analyzed by flow cytometry for the percentage of CD4+ KJ1-26+ cells as described previously (10). The remaining unla beled cell suspension was adjusted to 8.3–10^6 cells/ml in PBS. A portion of this suspension containing 2.5–3.0 x 10^6 CD4+ KJ1-26+ cells (0.3 ml) was then injected i.v. into each unirradiated BALB/c recipient.

Ag injections

Chicken OVA (0.016–2 mg; Sigma, St. Louis, MO) or Texas Red-labeled chicken OVA (Molecular Probes, Eugene, OR) were injected alone or with LPS (25 µg, serotype Escherichia coli 026:B6; Difco Laboratories, Detroit, MI) s.c. into three sites (0.03 ml/site) on the backs of recipient mice.

Abbreviations

Abbreviation used in this paper: MTOC, microtubule organizing center.
Frozen sections (6 μm) through the draining brachial lymph nodes were prepared as described by Pape et al. (13) and fixed in 2% formaldehyde. Slides were incubated with anti-FCR mAb (2.4G2) for 10 min to block Fc-binding sites, and then with avidin and biotin solutions (Vector Laboratories, Burlingame, CA) to block tissue biotin sites. In experiments designed to detect the DO11.10 TCR and IL-2, slides were then incubated in PBS containing 2% FCS and 0.5% saponin (Sigma) for 10 min, washed, and incubated for 30 min with biotin-labeled anti-IL-2 polyclonal Ab (R&D Systems, Minneapolis, MN) and digoxigenin-labeled KJ1-26 mAb. Slides were washed and then incubated for 30 min with sheep anti-digoxigenin Ab (Roche Biochemical, Indianapolis, IN) and streptavidin-labeled HRP (NEN, Boston, MA), both in TNB buffer (NEN). After two washes, the slides were incubated for 10 min with biotinyl tyramide (NEN) in amplification buffer (NEN), washed, and incubated for 30 min in TNB buffer with streptavidin-Cy3 (Catlog, San Francisco, CA), donkey anti-sheep-Cy5 (Jackson ImmunoResearch, West Grove, PA), and FITC-labeled anti-B220 mAb (BD PharMingen). In experiments designed to detect the DO11.10 TCR and Texas Red-labeled OVA, slides were blocked as described above and incubated with biotin-labeled KJ1-26 mAb for 30 min in TNB, streptavidin-labeled HRP for 30 min in TNB, and tyramide-Cy5 (NEN) for 10 min in amplification buffer (NEN). All slides were washed and then covered with Vectashield (Vector Laboratories) to preserve fluorescence.

Confocal laser scanning microscopy

Confocal microscopy and image analyses were performed as previously described (14) using a Bio-Rad MRC-1000 or 1020 confocal microscope equipped with a krypton/argon laser (Bio-Rad Life Science Group, Hercules, CA). Separate images for the FITC, Texas Red or Cy3, and Cy5 channels were collected for each section analyzed. Final image processing and assembly was performed using the Confocal Assistant (Minneapolis, MN) and Photoshop (Adobe Systems, San Jose, CA) software.

Polarity determinations

The relative polarities of the DO11.10 TCR or IL-2 were determined as follows. Photoshop software was used to view images of cells stained with KJ1-26 (detected with Cy5) and IL-2 (detected with Cy3) at 320 pixels/ inch in 67 × 67 pixel fields. The KJ1-26 image was pseudo-colored red, and then individual cells were divided into quadrants with a crosshair through the cell center. The crosshairs were positioned on the cells in the random positions in which they appeared in the field; no attempt was made to rotate each cell to maximize the degree of polarization. The red color in each quadrant was highlighted by selecting “Reds” using the Color Range function with a fuzziness value of 128. The Image/Histogram function was then used to obtain the number of pixels and the average pixel intensity in each quadrant. The pixel number and the average pixel intensity in each quadrant were multiplied to yield a quadrant value proportional to the intensity of staining in each quadrant. TCR polarity was then calculated by dividing the highest quadrant value by the lowest. The process was then repeated for the IL-2 image to calculate IL-2 polarity.

The distribution of the DO11.10 TCR relative to OVA-containing cells in mice injected with Texas Red-labeled OVA was determined as follows. Photoshop software was used to view images of lymph node sections containing conjugates between KJ1-26-stained cells (detected with Cy5) and cells that took up Texas Red, at 288 pixels/inch in 120 × 120 pixel fields. The KJ1-26 image was pseudo-colored green and the Texas Red-OVA image red, and the two images were overlaid to produce a dual color image containing yellow color at the point of contact between the two cell types. Each KJ1-26 image was then divided in half along an axis parallel to the yellow interface. The intensity of green color in the half of the image facing the Texas Red-labeled cell, or in the opposite half, was then calculated using the “Greens” color range function. Each KJ1-26 image was also divided in half along a vertical axis without regard to the yellow interface with the Texas Red-labeled cell, and the intensity of green color in the left and right halves was calculated using the “Greens” color range function.

Results

In vivo stimulation with Ag induces TCR redistribution

Previously we showed that IL-2 production in naive DO11.10 CD4 T cells in the draining lymph nodes peaks 12–14 h after s.c. injection of OVA plus the adjuvant LPS (11). Therefore, the in situ distribution of the TCR on DO11.10 T cells in the lymph nodes of adoptive recipients was examined around this time based on the assumption that Ag presentation and IL-2 production must occur within several hours of each other. As shown previously (13), naive DO11.10 T cells resided only in the T cell-rich paracortical regions of the lymph nodes after transfer (data not shown). The TCR was uniformly distributed in a ring-shaped pattern on naïve DO11.10 T cells in the absence of OVA (Fig. 1A), but was concentrated on one side of many DO11.10 cells in mice that were injected with OVA and LPS 12 h earlier (Fig. 1B).

The distribution of the TCR was quantified by partitioning the image of randomly positioned KJ1-26-stained T cells into quadrants, and then measuring the amount of KJ1-26 signal in each quadrant as shown in Fig. 1. The highest value was then divided by the lowest value, yielding a relative polarity score. If a cell expressed the TCR in a perfectly uniform fashion, then an equal signal would be present in each quadrant, yielding a relative polarity score of 1. The actual mean polarity score of unstimulated naïve DO11.10 cells was ~2, with >95% of the cells possessing values ≤5 (Fig. 2A). Based on this information, cells with relative polarity scores of >5 were counted as having a polarized TCR.

Injection of LPS alone did cause not cause TCR polarization (Fig. 2A). In contrast, ~50% of the DO11.10 T cells in the draining lymph nodes displayed a polarized TCR 12 h after injection of OVA and LPS (or OVA alone, data not shown), and this value increased to a maximum of 72% at 24 h (Fig. 2A). The average degree of TCR polarization in the population of DO11.10 cells, as

![FIGURE 1](http://www.jimmunol.org/). Identification of the cellular distribution of the TCR and IL-2 in Ag-specific CD4 T cells. High power views of several representative DO11.10 T cells from lymph node sections from mice that were injected s.c. with nothing (A) or 2 mg of OVA plus LPS (B). DO11.10 TCR staining is shown in the white, and IL-2 staining is overlaid in red. Two examples are shown to illustrate the quantification method: one cell from a naive recipient (A) and one cell from a recipient injected s.c. with 2 mg of OVA plus LPS 12 h before sacrifice (B). The positions of the crosshairs used to define the quadrants for each cell are shown along with the TCR (white) and IL-2 (red) intensity values for each quadrant (calculated as described in Materials and Methods). The relative polarity scores, calculated by dividing the highest quadrant value by the lowest, for the TCR and IL-2 are indicated below the relevant image.
measured by the mean relative polarity score, also increased with time after immunization, rising from a starting level of 2.0 ± 0.2 (±SEM) to 20.0 ± 3.5 at 12 h, 41.4 ± 4.5 at 24 h, and 34.0 ± 4.5 at 48 h. Therefore, TCR redistribution was apparent at the time of peak IL-2 production (12 h) (11) and was retained until at least 48 h after injection of OVA and LPS (Fig. 2A), when the cells enter the cell cycle (10, 15).

It was next determined whether or not the TCR was redistributed to the side of the DO11.10 T cell in contact with an APC. Texas Red-labeled OVA was injected into recipients of DO11.10 T cells to identify cells that took up OVA and thus were potential APC. Eighteen hours after injection, Texas Red-labeled cells appeared in the T cell areas of the lymph nodes near the follicles (data not shown). DO11.10 T cells were often found in close proximity to a Texas Red-labeled cell (Fig. 3A), and in 29/34 conjugates examined the DO11.10 TCR was higher in the half of the cell facing the Texas Red-labeled cell than the opposite half (Fig. 3B). These interactions were Ag specific because conjugates between Texas Red-labeled cells and adoptively transferred polyclonal T cells were not observed after injection of Texas Red-labeled OVA (data not shown). In addition, the polarization of the DO11.10 TCR toward the Texas Red-labeled cell was not due to chance because partitioning along a vertical axis that did not take into account the position of the Texas Red-labeled cell showed that 15/34 DO11.10 cells had the TCR concentrated on the left side and 19/34 on the right side (Fig. 3C). These results are consistent with the conclusion that the TCR becomes polarized toward the APC at early times during the primary response in vivo.

In situ detection of lymphokine production by Ag-specific CD4 T cells during a primary immune response

Lymph node sections were also stained with KJ1-26 and anti-IL-2 Ab to identify the location of the lymphokine within producing DO11.10 T cells. IL-2 was not detected in DO11.10 cells in mice that were not injected with OVA (Fig. 2C). About 20% of the DO11.10 cells stained with the anti-IL-2 Ab, 12 h after injection of OVA plus LPS (Fig. 2C) as expected from an earlier flow cytometry study (11). The IL-2-producing DO11.10 T cells were usually located in the T cell-rich paracortex, near the B cell-rich follicles (data not shown). IL-2 was always concentrated in a discrete area of the cytoplasm on one side of the DO11.10 T cell (Fig. 1B). Ten randomly selected IL-2+ DO11.10 T cells were analyzed by the quadrant method used above to assess the distribution of the TCR. Nine cells had IL-2 polarity scores >100 (see Fig. 1B for images of six of these cells), and one had a score of 55. In all cases, the area of the cell in which IL-2 was concentrated was also the area of highest TCR concentration (Fig. 1B). Therefore, naive CD4 T cells stimulated by Ag in vivo produced IL-2 in a highly polarized fashion, and the pole containing IL-2 localized with the TCR.

FIGURE 2. TCR redistribution in situ does not require CD28. A, The relative TCR polarity values for 74–86 individual KJ1-26− cells in lymph node sections from BALB/c recipients of naive DO11.10 T cells at the indicated times after injection of 2 mg of OVA plus LPS (•) or LPS alone (○). The percentage of cells with relative TCR polarity scores >5 are shown. Sections from several different animals in each group were included in the analysis. B, The mean relative TCR polarity values (±SEM) for 52–105 individual KJ1-26− cells in lymph node sections from BALB/c recipients of normal (○) or CD28-deficient (●) DO11.10 T cells 24 h after injection of the indicated doses of OVA (plus 25 µg of LPS) are shown. C, The fractions of KJ1-26− cells that also contained IL-2 in lymph node sections from BALB/c recipients of normal or CD28-deficient DO11.10 T cells 24 h after injection of 2 mg of OVA plus LPS (•) or nothing (○, barely visible) are shown.

FIGURE 3. The TCR polarizes to the side of the T cell contacting an APC. A shows a KJ1-26-stained (green) DO11.10 T cell interacting with a Texas Red-labeled cell (red) in the T cell area of a draining lymph node from a mouse that was injected 18 h earlier with Texas Red-labeled OVA. The amount of KJ1-26 signal (×10−5) on the left side of the same 34 DO11.10 cells facing a Texas Red-labeled cell vs the amount on the opposite half (see Materials and Methods for the calculation) is shown in B. The amount of KJ1-26 signal (×10−5) that was on the left side of the same 34 DO11.10 cells vs the amount on the right side is shown in C. The mean KJ1-26 value ± SD for the facing half (32,500 ± 18,400) was significantly greater (P < 0.0001, paired t test) than the mean value for the opposite side (20,600 ± 13,800). In contrast, there was no significant difference between the mean KJ1-26 value for the left (24,800 ± 16,900) and right sides (28,700 ± 17,700) (P > 0.1, paired t test).
CD28 to the site of TCR engagement (4, 5). However, CD28 is not absolutely required for TCR signaling because CD28-deficient T cells reorient the MTOC normally to the site of contact with peptide-MHC-bearing APC in vitro (18) and undergo TCR redistribution (Fig. 2B) and blastogenesis in response to Ag stimulation in vivo (11). This implies that enhancement of TCR clustering is not the only mechanism by which CD28 signaling enhances IL-2 production in vivo. Augmentation of the rate of IL-2 gene transcription or stabilization of IL-2 mRNA transcripts (6, 19, 20) are probably also involved.

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References