Dendritic Cells Sensitize TCRs through Self-MHC-Mediated Src Family Kinase Activation[1]

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It is unclear whether peptide-MHC class II (pMHC) complexes on distinct types of APCs differ in their capacity to trigger TCRs. In this study, we show that individual cognate pMHC complexes displayed by dendritic cells (DCs), as compared with nonprofessional APCs, are far better in productively triggering Ag-specific TCRs independently of conventional costimulation. As we further show, this is accomplished by the unique ability of DCs to robustly activate the Src family kinases (SFKs) Lck and Fyn in T cells even in the absence of cognate peptide. Instead, this form of SFK activation depends on interactions of DC-displayed MHC with TCRs of appropriate restriction, suggesting a central role of self-pMHC recognition. DC-mediated SFK activation leads to "TCR licensing," a process that dramatically increases sensitivity and magnitude of the TCR response to cognate pMHC. Thus, TCR licensing, besides costimulation, is a main mechanism of DCs to present Ag effectively. ***The Journal of Immunology,*** **2007, 178: 2262–2271.**

A fundamental question in immunology is how APCs manage to activate and deactivate T cells in an Ag-specific fashion. Dendritic cells (DCs)[3] are the most potent APCs for the elicitation of immunity (1) as well as for the induction of tolerance (2). The ability of DCs to activate T cells is linked to DC maturation, a process during which DCs accumulate abundant peptide-MHC class II (pMHC) complexes and costimulatory molecules (3). It is generally believed that high-copy display of pMHC complexes, along with exceptional costimulatory and adhesion capabilities, is the clue for the effective triggering of TCRs and subsequent T cell activation by DCs (4). The validity of this assumption is, however, complicated by the finding that immature DCs (iDCs), expressing very limited amounts of pMHC and costimulators, are also uniquely effective in the pMHC-dependent instruction of T cells to become anergic (5) or regulatory (6). Moreover, the absolute necessity of high level pMHC expression of DCs to induce or maintain T cell immunity is further questioned by observations that DCs of apparently immature phenotype (7) may drive protection against slowly replicating microbial agents (8). It is therefore conceivable that DCs use strategies beyond high pMHC expression and conventional costimulation or adhesion to elicit strong Ag-specific TCR signals.

Accordingly, we asked whether individual pMHC complexes displayed by monocyte-derived iDCs trigger higher numbers of cognate TCRs than individual pMHC complexes displayed by nonprofessional APCs by using a mechanism that acts independently of costimulation and adhesion. To address this question, we quantified the absolute number of triggered TCRs and of pMHC complexes on different APC types simultaneously. In this study, we show that individual pMHC complexes displayed by DCs, as compared with nonprofessional APCs, are intrinsically more efficient in productively triggering Ag-specific TCRs. We further demonstrate that this response is accomplished by the unique ability of self-pMHC complexes on DCs to activate Src family kinases (SFKs) in T cells. This effect leads to a dramatic gain in TCR responsiveness to cognate pMHC.

Materials and Methods

Cells, Ags, superantigens, and peptides

Human monocyte-derived DCs were generated as described (9, 10). The iDCs were harvested on day 7. Mature DCs (mDCs) were generated by culturing the cells for further 24 h in the presence of 50 ng/ml LPS (Sigma-Aldrich).

EBV-transformed lymphoblastoid B cells were generated as follows. PBMC were depleted of T cells through sheep erythrocyte rosetting, and the remaining B cell-rich fraction was resuspended in supernatant from the EBV-producing marmoset cell line, B 95-8. Cells were seeded in 96-well flat-bottom plates at a density of 200,000 cells/well. Fresh medium (10% FCS in RPMI 1640 with antibiotics) was added on the following days when needed. EBV-transformed lymphoblastoid B cells exhibited robust growth activity after 2–3 wk.

Tetanus toxoid (TT)-specific human $CD4^+$ T cell clones were generated according to standard procedures (11). Proof of specificity and clonality of T cell clones was based on Poisson distribution statistics ($p > 95\%$), homogeneous TCR down-modulation upon specific stimulation, and Ab-based TCR Vβ typing. HLA restriction was determined by inhibition of TCR down-modulation in the presence of 50 μg/ml anti-HLA-DP (NeoMarkers), of anti-HLA-DQ or anti-HLA-DR (both from BD Pharmingen) Abs, or by TCR down-modulation upon incubation with peptide-pulsed HLA-transfected mouse L cells. T cell clones used were as follows: 48.37 recognizing TT-derived peptide $TT_{830-843}$ on HLA-DRB1*11 or on HLA-DRB1*08; 48.35, 48.61, 48.69, 48.71, 48.72, 48.74, 48.75, 48.105 (TCR V$\beta 2^+$), and 48.112 recognizing

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[3] Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; mDC, mature DC; SFK, Src family kinase; pMHC, peptide MHC class II; HSA, human serum albumin; TT, tetanus toxoid; TSST-1, toxic shock syndrome toxin-1.

$TT_{830-843}$ on HLA-DRB1*11; 48.31 (TCR Vβ8(a)$^+$), 48.40, and 119.3 recognizing $TT_{947-967}$ on HLA-DPB1*04; 164.100 recognizing $TT_{830-843}$ on HLA-DRB1*0101; and 119.10 (TCR Vβ2$^+$).

TT C fragment was from List Laboratories. Peptides $TT_{830-843}$ (QYI KANSKFIGITE) and $TT_{947-967}$ (FNNFTVSFWLRVPKVSASHLE) were from PiChem. Biotinylated $TT_{830-843}$ (biotinyl-ε-aminocaproate–sGG GsGGGQYIKANSKFIGITE) and HLA-$A2_{103-117}$ (VGSDWRFLRGY HQYA) were from American Peptide Company. Biotinylated toxic shock syndrome toxin-1 (TSST-1) and biotinylated staphylococcal enterotoxin E were from Toxin Technology.

TCR down-modulation

TCR down-modulation was assessed essentially as described (12, 13). APCs were pulsed with peptide at 4°C in 0.1% human serum albumin (HSA)/RPMI 1640, washed several times in the cold, and mixed with T cells (APC to T cell ratio = 10:1). Cells were spun briefly and cocultured at 37°C for 90 min. The number of TCRs per cell was calculated from CD3 PE fluorescence level using QuantiBRITE PE beads (BD Biosciences). In costimulation blockade experiments, CTLA-4-Ig was added to a final concentration of 20 μg/ml. CD25 and CD69 expression on T cells were monitored in parallel with TCR down-modulation using mAbs after 4 and 20 h of coculture with APCs.

T cell proliferation

A total of 40,000 clonal T cells and 3000 APCs (irradiated with 30 Gy) pulsed with the indicated TT peptide concentration were cocultured in 96-well plates. Where indicated, CTLA-4-Ig was added to a final concentration of 20 μg/ml. After 24 h of culture, 1 μCi of [*methyl*-^{3}H]thymidine (GE Healthcare) was added per well, and proliferation was determined 18 h later using a Wallac 1205 Betaplate Liquid Scintillation counter.

T cell-APC conjugate formation

T cell-APC conjugate formation was performed essentially as previously described (14), with minor modifications. T cells were labeled with the green fluorescent dye CMFDA (5-chloromethylfluorescein diacetate, 50 nM in PBS; Molecular Probes) at 37°C for 10 min, followed by three washes in an excess of cold medium, and resuspended in 0.1% HSA/RPMI 1640. B cells and DCs were labeled similarly using the red fluorescent dye CMTMR (5- (and -6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; Molecular Probes) at 3 μM and 300 nM, respectively. Conjugate formation between dye-labeled T cells and APCs (mixed at a 1:10 ratio) was assayed both in end-over-end rotated cell suspensions as well as in centrifugation-pelleted cell mixtures by FACS analysis after various periods of incubation (cell pellets were resuspended by pipetting up and down five times). The percentage of T cells engaged in conjugates was calculated from the decrease of events (recording time-normalized) in the T cell gate.

Quantitation of cognate pMHC complexes and superantigen-MHC complexes

All procedures were performed at 4°C. After pulsing APCs with biotinylated $TT_{830-843}$ peptide biotinyl-ε-aminocaproate–sGGGsGGGQYIKANSKFIG ITE (15) at indicated concentrations for 30 min, cells were washed three times in 0.1% HSA/RPMI 1640 and once in PBS. Cell pellets (4×10^6 cells per condition) were lysed in 1% *N*-octylglucoside (Sigma-Aldrich) lysis buffer (50 mM Tris Cl (pH 7.8), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, and Complete Protease Inhibitor Cocktail (Roche)) for 60 min under continuous end-over-end rotation. After removal of nuclei and cell debris by brief centrifugation, lysates were mixed with 10 μg of DA6.147 mAb (anti-HLA-DRα cytoplasmic domain), a gift from P. Cresswell (Yale University School of Medicine, New Haven, CT) (16), or control Ab and rotated end-over-end for 3 h, followed by transfer to 100 μl (bed volume) of protein A-Sepharose (Sigma-Aldrich) prewashed in lysis buffer. MHC class II molecules were allowed to precipitate for 18 h under continuous rotation. Sepharose beads were then washed three times in lysis buffer, followed by the addition of 2 μl of ^{125}I-streptavidin (GE Healthcare) in 150 μl of lysis buffer per condition. Samples were rotated for 90 min and washed six times with lysis buffer, and bound radioactivity was determined using a Wallac 1470 Wizard automatic gamma counter. Background due to peptide nonspecifically sticking to APCs was virtually zero because equal counts were obtained with control Ab precipitates of peptide-pulsed and unpulsed APCs. Background resulting from nonspecific ^{125}I-streptavidin binding to Sepharose beads was subtracted. Recovery of HLA-DR molecules with DA6.147 immunoprecipitation was 90% complete for both DCs and B cells, as shown by densitometry of HLA-DR in Western blots of supernatants from DA6.147 and control Ab precipitates. Ligand numbers per cell were calculated from specific ^{125}I-streptavidin activity and number of input cells per condition.

HLA-DR1:HLA-$A2_{103-117}$ complexes on APCs were quantitated using the human mAb UL-5A1 (17) followed by biotinylated goat anti-human IgG (Jackson ImmunoResearch Laboratories) and streptavidin-PE (BD Biosciences) as second step reagents.

For quantitation of superantigen-MHC complexes, APCs were pulsed with biotinylated TSST-1 (biotin to TSST-1 ratio is 1) at various concentrations for 30 min at 4°C, and MHC class II-bound TSST-1 was labeled with an excess of PE-conjugated streptavidin and analyzed by cytofluorometry. Ligand numbers were calculated from mean fluorescence intensity values using QuantiBRITE PE beads. Similar results were obtained by assessment of MHC class II:TSST-1 complexes using cytofluorometry and MHC class II immunoprecipitation followed by ^{125}I-streptavidin-based detection (data not shown).

Western blotting

Western blotting of tyrosine-phosphorylated proteins was performed essentially as described (18). To assess CD3ζ phosphorylation, T cells and peptide-pulsed APCs were cocultured and lysed in 1% Brij97 (Sigma-Aldrich) lysis buffer (10 mM Tris Cl (pH 7.8), 150 mM NaCl, 10 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, and Complete Protease Inhibitor Cocktail (Roche)) at 4°C for 1 h. Solubilized proteins were resolved by 6–15% SDS-PAGE and transferred onto PVDF by semidry electroblotting, and tyrosine-phosphorylated proteins were detected using HRP-conjugated mAb PY20 (Transduction Laboratories) and ECL detection reagents (Amersham Biosciences). Tyrosine-phosphorylated CD3ζ was detected as 21-kDa phosphoprotein, as inferred from its coprecipitation with CD3ε (OKT3; Ortho Biotech) and inducibility by stimulation both with anti-CD3 (MEM-92, IgM) and peptide-loaded APCs. CD3ζ band intensities were quantitated by densitometry.

SFKs and their phosphorylation status were characterized in T cells stimulated by peptide-pulsed and unpulsed APCs. After coculture, cells were resuspended in cold 0.1% HSA/RPMI 1640 containing 20 mM NaF and 1 mM Na_3VO_4. APCs were effectively depleted by an excess of anti-CD40/anti-CD11b loaded (both from BD Pharmingen) Dynabeads pan mouse IgG magnetic beads (Dynal Biotech). Purified T cells were lysed in 1% Nonidet P-40 (Pierce) and 1% lauryl maltoside (Sigma-Aldrich). Lysates were separated by 8% SDS-PAGE under reducing conditions. In Western blots, binding of rabbit polyclonal Abs recognizing activation loop tyrosine-phosphorylated SFK (Cell Signaling Technology), corresponding to Lck phosphorylated Y394 and Fyn phosphorylated Y420, Lck (Cell Signaling Technology), and Fyn (Santa Cruz Biotechnology) was visualized with a HRP-conjugated mouse anti-rabbit IgG L chain mAb (Jackson ImmunoResearch Laboratories) and ECL detection. Band intensities were normalized for CD2 content for densitometric quantitation. The SFK inhibitors PP1 (Biaffin) or PP2 (Calbiochem) were added at the indicated concentrations to T cells 90 min before the addition of APCs.

Immunoprecipitation of SFK

Monoclonal murine Abs against Lck (LCK-01), Fyn (FYN-01), and phosphotyrosine (P-TYR-01, P-TYR-02), established in the laboratory of V. Horejsí (Academy of Sciences, Prague, Czech Republic), were coupled to CNBr-activated Sepharose 4 Fast Flow (Sigma-Aldrich) according to the manufacturer's instructions. T cells were cocultured with unloaded DCs, depleted from the latter as has been described, and lysed in lysis buffer containing 1% Nonidet P-40 (Pierce) and 1% lauryl maltoside (Sigma-Aldrich). Nuclei were removed by brief centrifugation. Lysates (~50 μl, corresponding to ~800,000 cells per condition) were rotated for 30 min with 30 μl (bed volume) of immunosorbent in spin columns (Pierce). Immunodepleted lysates and precipitated proteins (eluted with 0.1 M glycine NaOH (pH 11.5), 0.1% detergent) were mixed with reducing sample buffer, boiled, and subjected to electrophoretic separation and Western blotting.

SFK induction index

The SFK induction index was calculated as the product S1 and S2. S1 reflects the activation-related appearance of tyrosine-phosphorylated SFK above 56 kDa and was calculated as follows. Every value of the densitometric profile was multiplied by a factor between 0 (bottom) and 1 (top) according to the value's vertical position in the lane, and all products were summed up to generate a top-weighed score. A bottom-weighed score was calculated using the same procedure with factors arranged in the opposite direction (0, top; 1, bottom). S1 is the ratio of top-weighed vs bottom-weighed score. S2, reflecting the overall level of SFK activation loop tyrosine phosphorylation, is the CD2-normalized SFK activation loop tyrosine phosphorylation intensity of the lane of interest.

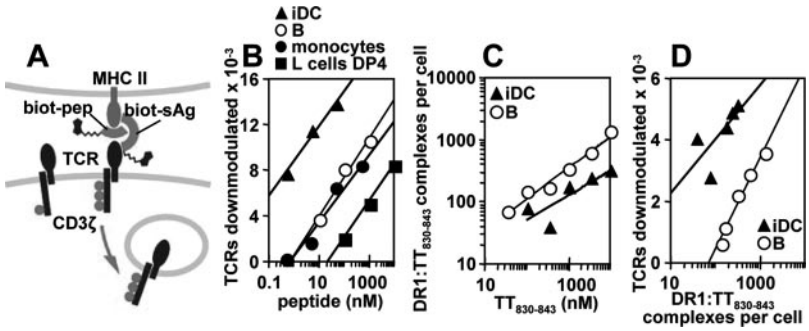

FIGURE 1. The pMHC complexes on DCs are uniquely efficient in down-modulating TCRs. *A*, Upon encounter of the TCR and cognate pMHC, CD3 chains (only CD3ζ is shown) are phosphorylated and the TCR-CD3 complex is internalized. The number of down-modulated TCR-CD3 complexes was determined by cytofluorometry. For ligand quantitation experiments, biotinylated peptide (biot-pep) and biotinylated superantigen (biot-sAg) were used. *B*, TCR down-modulation in T cells stimulated by peptide-pulsed iDCs, by autologous lymphoblastoid B cells (B) and monocytes, and by HLA-DP4-transfected mouse L cells (L cells DP4). Representative results obtained with T cell clone 48.31 ($\sim$25,000 TCRs in resting state) recognizing $TT_{947-967}$ presented by HLA-DP4. *C*, For quantitation of specific pMHC complexes, iDC and autologous lymphoblastoid B cells (B) were pulsed with various concentrations of biotinylated $TT_{830-843}$ peptide, and the number of surface-disposed HLA-DR1:$TT_{830-843}$ complexes was determined as described. *D*, TCR down-modulation of a HLA-DR1-$TT_{830-843}$-restricted T cell clone was measured and is shown as the function of the number of HLA-DR1-$TT_{830-843}$ complexes displayed by iDCs and B cells (B) as characterized in *C*. Data in *C* and *D* are representative of four experiments using DCs from three different donors and T cell clones specific for HLA-DR1-$TT_{830-843}$ and HLA-DP4-$TT_{947-967}$ complexes.

Results

pMHC complexes on DCs are uniquely efficient in down-modulating TCR

Upon encounter of TCR and cognate pMHC, CD3 chains are rapidly phosphorylated (19), and activated TCR-CD3 complexes are down-modulated (i.e., internalized) and degraded (20) (Fig. 1*A*). The number of down-modulated TCRs can be measured by cytofluorometry (12, 13). Using this approach, we compared the capacity of human monocyte-derived iDCs and various types of autologous APCs to down-modulate TCRs of human $CD4^+$ T cell clones specific for TT-derived peptides. APCs were pulsed with different concentrations of cognate peptide, cocultured with T cells, and TCR down-modulation was assessed. As shown in Fig. 1*B*, iDCs need much lower peptide concentrations than other syngeneic APC types (lymphoblastoid B cells, peripheral blood B cells (data not shown), monocytes) or MHC class II-transfected mouse L cells to down-modulate a given number of TCR. iDCs were superior to lymphoblastoid B cells in down-modulating TCRs independently of the time point analyzed (data not shown). iDCs require 50- to 200-fold lower peptide concentrations than lymphoblastoid B cells to down-modulate TCR. These results were obtained in experiments with several different T cell clones ($n = 14$) recognizing distinct pMHC complexes (peptide $TT_{947-967}$ presented by HLA-DP4 (Fig. 1*B*); $TT_{830-843}$ presented by HLA-DR1, HLA-DR8, or HLA-DR11 (data not shown)) and using various APC types from different donors ($n > 20$) matched for the presenting MHC class II allele. Among nonprofessional APCs, lymphoblastoid B cells (subsequently referred to as B cells) had the highest TCR down-modulation potency (Fig. 1*B*) and were therefore used for comparative analyses with DCs.

The observation that DCs use peptide for TCR down-modulation with higher efficiency than other investigated APC types prompted us to quantify surface-disposed pMHC complexes. We immunoprecipitated MHC class II from DCs and B cells pulsed with biotinylated $TT_{830-843}$ peptide and measured specific pMHC complexes by detection with ^{125}I-labeled streptavidin. Potential removal of the N-terminal biotin by proteases was avoided by coupling it to the peptide via the cleavage-resistant, flexible linker sGGGsGGG (s, D-serine; G, glycine) (15). APCs were peptide-pulsed at 4°C to load surface MHC selectively and to avoid generation of intracellular pMHC pools, which contribute to TCR down-modulation only after their export to the cell surface.

B cells formed only 2.0–3.5 times more HLA-DR1-$TT_{830-843}$ complexes than iDCs within the tested peptide dose range of 30–10,000 nM (Fig. 1*C*), although expressing substantially higher amounts of HLA-DR than iDCs at the cell surface (see below). The rather small difference in peptide loading by iDCs and B cells may be explained by the fact that iDCs, but less so B cells, express empty and thus peptide-receptive MHC class II dimers (21). B cells also formed 1.8-fold more pMHC complexes than iDCs when we pulsed a different peptide (HLA-$A2_{103-117}$) onto HLA-DR1-positive APCs and detected HLA-DR1-HLA-$A2_{103-117}$ complexes by a specific mAb (17) and cytofluorometry (data not shown). Prevalence of HLA-DR1-HLA-$A2_{103-117}$ display by B cells over iDCs was also seen in HLA-DR1-positive APCs that express HLA-A2 and load HLA-$A2_{103-117}$ via the endogenous pathway of pMHC formation (data not shown). Thus, the results of two detection assays probing two different pMHC complexes and two pathways of peptide loading show that DCs are not more efficient than lymphoblastoid B cells in generating surface-disposed pMHC complexes. Thus, the superior TCR down-modulation capacity of TT peptide-loaded iDCs is not a direct consequence of an excess in cognate pMHC display.

To analyze the relation between pMHC display and TCR down-modulation in greater detail, iDCs and B cells with defined numbers of surface TT pMHC complexes were used in TCR down-modulation experiments. Accordingly, individual pMHC complexes on iDCs, as compared with those on B cells, are much more efficient in down-modulating TCR (Fig. 1*D*). Extrapolation of regression curves suggests that TCR triggering can start at $\sim$1 pMHC complex per cell with iDCs, whereas B cells require $>$80 pMHC complexes to reach the threshold for TCR down-modulation.

TCRs down-modulated in amplified mode by DCs are signaling competent

We next investigated whether the high level of iDC-induced TCR down-modulation is linked to high level signaling via TCR. Early consequences of productive TCR activation, i.e., CD3ζ tyrosine phosphorylation and up-regulation of CD25 and CD69, were analyzed quantitatively in T cells stimulated by iDCs and B cells.

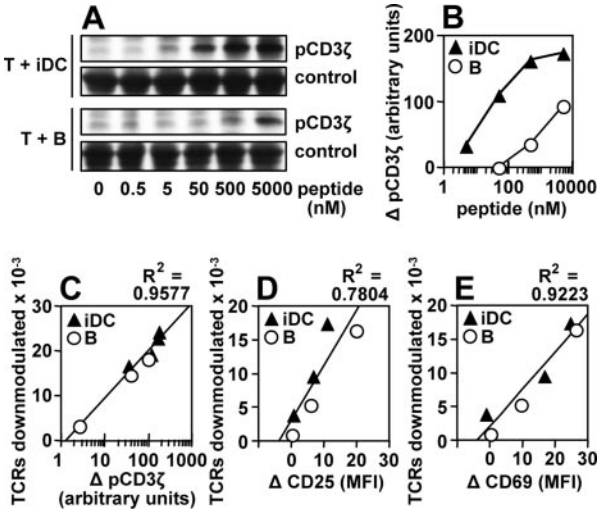

FIGURE 2. DCs are more efficient than B cells in inducing early TCR signaling events. *A* and *B*, T cells were cocultured for 15 min with peptide-pulsed iDCs and lymphoblastoid B cells (B), and CD3ζ tyrosine phosphorylation (pCD3ζ) was assessed in Western blotting (*A*) and quantitated by densitometry (*B*). *C–E*, TCR down-modulation, CD3ζ tyrosine phosphorylation, and TCR triggering-dependent expression of early activation Ags are interrelated quantitatively at ratios not influenced by the APC type. TCR down-modulation in T cells stimulated with peptide-loaded iDCs or B cells (B) is shown as the function of induced CD3ζ tyrosine phosphorylation (*C*) and up-regulation of CD25 (*D*) and CD69 (*E*) given as an increase of mean fluorescence intensity (MFI).

Expectedly, iDCs required ~100 times less peptide than B cells to induce TCR down-modulation (data not shown). Importantly, iDCs also required ~100 times less peptide to initiate CD3ζ phosphorylation (Fig. 2, *A* and *B*). Quantitative analysis also revealed that the amount of peptide-induced CD3ζ phosphorylation is directly linked to the number of down-modulated TCRs, regardless whether iDCs or B cells are used as APCs (Fig. 2*C*). Similarly, iDCs induce the early costimulation-independent T cell activation markers CD25 and CD69 (22–24) with ~100 times less peptide than B cells (data not shown), whereas a given number of down-modulated TCRs correlates with the same degree of activation marker up-regulation with both APC types (Fig. 2, *D* and *E*). Because TCR down-modulation cannot be dissociated from TCR signaling in our model, molecules that can uncouple TCR down-modulation and TCR activation, such as E3 ligases, seem to be regulated similarly by DCs and B cells (25, 26). Thus, measuring down-modulated TCRs appears suitable to assess biologically relevant TCR triggering in the comparative analysis of different APC types.

Amplified TCR triggering by DCs occurs independently of costimulation and of maturation-associated regulation of adhesion

We next asked whether amplified TCR triggering by DCs is regulated by DC maturation and well-defined costimulatory and adhesion molecules, by comparing the TCR triggering efficiency of iDCs (MHC class II^{low} $B7^{low}$ ICAM-1^{low}) and mDCs (MHC class II^{high} $B7^{high}$ ICAM-1^{high}) (Fig. 3*A*). iDCs were superior to mDCs in triggering TCRs (Fig. 3*B*), although expressing 3- to 5-fold less cognate pMHC complexes (data not shown). mDCs were 10-fold more effective TCR triggers than B cells (Fig. 3*B*), but displayed similar pMHC numbers as B cells (data not shown). Thus, it follows that amplified TCR triggering is a special feature of DCs, mostly exhibited by iDCs and reduced albeit not lost during the process of DC maturation.

The inverse regulation of TCR triggering capacity and costimulatory potency in the course of DC maturation already suggests that these functionalities can act independently of each other. To investigate this directly, we interfered with costimulatory molecule function and assayed in parallel the outcome on pMHC-induced TCR triggering and T cell proliferation. As shown in Fig. 3*B*, blocking the CD28-B7 costimulation pathway using CTLA-4-Ig did not affect TCR down-modulation by iDCs or mDCs. In sharp contrast, the enhanced capacity of mDCs to induce T cell proliferation was completely neutralized upon B7 blockade (Fig. 3*C*). Importantly, iDCs and B7-blocked mDCs induced stronger T cell proliferation than costimulation-competent B cells (Fig. 3*C*). This experiment directly shows the biological importance of amplified TCR triggering by DCs for the induction of T cell proliferation.

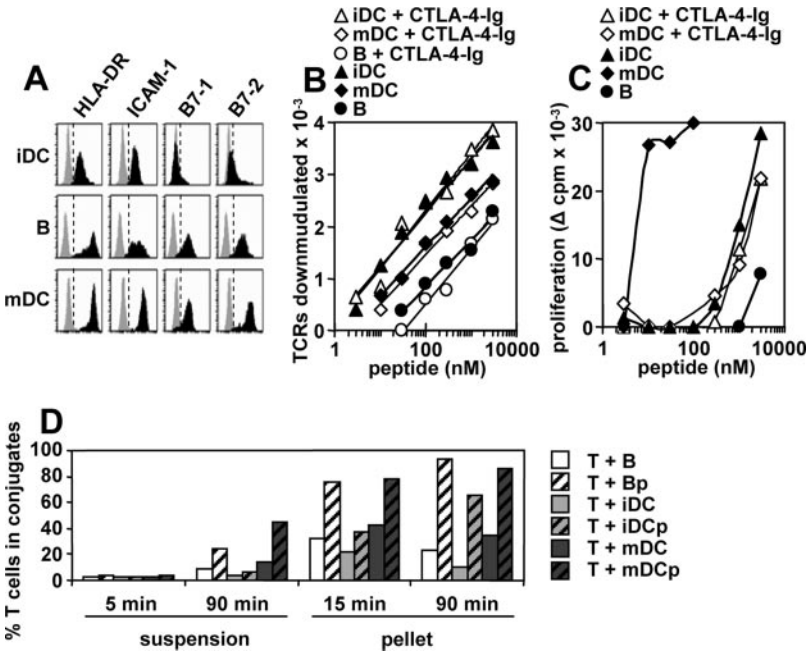

FIGURE 3. Amplification of TCR triggering and costimulation are two independent mechanisms of superior T cell stimulation by DCs. *A*, iDCs, B cells (B), and mDCs were analyzed for the expression of HLA-DR, ICAM-1, and costimulatory molecules (B7-1, B7-2; isotype: gray). *B* and *C*, $TT_{830-843}$-pulsed APCs were tested for their ability to induce TCR triggering (*B*) and T cell proliferation (*C*) in the absence and in the presence of CTLA-4-Ig. T cell clone 48.61 recognizes $TT_{830-843}$ on HLA-DR11. ICOS-Ig and PD-1-Ig were as ineffective as CTLA-4-Ig in inhibiting TCR down-modulation (data not shown). *D*, Conjugate formation is shown between fluorescently labeled T cells (T) and APCs (B cells (B), iDCs, mDCs, and 1 μM peptide-pulsed B cells (Bp), peptide-pulsed iDCs (iDCp), and peptide-pulsed mDCs (mDCp)) after various time periods in suspension or in centrifugation pellets.

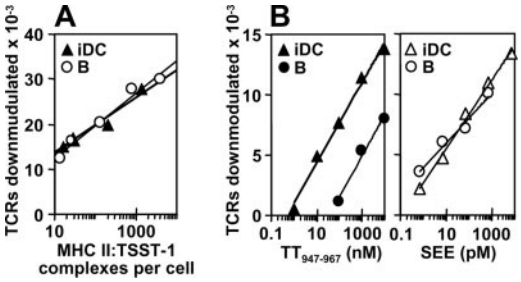

FIGURE 4. Lck-independent superantigen-mediated TCR triggering is equally effective with DCs and B cells. *A*, TCR down-modulation on a TCR V$\beta 2^+$ T cell clone in response to defined numbers of MHC class II-TSST-1 complexes (MHC II:TSST-1) on iDCs or B cells (B). *B*, Triggering of $TT_{947–967}$-specific V$\beta 8^+$ TCR on T cell clone 48.31 by cognate peptide (*left*) or superantigen (staphylococcal enterotoxin E (SEE)) (*right*) displayed on iDCs or B cells (B).

We next asked whether the superior TCR triggering potency of iDCs finds a correlate in a superior ability to form conjugates with T cells. As shown in Fig. 3*D*, iDCs formed less conjugates with peptide-specific T cells than did B cells or mDCs, irrespective of whether the APCs were peptide-pulsed or were not peptide-pulsed. This finding was seen both under the conditions used for the assessment of TCR down-modulation (APC-T cell interaction in pellets) as well as in cell suspensions. Thus, the higher TCR triggering potency of iDCs over B cells cannot be explained by a greater ability of iDCs to form stable contacts with T cells. We conclude that the amplification of TCR triggering by DCs and the DC maturation-associated functions of costimulation and adhesion are important but, at least at the early induction level, are independent mechanisms of DCs to ensure strong T cell activation.

DCs do not amplify TCR triggering in Lck-independent Ag presentation

Superantigen-MHC complexes induce TCR triggering, but, in contrast to pMHC, do not require the SFK Lck (27, 28). To define a potential critical role of Lck in DC-mediated TCR triggering amplification, we compared the TCR-activating potency of superantigen-loaded DCs and nonprofessional APCs. Individual superantigen-MHC complexes displayed by iDCs and B cells triggered an identical number of V$\beta 2^+$ TCRs (Fig. 4*A*), which is in sharp contrast to the observations with pMHC complexes. Moreover, using a double-specificity clone that recognizes both pMHC and superantigen-MHC by virtue of its V$\beta 8^+$ TCR, peptide-loaded iDCs triggered TCRs much more potently than peptide-loaded B cells (Fig. 4*B*, *left*), whereas both APC types triggered TCRs with equal efficiency when loaded with superantigen (Fig. 4*B*, *right*). Thus, DCs amplify TCR triggering in an Lck-dependent but not in an Lck-independent setting of Ag presentation.

DCs activate T cell SFKs in the absence of cognate peptide

Based on these findings, we reasoned that DCs may be particularly effective in activating SFKs in T cells. To investigate this possibility, T cells were cocultured with peptide-loaded and unloaded iDCs and B cells, and the T cell SFKs Lck and Fyn were characterized by immunoblotting. Characteristic of SFK activation is activation loop tyrosine phosphorylation of Lck Y394 and Fyn Y420 (29) and a shift in electrophoretic mobility of Lck from 56 to 59 kDa (30).

As shown in Fig. 5*A*, *top*, the activation-associated 59-kDa Lck species was induced when T cells contacted peptide-loaded iDCs and B cells. More interestingly, iDCs, and to a much lesser extent B cells, elicited prominent Lck band shifting also in the absence of cognate peptide. Induction of the 59-kDa Lck activation form by unloaded iDCs is an active process as it occurred during physical T cell-DC contact at 37°C only (Fig. 5*B*). In line with the Lck band shift results, unloaded iDCs induced strong activation loop tyrosine phosphorylation of SFK species of around 59 kDa (Fig. 5*A*, *bottom*). By immunoprecipitation, these phosphoproteins were identified as activation loop-phosphorylated Lck and Fyn (Fig. 5*C*). We also analyzed T cells contacting unloaded mDCs and found these cells to be almost equally potent inducers of SFKs as were iDCs (Fig. 5*D*). In aggregate, DCs can rapidly induce partial Lck and Fyn activation in the absence of cognate peptide ligands.

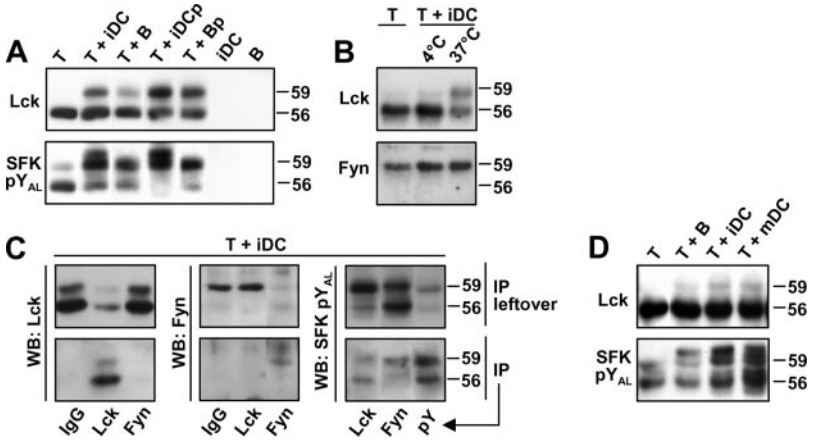

FIGURE 5. DCs activate T cell SFKs in an Ag-independent fashion. *A*, SFK activation is shown in clonal T cells (T) cocultured for 15 min with syngeneic APCs (iDCs, B cells (B), peptide-pulsed iDCs (iDCp), or peptide-pulsed B cells (Bp)) by immunoblotting. Characteristic of SFK activation is a shift from 56 kDa to higher molecular mass species (for Lck) and an increase in total SFK activation loop tyrosine phosphorylation (SFK pY_{AL}) (for both Lck and Fyn). After coculture, APCs were efficiently removed using immunomagnetic beads, and lysates of purified T cells were analyzed. Immunoblot band intensity was normalized for CD2. *B*, Induction of activation-associated Lck isoforms is an active process as it occurs only when T cells are cocultured with iDCs at 37°C. *C*, Unpulsed iDCs induce activation loop tyrosine phosphorylation of both Lck and Fyn in T cells. Lysates of T cells purified after coculture with unpulsed iDCs (T + iDC) were subjected to immunoprecipitation (IP) and immunodepletion (IP leftover) with mAbs directed against Lck, Fyn, and phosphotyrosine (pY). Western blotting (WB) was performed with polyclonal Abs specific for Lck (*left*), Fyn (*middle*), and SFK activation loop tyrosine phosphorylation (*right*). This analysis identifies DC-induced SFK activation loop tyrosine-phosphorylated bands as Lck and Fyn. *D*, Ag-independent SFK induction in T cells (T) cocultured with mDC in comparison to iDC and B cells (B).

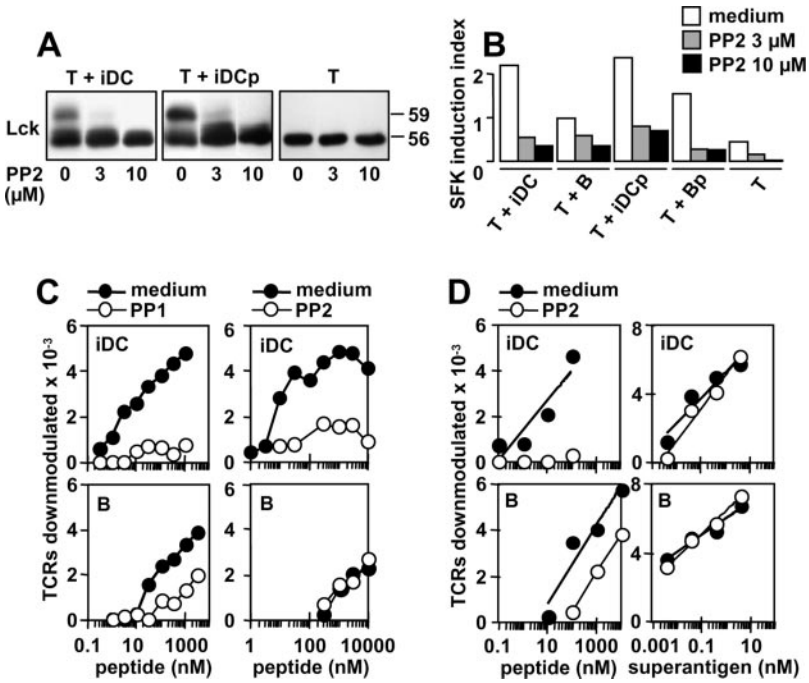

FIGURE 6. Attenuation of SFK activity in T cells abolishes the enhanced TCR triggering potency of DCs. *A*, Pharmacologic inhibition of kinase activity prevents the induction of activation-associated Lck isoforms. T cells were cultured with unloaded (iDC) or $TT_{830-843}$-loaded (peptide-loaded) iDCs (iDCp) in the absence or presence of the SFK-specific inhibitor PP2. Lck was analyzed in purified T cells by Western blotting. Similar results were obtained with B cells (data not shown). *B*, PP2 strongly attenuates APC-mediated induction of SFK mobility characteristics and activating tyrosine phosphorylation. T cells (T) contacting APCs (iDCs, B cells (B), peptide-pulsed iDC (iDCp), or peptide-pulsed B cells (Bp)) in the presence or absence of PP2 were analyzed by Western blotting. The SFK induction index takes into account both the dynamics of activation-associated electrophoretic mobility and the overall activation loop tyrosine phosphorylation of SFKs (see *Materials and Methods*). *C* and *D*, Attenuation of T cell SFK activity results in the loss of efficient pMHC-dependent TCR triggering by DCs, whereas superantigen stimulation is unaffected. *C*, TCR triggering in the absence or presence of the ATP-noncompetitive inhibitor PP1 (8 μM, T cell clone 119.3 recognizes HLA-DP4-$TT_{947-967}$ complexes) (*left*) or of the ATP-competitive inhibitor PP2 (3 μM, T cell clone 48.72 recognizes HLA-DR11:$TT_{830-843}$ complexes) (*right*). *D*, PP2 (1 μM) inhibits TCR triggering mediated by peptide-pulsed iDCs but not by peptide-pulsed B cells (B) (*left*) or by superantigen-pulsed (TSST-1) APCs (*right*) in a peptide-specific TCR Vβ2$^+$ T cell clone (48.105 recognizes HLA-DR11:$TT_{830-843}$ complexes).

Attenuation of T cell SFK activation abolishes the enhanced TCR triggering capacity of DCs

Treatment of T cells with the ATP-competitive SFK inhibitor PP2 (31) effectively attenuated SFK activation by DCs. PP2, at concentrations that did not interfere with tyrosine phosphorylation of non-SFK proteins (data not shown), inhibited the band shift of Lck induced by iDCs (Fig. 6*A*) and led to a drastic reduction of iDC-induced SFK activation loop tyrosine phosphorylation (Fig. 6, *A* and *B* and data not shown). However, basal SFK activation loop tyrosine phosphorylation of resting T cells was abolished under these conditions (Fig. 6*B*). T cell SFK activation by iDCs and B cells was equally effectively inhibited by PP2 (Fig. 6*B*). Pharmacologic interference can therefore be used to selectively attenuate the activation of the T cell SFKs Lck and Fyn.

This effect allowed us to investigate whether the special capacity of DCs to activate T cell SFKs leads to amplification of TCR triggering. Accordingly, peptide-pulsed iDCs and B cells were tested for TCR triggering performance in the absence and presence of PP2 or its ATP-noncompetitive structural analog PP1 (32). Upon T cell treatment with either inhibitor, the capacity of peptide-loaded iDCs to trigger TCRs was reduced drastically, indicating that the efficient SFK induction by DCs is of functional importance for T cell activation, whereas the effect on TCR triggering by B cells, requiring substantially higher peptide concentrations, was much less pronounced (Fig. 6, *C* and *D*, *left panels*). As a functional control, superantigen-dependent TCR triggering that is not amplified by DCs was insensitive to T cell SFK inhibition (Fig. 6*D*, *right*). Thus, our results strongly suggest that high level T cell SFK activation by DCs is functionally relevant and contributes to T cell activation by dramatically augmenting the pool of TCRs that are activated by cognate pMHC.

Self-MHC on DCs contributes to cognate peptide-independent SFK activation in T cells

We next aimed at identifying the molecular interaction partners that mediate Ag-independent SFK activation by DCs. Ag-independent SFK activation by DCs was strongly reduced in the presence of anti-MHC class II Abs (Fig. 7*A*). The mAb MEM-136 specific for peptide-HLA-DR/HLA-DP complexes reduced strikingly the robust T cell SFK phosphorylation induced by unpulsed iDCs and also abolished the relatively minor degree of SFK phosphorylation induced by B cells (Fig. 7*A*, *top*). The mAb FN1 recognizing a subset of MHC class II molecules that are localized in tetraspanin microdomains (33, 34) also strongly reduced T cell SFK phosphorylation induced by unpulsed iDCs (Fig. 7*A*, *bottom*). Both mAbs were highly efficient in preventing TCR down-modulation by peptide-loaded iDCs and B cells, indicating their ability to functionally interfere with TCR recognition of cognate peptide-bearing MHC complexes (Fig. 7*A*; data not shown). In aggregate, these data suggest that noncognate pMHC complexes are critical ligands for T cell SFK activation by DCs.

To see whether the TCR and its MHC restriction is important for SFK activation by noncognate pMHC complexes, we studied T cells contacting Ag-unloaded iDCs and B cells from donors

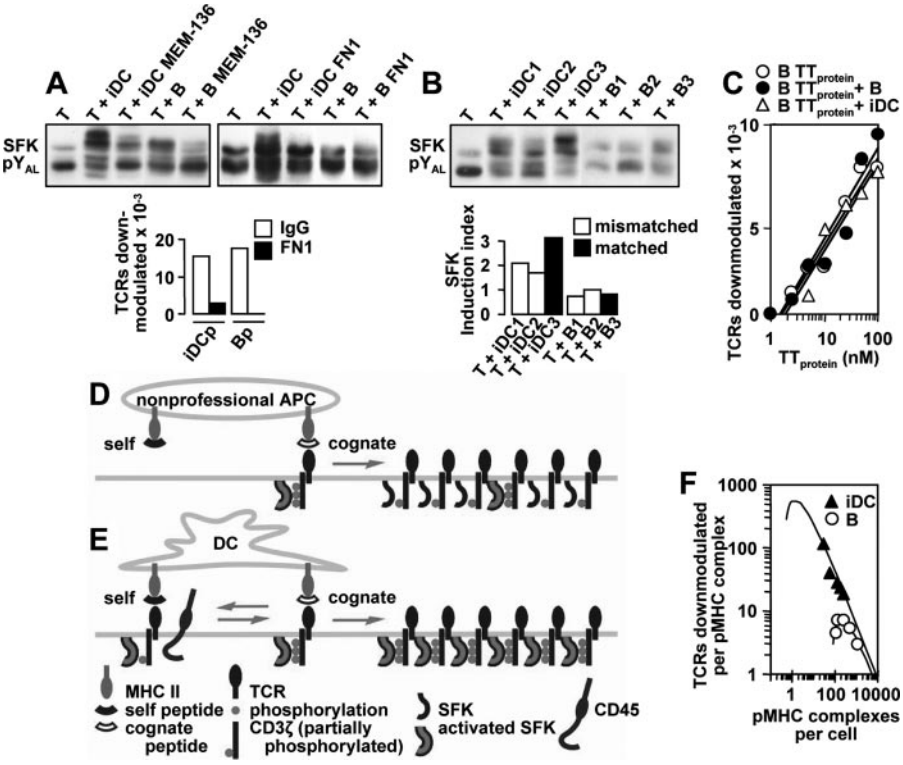

FIGURE 7. Potent T cell SFK activation by unloaded DCs is mediated by MHC class II molecules. *A*, Anti-MHC class II mAbs MEM-136 and FN1 (anti-CDw78, recognizing tetraspanin-associated MHC class II) prevent Ag-independent SFK activation in T cells cocultured with iDCs (*left*). MEM-136 and FN1 efficiently block cognate peptide-mediated TCR triggering with peptide-pulsed iDCs (iDCp, 10 nM) and peptide-pulsed B cells (Bp, 1000 nM) (data shown for FN1 only). *B*, MHC class II allele restriction of DC-mediated, Ag-independent SFK activation demonstrated in a HLA-DR11-restricted T cell clone after coculture with unloaded iDCs and lymphoblastoid B cells (B) from different donors (1, 2, and 3). Only donor 3 expressed HLA-DR11. The SFK induction index for the SFK activation loop tyrosine phosphorylation (SFK pY_{AL}) blot (*top*) is shown at the *bottom*. *C*, TCR triggering by Ag-loaded B cells in the presence of unloaded DCs. B cells were pulsed with TT C fragment protein ($TT_{protein}$) to avoid carry over of peptide to unpulsed iDCs. TCR triggering was assessed with pulsed B cells alone (B $TT_{protein}$), pulsed B cells in the presence of unpulsed B cells (B $TT_{protein}$ + B, ratio 2:1), or pulsed B cells in the presence of unpulsed autologous iDCs (B $TT_{protein}$ + iDC, ratio 2:1). *D* and *E*, Model of DC-mediated TCR licensing and its functional consequences. T cell SFKs are poorly responsive to the TCR encounter of self-pMHC displayed by nonprofessional APCs, whereas TCR interaction with self-pMHC displayed by allele-matched DCs results in strong activation of T cell SFKs (*left*). In the absence of cognate pMHC, SFK activation is not followed by productive TCR signaling. However, activated SFK increases the probability for productive signaling when TCRs contact cognate pMHCs (*right*). We propose the term TCR licensing for this mechanism. *F*, Number of TCRs down-modulated per pMHC ligand in relation to pMHC density on iDCs or B cells (B) (shown using experimental data from Fig. 1*D*, covering a range from 38 to 1309 pMHC complexes per cell, with continuous lines indicating extrapolation).

matched or mismatched for the restricting MHC class II allele. T cell SFKs were activated strongly only by matched donor iDCs (Fig. 7*B*). Interestingly, the limited SFK phosphorylation with MHC class II-mismatched iDCs was always more pronounced than that induced by B cells arguing for, although limited, a contribution of DC molecules other than MHC class II for cognate peptide-independent SFK activation. We thus conclude that the interaction of appropriately restricted TCRs with self peptide-bearing MHC class II molecules is a main mechanism for cognate peptide-independent T cell SFK activation by DCs.

Finally, we asked whether SFK activation by unpulsed DCs transactivates T cells for high level TCR triggering by cognate pMHC displayed on B cells. B cells were pulsed with full protein Ag to avoid carry over of peptide and cocultured with nonpulsed autologous iDCs and T cells. Importantly, the ability of cognate pMHC on B cells to trigger TCRs was not affected by the presence of only self- but not cognate pMHC-displaying iDCs (Fig. 7*C*) (wherein the very same iDCs were capable of amplified-mode TCR triggering; data not shown). Thus, self-pMHC recognition leads to TCR triggering amplification only if SFK-activating self-pMHC complexes and cognate pMHC complexes are displayed by one and the same DC.

Discussion

Recent evidence suggests that self-pMHC-bound complexes can augment $CD4^+$ (35) or $CD8^+$ (14) T cell responses to cognate peptide. It remains to be resolved in detail how this synergism of self- and cognate peptide recognition operates at the molecular level and which APC types are "professional" in mediating this effect. In this study, we show that self-pMHC displayed by human monocyte-derived DCs, in contrast to autologous nonprofessional APCs, can potently activate the SFKs Lck and Fyn in T cells. The critical role for self-pMHC in this process is inferred from our observations that Ag-independent SFK activation by DCs is sensitive to MHC class II blockade and fully operative with MHC class II-matched DCs only. As we further show, this unique Ag-independent SFK activating ability of DCs greatly amplifies the potency of individual cognate pMHC molecules to activate a large number of TCR productively.

As shown in our study, TCR contact with noncognate (self) pMHC-loaded molecules on nonprofessional APCs does not activate T cell SFKs remarkably (Fig. 7*D*). In sharp contrast, TCR contact with self-pMHC molecules displayed on DCs results in strong T cell SFK activation (Fig. 7*E*), involving both Lck and

Fyn. Ag-independent T cell SFK activation by DCs has been observed previously (36), but the functional consequences for T cell activation remained unclear. In this study, we show that Ag-independent DC-mediated SFK activation does not result in full TCR activation, as T cells fail to undergo substantial phosphorylation of CD3ζ or TCR down-modulation (37) (Fig. 2*A*). Importantly, the functional impact of self-pMHC-mediated activation of SFK by DCs becomes apparent when T cells encounter cognate pMHC simultaneously. Only then, self-pMHC-mediated activation of SFKs results in massively amplified CD3ζ phosphorylation and downstream TCR signaling (Fig. 7*E*). For this mechanism, we propose the term "TCR licensing."

The functional impact of DC-mediated TCR licensing is illustrated by the dramatic increase in TCR sensitivity to cognate peptide, as evident from the shift of the dose-response curve to 10- to 100-fold lower pMHC levels. This condition also allows cognate pMHC on DCs to reach its maximal TCR triggering capacity of several hundred TCRs (per 2 h) at a calculated value of a single or very few cognate pMHC complexes per cell (Fig. 7*F*). In contrast, B cells lacking TCR licensing trigger only around 10 TCRs per individual cognate pMHC with an optimal density for this effect of a few hundred cognate pMHC complexes per cell.

It may seem unexpected that DCs can potently modulate TCR sensitivity because B cells have been shown to elicit at least certain sustained T cell responses with few cognate ligands (~10 pMHC) in the T cell-B cell contact zone (15). Some 80 cognate pMHC complexes per B cell surface were required to reach the TCR triggering threshold in our experiments. This apparent difference finds explanation by the fact that a B cell will have a severalfold higher number of pMHC complexes on the entire cell surface than in the T cell-B cell contact zone. The low TCR triggering threshold of one or very few cognate pMHC complexes on DCs is likely relevant because it will allow the recognition of cognate pMHC of the lowest abundance. It will therefore increase considerably the repertoire breadth of pMHC species recognizable by T cells. In addition, TCR licensing endows DCs with amplification capabilities that are not binary but dynamic, allowing them to transduce a wide range of cognate pMHC ligand numbers into an amplified TCR triggering response.

Our measurements suggest that physical TCR-pMHC interactions on both DCs and B cells occur in a serial fashion but do not entirely exclude that both APC types can induce bystander activation of nonengaged TCRs. However, the assumption of increased bystander TCR activation by DCs as the functional consequence of TCR licensing would imply that TCR triggering with DCs is multiplied by a factor (making the slope of the peptide-TCR triggering dose-response curve steeper), whereas the pMHC threshold number for TCR triggering should be the same in DCs and B cells. This assumption clearly contrasts the results of our experiments, which show a close to parallel shift in the dose-response curves. Moreover, an up-regulated serial TCR triggering capacity of DCs is equally unlikely. This reasoning is evidenced by the fact that Lck activation results in enhanced TCR signaling in response to TCR agonists that are fully incapable of serial triggering (37, 38). Thus, it follows that up-regulation of the serial TCR triggering rate is unlikely the mechanistic reason for superior T cell stimulation by SFK-mediated TCR licensing. However, our data show that it is the productivity of serial TCR triggering that clearly differs between DCs and B cells (Fig. 7*F*). This finding strongly implies that self-pMHC-mediated SFK activation by DCs sensitizes individual TCRs for a high probability of full activation after encounter of a cognate pMHC ligand. Thus, TCR sensitivity tuning by DCs is compatible with an increased efficacy at the level of kinetic proofreading (39).

Our data show that a spatially and timely controlled cooperativity between self- and cognate pMHC is required to allow DCs to exert their high TCR triggering capacity. For example, TCR triggering was not amplified in T cells contacting simultaneously self-pMHC-displaying iDCs and cognate pMHC-displaying B cells (Fig. 7*C*). Moreover, DC-T cell conjugation and subsequent DC removal was followed by rapid reversal of SFK induction, and T cells consequently failed to exhibit amplified TCR triggering (data not shown). These findings imply that the signals induced by self- and cognate pMHC cannot be simply separated in space or in time to allow TCR licensing to proceed, but must take place in an organized, possibly synapse-like, cell-cell contact (40). In fact, recent elegant studies demonstrated that soluble self/cognate pMHC heterodimers can activate T cells, whereas soluble cognate pMHC monomers were ineffective (41, 42). These and our results suggest that the intimate association of self- and cognate pMHC-bearing complexes is a driving principle for efficient TCR activation. It is furthermore conceivable that cooperativity between self- and cognate pMHC occurs only in specialized membrane microdomains carrying densely packed MHC complexes that, in fact, have been identified on DCs but less so on other APCs (3). In particular, tetraspanin microdomains were found to contain cognate pMHC complexes with strong TCR triggering potential in DCs and B cells (33), although the underlying mechanism remained to be elucidated. In this study, we show that self-pMHC-mediated SFK activation is essentially aborted by the tetraspanin marker Ab FN1, which recognizes multimeric but not monomeric MHC molecules. This further supports the requirement for compartmentalized delivery of the self-pMHC- and the cognate pMHC-induced signals for strong T cell stimulation and urges for experiments that aim at fully resolving the molecular contribution of tetraspanin domains to this event.

As a nonmutually exclusive explanation, structural features of MHC class II may exist that are specific for DCs but are not found on other APCs (43). Such a condition may result in an altered MHC class II affinity for TCRs and for CD4 molecules resulting in improved Lck activation. We also have evidence that a fraction of Ag-independent SFK activation by DCs occurs in an apparently MHC class II-independent fashion. This evidence derives from the observations that MHC class II-mismatched DCs can induce some degree of SFK activation in T cells and that mAbs to MHC class II reduce but do not completely abolish SFK activation. Thus, it will be interesting to investigate whether non-MHC ligands of CD4 are expressed on DCs and contribute to Lck activation and TCR licensing. A candidate molecule for this process is the CD4 ligand IL-16 (44), which is expressed by iDCs but down-regulated during DC maturation (45, 46). Other possible mechanisms are SFK activation in a CD4-independent as well as MHC-independent fashion, such as triggering of the T cell-expressed tetraspanin molecule CD81 (47). Finally, it needs to be explored whether DCs can MHC-independently activate protein kinase C or MAPK in T cells and thereby activate SFK in an alternative fashion (48, 49).

We also entertained the idea that adhesion and costimulatory molecules contribute to T cell SFK activation by iDCs. This possibility is conceivable because CD3ζ phosphorylation is increased when APCs display ICAM-1 (50) and could, in theory, be indirectly regulated by CD28 signaling. Our experimental results clearly indicate that neither of the two possibilities are fully explanatory for T cell SFK activation by iDCs. This indication is best illustrated by the findings 1) that T cell conjugation (a large part of which is ICAM-1-dependent; data not shown) with iDCs is less

prominent than with B cells and 2) that B7 blockade with CTLA-4-Ig does not affect the strong SFK-mediated TCR down-modulation capacity of iDCs. Surprisingly, DCs upon maturation are somewhat less potent in triggering TCRs while maintaining their capacity to Ag-independently activate T cell SFK. At the functional level, this relative deficit of mDCs is more than compensated by the up-regulation of B7 costimulatory molecules resulting in vigorous Ag-dependent T cell proliferation (Fig. 3, *B* and *C*). Although of unknown significance, it is conceivable that the inhibitory costimulatory molecule PD-L1/B7-H1, which is up-regulated during DC maturation (51), counteracts TCR triggering by mDCs by limiting phosphorylation of CD3ζ and Zap70 (52).

What is the biological role of TCR licensing? As this mechanism, independently of conventional costimulation, reduces pMHC threshold numbers and allows T cells to "see" Ags on DCs that otherwise would be invisible, it may be critical for the sensing of pathogens that are displayed in limited pMHC copy numbers, e.g., due to active inhibition of Ag presentation or low pathogen replication (53). It is also conceivable that TCR sensitivity tuning brings about an increased risk of autoimmune sensitization. This response may be compensated by a critical role of TCR licensing for the establishment and maintenance of tolerance by iDCs. Accordingly, the described mechanism of "silent" sensitivity tuning during the communication between immune cells may be critical to keep the immune system alert for a rapid decision whether to react or not to react. It can also be envisaged that the discovery of TCR licensing offers novel possibilities for the therapeutic regulation of immunity.

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Disclosures

The authors have no financial conflict of interest.

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