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Early Events of TCR Signaling Are Distinct in Human Th1 and Th2 Cells

Sigrid Hannier,1 Christina Bitegye, and Stéphane Demotz2

To study the requirements for activation of human Th1 and Th2 cells, soluble peptide/DR1 complexes were prepared from naturally expressed DR1 protein. When immobilized, this material induced T cell activation, as revealed by CD25 up-regulation. Unexpectedly, Th2 cells required a higher density of peptide/DR1 complexes than Th1 cells to initiate CD25 up-regulation. Similar findings were obtained with immobilized or soluble and cross-linked anti-CD3 mAb. In contrast, peptide/DR1 complexes displayed the surface of nonprofessional APC similarly induced CD25 up-regulation in Th1 and Th2 cells. Signaling events distinguishing human Th1 and Th2 cells following TCR engagement by anti-CD3 mAb were then studied. It was observed that upon TCR triggering, the overall tyrosine phosphorylation profiles were fainter in Th2 than in Th1 clones. Similar results were obtained with Th1- and Th2-polarized polyclonal lines. Varying the dose of anti-CD3 mAb, the kinetics of activation, and coengagement of CD3 and CD28 failed to increase tyrosine phosphorylation in Th2 cells to levels reached in Th1 cells. In contrast, treatment with the tyrosine phosphatase inhibitor phenylarsine oxide resulted in similar tyrosine phosphorylation levels in Th2 and Th1 cells. These findings indicated that Th2 cells had an intrinsically lower TCR-induced tyrosine phosphorylation capacity than Th1 cells, which might be controlled by Th1- and Th2-specific phosphatase profiles. Finally, a weaker association was found between ZAP-70 and CD3ζ in Th2 than in Th1 cells after TCR engagement. Taken together, these results constituted evidence that early events in the TCR signaling cascades are distinct in human Th1 and Th2 cells. The Journal of Immunology, 2002, 169: 1904–1911.

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CD3 mAb. A biochemical analysis of TCR signaling events induced by anti-CD3 Ab revealed fainter tyrosine phosphorylation profiles and a weaker CD3/zAP-70 association in human Th2 than in Th1 cells. Distinct proximal events of the TCR signaling cascades between Th1 and Th2 cells might be due to distinct profiles of phosphatases found in Th1 and Th2 cells.

Materials and Methods

Cell clones and lines

PBMC were obtained by Ficoll-Paque (Amersham Pharmacia Biotech, Orsay, France) density gradient centrifugation of heparinized blood from healthy donors. Measles virus fusion protein-derived peptide 254–268 (F254)-specific, HLA-DRB1*0101-restricted Th1 and Th2 clones were isolated from PBMC (1.5 × 10⁶ cells/200 μl) cultured for 1 wk in the presence of 100 ng/ml of F254 peptide. The cultures were performed under Th1- and Th2-polarizing conditions as previously described (18). Briefly, for differentiation of Th1 cells, cultures were supplemented with 2 ng/ml IL-12 (BD Biosciences, San Jose, CA) and 125 ng/ml anti-IL-4 mAb (clone SD4-8; BD Biosciences). For differentiation of Th2 cells, cultures were supplemented with 7 mg/ml IL-4 (R&D Systems, Minneapolis, MN) and 2 μg/ml anti-IL-12 mAb (clone C8.6; BD Biosciences). Isolation and maintenance of the Th clones were previously reported in detail (19). The three Th1 (F254.7.2, SDMV3.13, and Th1 no. 4) and three Th2 (FR6.6, SDMV3.5, and Th2 no. 1) clones used in this study were isolated from independent cultures, and from two donors.

T cell lines were established by culture of PBMC at 10⁶ cells/ml for 2 wk with 10 ng/ml toxic shock syndrome toxin superantigen (Sigma, Saint Quentin Fallavier, France) in the presence of cytokines and mAb for differentiation into Th1 and Th2 cells, as described for the T cell clones.

Th1 and Th2 polarization was determined by ELISA. Cells producing IFN-γ, but not IL-5, were considered Th1, and cells producing IL-5, but not IFN-γ, were considered Th2. Since differentiation of Th2 cells was conducted in medium containing IL-4, IL-5, rather than IL-4, was determined to establish Th phenotypes.

T cells were cultured in RPMI medium (Life Technologies, Cergy Pontoise, France) supplemented with glutamine, sodium pyruvate, nonessential amino acids, kanamycin, 5% decomplemented AB human serum, and 100 U/ml IL-2 (Tebu, Le Perray en Yvelines, France). Th1 and Th2 cells were simultaneously restimulated for expansion, and were used at least 15 days after restimulation.

The HLA-DR1-transfected HeLa cell line was cultured in RPMI medium supplemented with glutamine, sodium pyruvate, nonessential amino acids, kanamycin, 10% decomplemented FCS, and 1 mg/ml gentamicin (Life Technologies) (20).

Antibodies and chemicals

For T cell stimulation, anti-human CD3 mAb TR66 (mouse IgG1) was a gift from Dr. Lanzavecchia (Biomedical Research Institute, Bellinzona, Switzerland). Anti-human CD28 mAb (clone CD28.2, mouse IgG1) and goat anti-mouse IgG Ab were obtained from Tebu. For Western blotting analyses, anti-phosphotyrosine (4G10) and anti-Lck (clone 3A5) mAb were purchased from Euromedex (Souffelweyersheim, France); anti-p38 (clone A-12), anti-C3δ (clone 6B10.2), anti-ZAP-70 (clone LR), anti-Src homology 2 containing protein tyrosine phosphatase (SHP)-1 (clone C-19), and anti-SHP-2 (clone C-18) mAb were obtained from Tebu. Anti-CD45 (clone BERA-55) mAb was obtained from Sigma. HRP-labeled goat anti-mouse IgG and goat anti-rabbit IgG Ab were from Amersham Pharmacia Biotech (Uppsala, Sweden). Phenylarsine oxide and okadaic acid were purchased from Calbiochem (La Jolla, CA). For flow cytometric analysis, anti-CD25 (clone M-2A5) and isotype-matched IgG1 (clone A112-2) mAb were obtained from BD Biosciences.

Preparation of soluble F254 peptide-loaded HLA-DR1 protein

DR1 protein was purified by affinity chromatography on anti-DR mAb L243-coupled Sepharose as previously described (21). For peptide loading, DR1 was incubated for 2 days at 37°C at pH 5 in the presence of a 60- to 100-fold molar excess of synthetic F254 peptide (sequence 254–268 of the measles virus fusion protein). Synthesis and purification of the F254 peptide were previously reported (20). DR samples were then dialyzed against 0.1% sodium deoxycholate in 20 mM Tris, pH 8.0. Papain was activated by incubation for 10 min at 37°C in 1 mM EDTA, 1 mM DTT, and 20 mM Tris, pH 8.0. Digestion was performed by incubation of 7 vol DR1 protein and 3 vol activated papain for 1 h at 37°C. The reaction was terminated by the addition of 1 vol of a protease inhibitor mixture (Mini Complete; Roche Diagnostics, Mannheim, Germany). DR1 material was subsequently chromatographed over a Superose 12 column (Amersham Pharmacia Biotech) equilibrated in PBS. DR material was analyzed by SDS-PAGE on 12% gels, followed by Coomassie and silver staining (Accurate Chemical & Scientific, Westbury, NY).

Flow cytometric analysis

TCR engagement was performed using plastic-bound F254 peptide/DR1 complexes or anti-CD3 mAb TR66. Immobilization of TCR ligands was obtained by a 4- to 6-h incubation in PBS at 4°C in wells of microtiter plates, alternatively, soluble anti-CD3 mAb, followed by the addition of goat anti-mouse IgG Ab, were used at the indicated concentrations. In experiments using APC for stimulation, T cells (10⁵) were combined with irradiated (5000 rad) DR1-transfected HeLa cells (2 × 10⁵) in the presence of graded concentrations of synthetic F254 peptide. Direct immunofluorescence determination was performed 24 h later. PE-labeled anti-CD25 mAb and isotype-matched control were incubated with 3 × 10⁶ cells/ml for 1 h at 4°C in PBS, 0.1% sodium azide, and 1% FCS, then washed twice in PBS. Mean fluorescence was measured by flow cytometry on an EPICS XL cytometer (Coulter, Miami, FL).

Cell stimulation and lysis

Cells were washed twice, resuspended at 12 × 10⁶ cells/ml in prewarmed RPMI and incubated in a water bath at 37°C for 15 min. Stimulation was then performed on 6 × 10⁶ cells/ml by point of addition of soluble TR66 mAb, in the presence or the absence of anti-CD28 mAb, followed by addition of goat anti-mouse IgG Ab and incubation at 37°C for the indicated periods of time. Stimulation was stopped by centrifugation, followed by solubilization of the cell pellets for 45 min on ice in 100 μl lysis buffer (1% Igepal (Sigma) in 20 mM Tris (pH 7.5) and 140 mM NaCl, supplemented with 1 mM EDTA, 1 mM sodium orthovanadate, and a mixture of protease inhibitors (Mini Complete; Roche Diagnostics) used according to the manufacturer’s instructions). Each condition of stimulation was simultaneously performed on Th1 and Th2 cells. After removal of nuclei and cellular debris by centrifugation, the protein concentration was determined (bicinchoninic acid; Pierce, Interchim, Asnières, France), and constant amounts of protein sample were diluted in Laemmli sample buffer (Bio-Rad, Marne la Coquette, France) with 5% 2-ME and boiled for 3 min before SDS-PAGE analysis.

Lipid raft fractionation

Cell lysates (15 × 10⁶ cells/point) were prepared as described above in 1 ml lysis buffer, except that 1% Triton X-100 was used as a detergent. The samples were centrifuged through a sucrose step gradient as described by Xavier et al. (22). Briefly, following 30-min incubation on ice, the lysates were gently mixed with 1 vol 85% sucrose in lysis buffer. The samples were placed in the bottom of a tube, overlaid by 6 ml 35% sucrose, and 100 μl 100% TCA in water, and the pellet was washed twice with acetone. The pellets were resuspended in Laemmli sample buffer plus 5% 2-ME and boiled for 3 min before SDS-PAGE analysis. One-fifth of each fraction was loaded on 12% SDS-PAGE gels.

Immunoprecipitation

Cell lysates (6 × 10⁶ cells/point in 100 μl lysis buffer) were prepared as described for lipid raft fractionation. Following incubation for 2 h on ice with the indicated mAb, immunoprecipitated material was recovered with 20 μl protein G bound on agarose beads (France Biochem) for 1 h at 4°C under continuous agitation. After three washings in lysis buffer, immune complexes were eluted by boiling for 3 min in Laemmli sample buffer and 5% 2-ME before SDS-PAGE analysis.

Immunoblotting

Equal amounts of protein, as determined by Bradford assay (Bio-Rad), were run on 12% SDS-PAGE gels and then electrotransferred onto nitrocellulose (Bio-Rad). The membranes were incubated with 0.1% Triton X-100 and 3 vol activated papain for 1 h at 37°C. The reaction was terminated by the addition of 1 vol of a protease inhibitor mixture (Mini Complete; Roche Diagnostics, Mannheim, Germany). DR1 material was subsequently chromatographed over a Superose 12 column (Amersham Pharmacia Biotech) equilibrated in PBS. DR material was analyzed by SDS-PAGE on 12% gels, followed by Coomassie and silver staining (Accurate Chemical & Scientific, Westbury, NY).

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Abbreviations used in this paper: SHP Src homology 2 containing protein tyrosine phosphatase; FAP-1, Fas-associated phosphatase 1.

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and 1% gelatin. Revelation was performed using ECL (Amersham Pharmacia Biotech). Stripping was performed by incubation of nitrocellulose for 30 min at 50°C in 62.5 mM Tris (pH 6.7), 100 mM 2-ME, and 2% SDS, followed by four washings in TBS containing 0.1% Tween 20.

Results

Isolation and purification of soluble HLA-DR1 heterodimers

To study the TCR-mediated activation requirements of Th1 and Th2 lymphocytes under defined experimental conditions closely mimicking the physiological situation, soluble peptide/MHC class II complexes were chosen as a stimulus of choice. Rather than using a classical strategy based on the expression of soluble recombinant MHC class II heterodimers, an alternative approach was followed, which consisted of the biochemical manipulation of naturally expressed DR1 protein. Following purification by affinity chromatography from EBV-B cells, DR1 protein was loaded with synthetic F254 peptide by incubation under optimal conditions using a large molar excess of peptide. Then, a method was established for the production of soluble DR1 material devoid of detergent. Samples of DR1 protein were treated with increasing concentrations of preactivated papain, and the extent of digestion was evaluated by analysis of Coomassie-stained SDS-PAGE gels. By increasing the papain concentration, a concomitant fraction of polypeptide corresponding to the DR1 α- and β-chains showed increased mobility, indicating that these conditions resulted in limited digestion of the DR1 protein. Treatment with 40 ng/ml papain led to a complete shift in mobility of both DR1 chains. When higher concentrations of papain were used, the DR1 material was progressively more degraded, up to a point where digestion was complete (Fig. 1A). Based on this initial experiment, a large sample of DR1 (300 μg) was treated with 40 ng/ml papain and fractionated by size exclusion chromatography. Material was eluted as an isolated peak with an apparent m.w. similar to that of BSA, which was expected to correspond to solubilized DR1, without the transmembrane and cytoplasmic domains (Fig. 1B). This assumption was further supported by the analysis of material from this peak on silver-stained SDS-PAGE gels. Two bands of lower m.w. than the undigested DR1 α- and β-chains were resolved (Fig. 1C). In addition, this chromatography was expected to produce material devoid of papain, Ag peptide, and detergent. Therefore, this procedure resulted in the isolation of soluble, peptide-loaded DR1 protein suitable for cellular assays.

CD25 up-regulation is more sensitive to immobilized TCR ligands in Th1 than in Th2 cells

Isolated F254 peptide/DR1 complexes offered the opportunity to compare activation of Th1 and Th2 cells following engagement of the TCR by their physiological ligands in the absence of the contribution of accessory and adhesion molecules. As assessed by the percentage of CD25 up-regulation relative to CD25 expression by their physiological ligands in the absence of the concomitant digestion, the DR1 material was chromatographed on a Superose 12 column at 0.5 ml/min PBS. C, Silver-stained SDS-PAGE gel of papain-treated F254 peptide/DR1 complexes. Material eluted from the Superose 12 column with an apparent molecular mass of 66 kDa was analyzed on silver-stained 12% SDS-PAGE gels. –, Untreated F254 peptide/DR1; +, papain-treated (40 ng/ml) F254 peptide/DR1. The molecular mass of a 35-kDa protein standard is indicated.

FIGURE 1. Preparation and isolation of soluble F254 peptide/DR1 complexes. A, Papain digestion of DR1 molecules. Aliquots of DR1 protein (3 μg) were treated with increasing concentrations of papain, and the samples were analyzed on Coomassie-stained 12% SDS-PAGE gels. The molecular masses of protein standards are indicated. B, Size exclusion chromatography of papain-treated F254 peptide/DR1 complexes. Following digestion, the DR1 material was chromatographed on a Superose 12 column at 0.5 ml/min PBS. C, Silver-stained SDS-PAGE gel of papain-treated F254 peptide/DR1 complexes. Material eluted from the Superose 12 column with an apparent molecular mass of 66 kDa was analyzed on silver-stained 12% SDS-PAGE gels. –, Untreated F254 peptide/DR1; +, papain-treated (40 ng/ml) F254 peptide/DR1. The molecular mass of a 35-kDa protein standard is indicated.

regulation by Th1 than by Th2 cells (Fig. 2B). Likewise, stimulation with soluble anti-CD3 mAb cross-linked with anti-mouse IgG Ab resulted in a 10-fold more sensitive dose-response in Th1 than in Th2 cells (Fig. 2B). However, it could not be formally excluded that a fraction of soluble Abs might be adsorbed on the plastic wells and contributed to T cell activation. Nevertheless, in both conditions CD25 up-regulation was triggered by less anti-CD3 mAb in Th1 than in Th2 cells. These results indicated that TCR engagement resulted in distinct signaling events in Th1 and Th2 cells regardless of the form of the anti-CD3 mAb, immobilized or soluble and cross-linked. Similar results were obtained with the other Th1 and Th2 clones used in this study (data not shown).

F254 peptide/DR1 complexes displayed on HeLa cells elicit similar CD25 up-regulation in Th1 and Th2 cells

Remarkably, when Th1 and Th2 cells were triggered with F254 peptide-pulsed DR1-transfected HeLa cells, the two Th subsets did not exhibit marked differences in their sensitivities. The most sensitive clones were the Th2 FR6.6 and Th1 6396 F254.7.2 clones. The four other Th1 and Th2 clones showed similar Ag dose responses (Fig. 3). Stimulation by F254 peptide-pulsed HeLa cells
served with purified DR1 material were probably not due to distinct patterns of CD25 up-regulation (data not shown).

Since HeLa cells are nonprofessional APC that are largely devoid of costimulatory activity, it indicated that the different thresholds of activation observed with purified DR1 material were probably not due to distinct costimulation requirements. Rather, these results suggested that Th2 cells might rely on a higher level of TCR aggregation for activation than is the case for Th1 cells.

FIGURE 2. CD25 up-regulation was less sensitive to engagement by immobilized TCR ligands in Th2 than in Th1 cells. A, CD25 up-regulation by Th1 and Th2 cells after stimulation with immobilized F254 peptide/DR1 complexes. Increasing amounts of soluble F254 peptide/DR1 complexes were incubated in wells of microtiter plates for 4–6 h at 4°C. After washing, T cells were cultured overnight, and CD25 expression was determined by immunofluorescence. Results are expressed as the percentage of CD25 up-regulation relative to CD25 expression by nonstimulated cells. Similar results were obtained in three independent experiments. B, CD25 up-regulation after anti-CD3 mAb stimulation. Anti-CD3 mAb was either immobilized in graded amounts in wells of microtiter plates (coated; nanograms per milliliter) or added to the culture medium together with goat anti-mouse IgG Ab (soluble; micrograms per milliliter). T cells were cultured overnight, and CD25 expression was determined by immunofluorescence. Results are expressed as the percentage of CD25 up-regulation relative to CD25 expression by nonstimulated cells. Similar results were obtained in three independent experiments.

resulted in profiles of cytokine production that paralleled the patterns of CD25 up-regulation (data not shown). Since HeLa cells are nonprofessional APC that are largely devoid of costimulatory capacity, it indicated that the different thresholds of activation observed with purified DR1 material were probably not due to distinct costimulation requirements. Rather, these results suggested that Th2 cells might rely on a higher level of TCR aggregation for activation than is the case for Th1 cells.

FIGURE 3. CD25 up-regulation driven by peptide-pulsed nonprofessional APC is as sensitive in Th2 as in Th1 cells. Increasing amounts of F254 peptide were incubated for 3 h at 37°C with DR1-transfected HeLa cells. Then the cells were washed, irradiated (5000 rad), and cultured overnight with T cells. CD25 expression was determined by immunofluorescence. Results are expressed as the percentage of CD25 up-regulation relative to CD25 expression by nonstimulated cells. Similar results were obtained in a duplicate experiment.

to overall fainter phosphotyrosine levels in Th2 than in Th1 polyclonal lines, indicating that following comparable levels of TCR ligation, a more sustained cascade of signaling events was generated in Th1 than in Th2 cells (Fig. 4B). Tyrosine phosphorylation profiles were also fainter in Th2 than in Th1 cells regardless of the time following TCR engagement. Maximal intensity in both Th1 and Th2 cells was observed at 150 s after TCR ligation, followed by decay of the phosphotyrosine signal (Fig. 4C). This finding was observed for three Th1 and three Th2 clones (data not shown). Together these data established that anti-CD3 mAb-mediated TCR engagement resulted in a lower phosphotyrosine content in Th2 than in Th1 cells. These differences could not be explained by distinct optimal intensities of stimulation or by different response kinetics. Rather, they indicated that Th2 cells were inherently less capable than Th1 cells of anti-CD3 mAb TCR-induced tyrosine phosphorylation.

CD3 and CD28 coengagement fails to increase tyrosine phosphorylation in Th2 cells to levels reached in Th1 cells

It is well documented that signaling pathways emanating from the TCR and CD3 complex alone are not sufficient for T cell activation. Simultaneous engagement of costimulatory molecules, in particular CD28, is required to achieve full activation of T cells. Therefore, it was evaluated whether simultaneous engagement of CD3 and CD28 increased tyrosine phosphorylation in Th2 cells to levels matching those attained in Th1 cells. First, immunofluorescence staining established that expression levels of CD28 were similar in Th1 and Th2 clones (data not shown). Then, it was found that CD3 and CD28 coengagement with mAb did not augment the intensity of the overall phosphotyrosine profiles in Th2 cells to levels attained in Th1 cells. A slight increase in the phosphorylation of a 38-kDa protein, which might correspond to p38 mitogen-activated protein kinase, was nevertheless noted (Fig. 5). Similar results were found with three pairs of Th1 and Th2 clones (data not shown). Since strong TCR engagement as well as ligation of the costimulatory molecule CD28 failed to induce tyrosine phosphorylation levels in Th2 cells matching those observed in Th1 cells, it was next questioned whether these differences might be due to a greater ability of Th2 cells to dephosphorylate tyrosine residues.

The tyrosine phosphatase inhibitor phenylarsine oxide reverts faint phosphotyrosine profiles of Th2 cells

An intrinsic low kinase activity in Th2 compared with Th1 cells might explain the faint intensity of the phosphotyrosine profiles after TCR engagement in Th2 cells. Alternatively, the phosphatase activity might be higher in Th2 than in Th1 cells. As a result, tyrosine phosphorylation would be more inhibited in Th2 than in Th1 cells. To discriminate between these two hypotheses, Th1 and Th2 cells...
were stimulated with anti-CD3 mAb in the presence or the absence of phosphatase inhibitors. Remarkably, the tyrosine phosphorylation profiles in Th2 cells treated with the tyrosine phosphatase inhibitor phenylarsine oxide were as intense as those in Th1 cells similarly treated, while the serine and threonine phosphatase inhibitor okadaic acid failed to restore intense tyrosine phosphorylation profiles in Th2 cells after TCR engagement (Fig. 6A). Analysis of three Th1 and three Th2 clones gave the same results (data not shown). This circumstantial evidence indicated that Th2 cells are endowed with as high a tyrosine phosphorylation capacity as Th1 cells. It therefore indirectly suggested that distinct phosphatases of higher activity or expressed at higher levels made Th2 cells less reactive than Th1 cells to TCR-mediated stimulation. To evaluate whether higher overall expression levels of phosphatases in Th2 than in Th1 cells might explain the distinct profiles of tyrosine phosphorylation in these two cell types, the relative amounts of CD45, SHP-1, and SHP-2 in Th1 and Th2 cells were assessed by Western blotting. Expression levels of these three tyrosine phosphatases were similar in Th1 and Th2 cells before and after TCR engagement (Fig. 6B). The absence of differences in total phosphatase expression levels between Th1 and Th2 cells suggested that the fainter phosphotyrosine profiles in Th2 than in Th1 cells might be alternatively explained by distinct enzymatic activities of phosphatases involved in the TCR signaling pathway or by distinct subcellular localization of phosphatases in the two Th subsets. The latter possibility was assessed by evaluating whether tyrosine phosphatases were excluded from lipid rafts to a comparable extent in Th1 and Th2 cells before TCR engagement (Fig. 6C). Collectively, these results indicated that for the three abundant phosphatases analyzed and previously shown to be implicated in TCR-mediated signaling pathways, the fainter phosphotyrosine profiles in Th2 than in Th1 cells cannot be explained by distinct expression levels before and after TCR engagement.
Stimulation was performed using 6/HL9262 larsine oxide (and coimmunoprecipitated ZAP-70 protein was then evaluated by association between CD3 us to assess whether one of the earliest signaling events, the as-signaling cascades differ at an early step. This possibility prompted Th2 cells. This observation suggested that the Th1 and Th2 TCR profiles in Th2 than in Th1 cells, no individual bands were found fi
Beside the overall fainter intensity of tyrosine phosphorylation Th1 cells
Weaker association between ZAP-70 and CD3 engagement or subcellular localization before TCR engagement.

**FIGURE 6.** Phenylarsine oxide treatment induces intense tyrosine phosphorylation in both Th1 and Th2 cells. A, Th1 and Th2 clones were pretreated with medium alone (a), 1 μM okadak acid (b), or 50 μM phenylarsine oxide (c and d; d is the same as c, but less exposed) for 30 min. Stimulation was performed using 6 μg/ml anti-CD3 mAb plus 10 μg/ml goat anti-mouse IgG Ab for 2 min. Then lysates were prepared and electrophoresed as described, and tyrosine phosphorylation was revealed by Western blotting. Similar results were obtained in two independent experiments with three Th1 and three Th2 clones. B, CD45, SHP-1, and SHP-2 expression was assessed in whole lysates by Western blotting before and after TCR engagement. C, CD45, SHP-1, SHP-2, and Lck expressions were assessed by Western blotting in nonstimulated T cells fractionated on sucrose gradients. Lipid rafts were present in fraction 8, as revealed by the presence of Lck. Similar results were obtained in two independent experiments with two Th1 and two Th2 clones.

after TCR engagement or subcellular localization before TCR engagement.

**Weaker association between ZAP-70 and CD3ξ in Th2 than in Th1 cells**

Beside the overall fainter intensity of tyrosine phosphorylation profiles in Th2 than in Th1 cells, no individual bands were found to exhibit a markedly different ratio of intensity between Th1 and Th2 cells. This observation suggested that the Th1 and Th2 TCR signaling cascades differ at an early step. This possibility prompted us to assess whether one of the earliest signaling events, the association between CD3ξ and ZAP-70, was different in Th1 and Th2 cells. CD3ξ material was immunoprecipitated from both Th1 and Th2 cells before and after TCR engagement. The presence of coimmunoprecipitated ZAP-70 protein was then evaluated by Western blotting (Fig. 7). Following TCR engagement, phosphorylated ZAP-70 was associated with CD3ξ in activated Th1 cells. By contrast, in Th2 cells no ZAP-70 protein was detected in the material immunoprecipitated by anti-CD3ξ mAb. In a control experiment using anti-TNF mAb instead of anti-CD3ξ mAb, no ZAP-70 was detected by Western blotting. Similar results were observed in three Th1 and three Th2 clones (data not shown). A lower association between CD3ξ and ZAP-70 in Th2 cells compared with Th1 cells by controlling the TCR signaling cascades might at least partly determine the overall fainter phosphorylation profiles in Th2 than in Th1 cells.

**Discussion**

The observations reported here constitute evidence that TCR sig-naling is distinct in human Th1 and Th2 cells. Analysis of up-regulation of the activation marker CD25 revealed that Th2 cells are less sensitive than Th1 cells upon TCR engagement with immobilized peptide/MHC class II complexes or anti-CD3 mAb. This lower sensitivity of Th2 than Th1 cells was paralleled by an overall weaker tyrosine phosphorylation intensity in the former than in the latter cells. Distinct tyrosine phosphatase profiles might differentially control the Th1 and Th2 signaling cascades, as phenylarsine oxide treatment restored a high level of tyrosine phosphorylation in Th2 cells. Finally, TCR signaling in Th1 and Th2 differs at an early step of the cascade, as documented by a weaker association between CD3ξ and ZAP-70 in Th2 than in Th1 cells.

MHC class II protein preparations isolated from B cells were biochemically manipulated to obtain soluble, largely monomeric, Ag peptide/MHC class II complexes. The absence of detergent in this material made it suitable for cellular assays. In addition, this procedure offered the clear advantage that it did not require the development of expression vectors for each allelic form of MHC class II molecules. Since EBV-transformed B cells express high levels of DR protein, soluble peptide/DR complexes can be readily obtained in large quantities (300 μg DR protein/1 EBV-B cell culture following this procedure). In contrast with MHC class II protein produced by recombinant techniques in insect cells or bacteria, MHC class II molecules isolated from B cells are not empty, but are occupied by cell- and medium-derived material. For that rea-son, it is expected that complete loading with Ag peptide cannot be attained, since, depending on the MHC class II alleles, occupancy with exogenously added peptides varies between <5% and up to 20% (24).

This study provided evidence that activation of Th1 cells was elicited at lower densities of immobilized peptide/MHC class II complexes, or anti-CD3 mAb than that for Th2 cells. In contrast,
Th2 cells were as sensitive as Th1 cells upon TCR engagement with peptide/MHC class II complexes displayed by transfection at the surface of nonprofessional APC. These findings could be rationalized along two lines of reasoning. First, immobilized peptide/MHC class II complexes and anti-CD3 mAb cannot be clustered by T cells, while peptide/MHC class II molecules expressed on APC are laterally mobile and can be capped at the area of contact with T cells. Our observations might therefore suggest that Th2 cells require a greater degree of TCR clustering for activation than is the case for Th1 cells. Second, APC can provide Th2 cells with accessory signals needed for activation. DR1-transfected HeLa cells were used as a model of nonprofessional APC. It was assumed that these cells were largely devoid of secondary signaling molecules. In particular, they lack the expression of CD80, CD86, and CD40 molecules (25). In addition, it was found that CD3 and CD28 coengagement failed to increase tyrosine phosphorylation in Th2 cells to levels reached by Th1 cells. These results indicated that the lower activation ability of Th2 than of Th1 cells was intrinsic to the TCR signaling cascades, rather than due to the absence of CD28 costimulatory signals. Together, our results favored the idea that activation of Th2 cells might require more robust TCR-mediated signals than is the case for Th1 cells. Interestingly, it was recently shown in the murine system that TCR complexes of Th1 cells are efficiently recruited into rafts at the area of contact with peptide/MHC class II complexes, while such marked TCR partitioning does not occur in Th2 cells (26). Our results might suggest that these differences in plasma membrane dynamics are also found between human Th1 and Th2 cells.

The differential reactivity of human Th1 and Th2 cells observed in this study could be explained by distinct features in the proximal events of the TCR signaling machinery, as revealed by a weaker association between CD3ζ and ZAP-70 in human Th2 than in Th1 cells. In the mouse model a looser interaction between CD3ζ and ZAP-70 in Th2 than in Th1 cells was similarly reported by Tamura et al. (15). These authors suggested that the lower activities of Fyn and ZAP-70 observed in Th2 cells could explain this weaker association. As postulated in the mouse system, the weaker association between CD3ζ and ZAP-70 in human Th2 cells than in Th1 cells might be due to a weaker activity of Fyn and ZAP-70 kinases. Interestingly, the activities of Fyn and of ZAP-70 kinases were reduced in human Th cells activated by altered peptide ligands (APLs) in Th2, but not in Th1, clones (32). Fas-associated phosphatase 1 (FAP-1) mRNA was expressed at high levels in Th2, but not in Th1, cells (33). It was suggested that FAP-1, a membrane tyrosine phosphatase, is involved in resistance to activation-induced cell death in Th2 cells, suggesting a role for FAP-1 in TCR signaling cascades.

Identification and characterization of molecules such as phosphatases that differentially control early steps of TCR signaling cascades in Th1 and Th2 cells might provide new avenues for the pharmacological manipulation of dysregulated Th cell functions. Future work would aim at evaluating whether interfering with early steps of the TCR signaling cascades distinguishing Th1 and Th2 cells might result in a beneficial impact on various immune-related pathological manifestations, such as autoimmune diseases and allergic reactions.

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