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Strong and Durable TCR Clustering at the T/Dendritic Cell Immune Synapse Is Not Required for NFAT Activation and IFN-γ Production in Human CD4+ T Cells1,2

Nicolas Blanchard, Maud Decraene, Kun Yang,3 Francesc Miro-Mur, Sebastian Amigorena, and Claire Hivroz4

The exact function of TCR clustering and organized macromolecular patterns at the immune synapse between APCs and T lymphocytes is unclear. Using human immature or mature dendritic cells (DCs) and autologous CD4+ effector T cells, we demonstrate that, within a given conjugate, mature DCs induce strong and long-lasting TCR clustering and protein kinase C-θ translocation in a superantigen dose-dependent manner. Moreover, mature DCs promote CD43 exclusion in a dose-independent manner. In contrast, immature DCs are less potent at inducing these molecular rearrangements. Using these models to correlate T cell functions with the frequency, the intensity, and the duration of TCR clustering, we show, in Jurkat T cells, that weak and transient TCR clustering is sufficient to promote TCR down-modulation, protein kinase C-θ translocation at the synapse, and substantial NFAT transcriptional activation. Moreover, we show, in CD4+ T cell blasts, that strong TCR clustering is required for neither TCR down-modulation nor optimal IFN-γ production. Together, our results demonstrate that some CD4+ functional responses, such as cytokine production, are independent of central supramolecular activation cluster formation. The Journal of Immunology, 2004, 173: 3062–3072.

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5 Abbreviations used in this paper: PKC-θ, protein kinase C-θ; SMAC, supramolecular activation cluster; cSMAC, central SMAC; CD2AP, CD2 adaptor protein; YFP, yellow fluorescent protein; DC, dendritic cell; TSST-1, toxic shock syndrome toxin 1; SEE, Staphylococcus enterotoxin E.
wherein signal strength drives T cells through hierarchical thresholds of activation leading to proliferation, differentiation, and death (19, 20). Stimulation strength depends on several parameters, such as Ag concentration, duration of Ag presentation by APCs, and the cellular and molecular environment in which Ag is presented (21–23). Thus, the history of the T cell, the cytokine context, and the nature of the APC encountered shape the T cell response and drive the decision toward immunity or tolerance. For instance, immature and mature DCs display highly different T cell immunostimulatory properties, which may result in immunity or tolerance induction (for review, see Refs. 24–26). We took advantage of marked differences in terms of synapse formation and T cell activation to decipher the links between the molecular rearrangements at the T/DC interface and various T cell functions.

Human immature or mature monocyte-derived DCs loaded with superantigens were used to activate autologous T cell blasts or Jurkat T cells. We studied TCR clustering, PKC-θ translocation, and CD43 exclusion in T cell blasts and Jurkat T cells. We also measured NFAT transcriptional activity in Jurkat cells, TCR down-modulation, and IFN-γ production in T cell blasts. Using confocal imaging and long-term time lapse videomicroscopy, we show that immature DCs induce TCR clustering and CD43 exclusion in a lower percentage of T cells than mature DCs. Moreover, TCR clustering, when induced with immature DCs, was less intense and more transient. This was not a consequence of a differential TCR engagement, because similar early TCR down-modulation is induced by both immature and mature DCs. In Jurkat T cells, weak and transient TCR clustering was sufficient to promote PKC-θ recruitment and NFAT activation. Moreover, in T cell blasts, strong TCR clustering was required for neither TCR down-modulation nor optimal IFN-γ production.

**Materials and Methods**

**Plasmids and transfection**

The ζ-EGFP plasmid described in Ref. 27 was modified to introduce a longer linker (three repeated motifs of four glycine and one serine) between ζ and the GFP. The NFAT-YFP construct was derived from the NFAT-GFP plasmid (from Dr. N. Clipstone, Northwestern University, Chicago, IL) containing four NFAT binding sites and a minimal SV40 promoter, through insertion of the yellow fluorescent protein (YFP) gene between the S' HindIII and S' NotI restriction sites.

Wild-type Jurkat cells (clone 20; obtained from Dr. A. Alcover, Pasteur Institute, Paris, France) were grown in Glutamax-containing RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (European Grade; Biological Industries, Beit Haemek, Israel). Jurkat cells were electroporated at 260 V and 1200 μF using the Optimix kit according to the manufacturer's instructions (EquiBio, Needham Heights, MA). Stable clones expressing ζ-GFP or the YFP reporter gene were obtained by continuous growing in the presence of 1 mg/ml neomycin.

**Dendritic cells (DCs) and T cell blasts preparation**

Monocytes were purified from human PBMCs by adherence on plastic flasks. Immature DCs were generated by culturing monocytes in RPMI 1640 supplemented with 100 ng/ml GM-CSF (Leukomax; Novartis, East Hanover, NJ) and 40 ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 5–6 days. Maturation was induced for 24 h in the presence of 10 ng/ml TNF-α (R&D Systems) and 1 μg/ml PGE2 (Sigma-Aldrich, St. Louis, MO). Autologous superantigen-reactive T cell blasts were obtained by activating PBMCs from the same donor with 0.1 μg/ml toxic shock syndrome toxin 1 (TSST-1; Toxin Technology, Sarasota, FL) for 3 days. Then, IL-2 at 20 U/ml was added for 6 more days. Superantigen loading on DCs was performed at 37°C for 1 h.

**Abs and reagents**

The following murine mAbs were used: anti-CD1a-FITC, anti-CD14-PE, anti-CD86-FITC, anti-HLA-DR-FITC, anti-CD80-PE, anti-CD83-PE, anti-CD3-PE, anti-CD11c-allophycocyanin (all from BD Pharmingen, San Diego, CA). Human immature and mature DCs were labeled with anti-CD11c-PE, anti-CD14-PE, anti-CD3-PE, anti-CD86-PE, anti-CD80-PE, and anti-CD83-PE (BD Pharmingen, San Diego, CA) and subjected to flow cytometry. Mouse M104 monoclonal antibody against human CD43 was purchased from BD Pharmingen. Human wild-type Jurkat cells were electroporated using a BD Bio vector delivery system. 

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CA); anti-TCRVß2-FTTC and anti-IFN-γ-PE (from Beckman Coulter, Fullerton, CA); anti-ζ (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PKCa (BD Transduction Laboratories, Lexington, KY); anti-CD43 (kind gift of Dr. F. Sanchez-Madrid, Universidad Autonoma de Madrid, Madrid, Spain); the anti-human MHC class II L243 (purified by Dr. W. Faigle, Institut Cochin, Paris, France), Cy5-conjugated F(ab')2 goat anti-mouse, PE- coupled anti-mouse Abs and streptavidin were from Jackson Immuno- Research Laboratories (West Grove, PA), and Alexa 488-conjugated F(ab')2, anti-mouse Abs and CFSE were purchased from Molecular Probes (Eugene, OR).

Immunolabeling and fluorescence microscopy

DCs were pulsed with various concentrations of superantigen, washed, resuspended in RPMI 1640, and settled onto a poly-L-lysine-coated cover-slips for 15 min. T cells were washed in RPMI medium with PBS plus 0.4% BSA, cells were labeled with anti-IFN-γ, anti-MHC class II, rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) for 45 min at 37°C. Remaining cells were put back in culture. CFSE labeling analysis was performed immediately after depletion and at day 3.

Statistics

All p values were calculated with the unpaired Student’s t test assuming equal variances. Significant differences are symbolized by the following: *p < 0.05; **p < 0.01. Linear correlation between two parameters was assessed through the Pearson coefficient.

Results

Generation and phenotype of blast T cells and DCs

Human T cell blasts were obtained by stimulation of PBMCs with the TSST-1 superantigen, which binds to TCR Vß2 and specifically activates this TCR Vß2+ subset. At day 9, >95% of the cells were CD3+ (data not shown), and the majority was CD4+ Vß2+ (~70% as opposed to ~4% before expansion, according to the donor; Fig. 1A and data not shown). Autologous immature DCs were obtained by differentiation of the monocytes from the same donor with GM-CSF and IL-4 for 5–6 days. Maturation of DCs was induced by a 24-h incubation with a mixture of PGE2 and TNF-α. As previously described (28), this treatment reproducibly increased the surface expression of CD80, CD86, CD40, CD83, and MHC class II molecules (HLA-DR, 3.6-fold increase; SD, 1.2; n = 17), whereas it slightly down-regulated CD1a expression (Fig. 1B). Immunostimulatory capacity of both types of DCs was compared in an allogeneic MLR. PGE2/TNF-α-treated DCs were able to induce a stronger proliferation of allogeneic PBMCs, compared with immature DCs (data not shown). We also verified that mature DCs were more immunocompetent than immature DCs in the model we used in this study (autologous T cell blasts). We determined T cell blast proliferation induced by immature or mature DCs, using CFSE labeling and flow cytometry analysis. To avoid DC maturation during the 3-day coculture (29), DCs were depleted after 6 h of contact. This was achieved by two rounds of complement-mediated lysis after MHC class II labeling of DCs, which expressed much more MHC class II than T cell blasts (data not shown). The proportion of live DCs dropped off from ~35% of total cells to <4% (data not shown). At day 3, CFSE profiles (Fig. 1, C and D) show a weak proliferation (1–2 cycles) of T cell blasts in the absence of TSST-1. Addition of TSST-1 induced, in a dose-dependent manner, more T cells to divide. T cells activated by TSST-1-pulsed immature DCs stopped dividing earlier than those activated by mature DCs, as shown by the proportion of cells in the fourth cell cycle (37 vs 5%), demonstrating that mature DCs were more potent than immature DCs at inducing autologous T cell blast proliferation.

These functional responses, as well as the phenotypes described above, confirm that DCs obtained in these conditions are conventional immature and mature DCs.

FIGURE 2. Stronger TCR enrichment and higher rate of CD34 exclusion at the synapse formed with mature DCs. A–E, DCs pulsed with TSST-1 or SEE were mixed for 40 min with primary blast T cells (A, B, and C) or Jurkat cells (D and E), respectively. Subcellular localization of ζ (A), TCR Vß2 (B), ζ-GFP (D), and CD43 (C and E) was analyzed by confocal microscopy through immunofluorescent labelings or intrinsic fluorescence of the GFP. On each panel, the left image presents a representative conjugate showing no clustering of ζ, Vß2, or ζ-GFP, or no exclusion of CD43, whereas the right image shows one representative conjugate exhibiting clustering of ζ, Vß2, or ζ-GFP, or exclusion of CD43 (open arrowhead). Scoring of cells exhibiting TCR/ζ clustering or CD43 exclusion was done blindly by two distinct observers on conjugates randomly chosen from transmission images (histograms). Error bars represent the SD from 2 to 5 independent experiments, and 40–100 conjugates have been observed in each condition. Scale bar, 5 μm. F and G, Fluorescence intensity of TCR Vß2 (F) or ζ-GFP (G) in a fixed region of the synapse was divided by the average intensities measured in three regions outside the synapse. These values were intermediate, averaged, and plotted on the diagrams. Each plot symbolizes one conjugate with immature DCs (○) or mature DCs (■). More than 20 blast T cell/DC conjugates and >35 Jurkat/DC conjugates from two independent experiments were evaluated for each condition. —, Represents the mean from all of the conjugates.
Synapse formation between T cells and DCs

To study whether the patterning of surface molecules at synapses between human effector T cells and superantigen-loaded DCs depends on DC maturation, we assessed the distribution of the TCR and CD43 after 40 min of T/DC contact. To do so, we detected the \( \xi \) and V\( \beta \)2 subunits of the TCR (Fig. 2, A and B) and CD43 (C) by immunofluorescent labeling. Scoring of the conjugates displaying \( \xi \) or V\( \beta \)2 clustering at the contact zone revealed a superantigen dose dependence for TCR clustering. At high concentration of TSST-1, the frequency of blast/mature DC conjugates exhibiting TCR V\( \beta \)2 accumulation was around twice higher than the frequency of blast/immature DC conjugates (75.5 vs 37%; Fig. 2B).

At \( 10^{-2} \mu g/ml \), the proportion of conjugates showing TCR accumulation was weaker and similar for immature and mature DCs (20.5 vs 20.5%; Fig. 2B). CD43 was excluded more frequently in response to mature DCs than to immature DCs (69 vs 39.5% at dose of 1 \( \mu g/ml \)). These percentages roughly correspond to those found for V\( \beta \)2 clustering, suggesting that TSST-1-induced CD43 exclusion takes place in the V\( \beta \)2\(^{+} \) and not in the V\( \beta \)2\(^{−} \) T cell blasts. However, we cannot exclude that a certain proportion of the conjugates showing CD43 exclusion is formed with V\( \beta \)2\(^{−} \) cells. As opposed to TCR V\( \beta \)2 clustering, CD43 exclusion was also observed in the absence of TSST-1.

Similar experiments were then performed using \( \xi \)-GFP-expressing Jurkat cells and DCs loaded with the Staphylococcus enterotoxin E (SEE) superantigen, which binds to and activate the TCR V\( \beta \)8 expressed by Jurkat cells. Like B cells (27), DCs induced a dose-dependent \( \xi \) clustering (Fig. 2D). At \( 1 \mu g/ml \) SEE, mature DCs drove \( \xi \) clustering more frequently than immature DCs, whereas the rate of clustering was very low at \( 10^{-2} \mu g/ml \) (Fig. 2D). Similarly to the observations made on T cell blasts, CD43 exclusion occurred more frequently in Jurkat/mature DCs conjugates at high SEE concentration but also in the absence of SEE (Fig. 2E).

To confirm these results, we quantified the enrichment of TCR V\( \beta \)2 in V\( \beta \)2\(^{+} \) blast T cells (Fig. 2F) or the enrichment of \( \xi \)-GFP in Jurkat cells (G) at the DC contact site relative to the T cell periphery. At high superantigen concentrations, we observed a significantly stronger accumulation of the TCR (2.2-fold; \( p = 0.005 \)) and \( \xi \) (2.8-fold; \( p = 10^{-8} \)) in synapses formed with mature DCs than with immature DCs. In contrast, at lower superantigen concentration or without superantigen, the difference in TCR and \( \xi \) accumulation at the synapse formed with mature and immature DCs was much lower (Fig. 2, F and G). Measuring the accumulation of \( \xi \) in blast T cells gave similar results (data not shown).

Strikingly, as compared with internal controls that were in average equal to 1 (data not shown), levels of TCR and \( \xi \) enrichment at the synapse were higher than 1 even without superantigen. This basal enrichment in the absence of superantigen is likely to reflect the previously described Ag-independent synapses (17) for autologous blast T cells and/or an effect of allogeneic MHC recognition for Jurkat cells.

Together, these results demonstrate, first, that the percentage of T cells presenting with TCR clustering and the intensity of this clustering are a function of both superantigen concentration and DC maturation state; and second, that exclusion of CD43 mainly relies on DC maturation state and not on the presence of superantigen. In summary, DC maturation controls the surface molecular patternings occurring at the T/DC synapse and mature DCs loaded with high superantigen concentrations preferentially promote intense TCR accumulation.

**FIGURE 3.** TCR down-modulation induced by immature and mature DCs. A. After 40 min of contact with autologous TSST-1-pulsed DCs, surface TCR expression on T cell blasts was analyzed by gating on V\( \beta \)2\(^{+} \) cells. B. Percentage of TCR V\( \beta \)2 remaining at the surface of blast T cells stimulated by immature (○) or mature DCs (■) is plotted vs TSST-1 concentration. Mean fluorescence intensities are normalized with respect to the mean fluorescence intensity measured in blast T cells interacting with immature DCs in the absence of TSST-1. One representative experiment of four is shown.

**TCR down-modulation induced by DCs**

Surface TCR down-regulation reflects the level of TCR triggering by its ligand on the APC (15). To see whether TCR down-modulation would be enhanced in the conditions where a strong TCR clustering is observed, we measured at 40 min both TCR V\( \beta \)2 down-modulation and TCR clustering in the same T cell blasts interacting with autologous DCs pulsed with increasing amounts of TSST-1. Surface expression of TCR V\( \beta \)2 was assessed exclusively on V\( \beta \)2\(^{+} \) cells, as depicted on Fig. 3A. Down-modulation was undetectable at \( <10^{-2} \mu g/ml \) TSST-1, reached \( \approx \)35% at the highest concentration, and remained similar in response to both types of DCs (Fig. 3B). These results indicate that the number of TCRs triggered by immature and mature DCs was identical at this early time point, whereas the TCR enrichment was higher with mature DCs at high superantigen concentration (see Fig. 2F).

**PKC-\( \theta \) translocation induced by DCs**

The mature immune synapse is not only characterized by TCR clustering but also by PKC-\( \theta \) translocation at the contact zone (6). To detect PKC-\( \theta \) localization, we performed immunofluorescent labelings after 40 min of contact between \( \xi \)-GFP-expressing Jurkat cells and immature or mature DCs (Fig. 4A). We then scored the percentage of conjugates showing PKC-\( \theta \) translocation (Fig. 4B).

PKC-\( \theta \) translocation was SEE-dependent, and percentages of T/DC conjugates showing translocation reached a plateau of 90% at \( 10^{-3} \mu g/ml \). At \( 10^{-2} \mu g/ml \) mature DCs induced PKC-\( \theta \) translocation in a higher percentage of conjugates than immature DCs,
but this difference vanished at higher concentrations. We then quantified the enrichment of PKC-θ at the T/DC junction relative to the T cell periphery (Fig. 4C), as described above for Vβ2 and ζ. Very weak in the absence of SEE, the enrichment significantly increased with SEE concentration to reach values $\geq 3.2$-fold. Differences in PKC-θ enrichment induced by immature and mature DCs were significant at $10^{-3} \mu$g/ml but not at higher doses. These results indicate that optimal surface translocation of PKC-θ requires neither TCR clustering nor CD43 exclusion (see the results at $1 \mu$g/ml superantigen with immature DCs on Fig. 2, D and E).

**Characterization of TCR dynamics between live T cells and DCs**

Results presented in Fig. 2 describe the levels of mature synapse formation after 40 min of contact. To better examine the temporal evolution of TCR clustering and the dynamics of the T/DC interactions, we performed 6-h time-lapse videomicroscopy experiments on ζ-GFP-expressing Jurkat cells interacting with mature DCs (Fig. 5A and supplemental movie 1) or immature DCs (B and supplemental movies 2 and 3) pulsed with 1 μg/ml SEE. As previously described, ζ localizes at the plasma membrane and in intracellular compartments found in the centrosome region (27). Following interaction with a mature DC, both polarization of intracellular ζ, reflecting centrosome reorientation toward the DC, and clustering of ζ at the synapse occurred (Fig. 5A and supplemental movie 1). After a few hours, while the two cells were tearing apart, it was not rare to see transfer of GFP material from the synaptic zone onto the DC. At high SEE concentration, the vast majority of T cells had clustered their TCR as soon as 10 min after the first contact (Fig. 5C, right panel). Lower SEE concentrations also promoted ζ clustering, but with a delayed kinetics (Fig. 5C, left panel). In contrast, although T cells interacted with and did form conjugates with immature DCs, ζ clustering was not necessarily observed (Fig. 5B and supplemental movies 2 and 3). More than 50% of T cells in conjugates at $10^{-2} \mu$g/ml and one-third at $1 \mu$g/ml SEE did not display ζ clustering after 4 h of interactions, regardless of their static or dynamic status (Fig. 5C). Furthermore, at high SEE concentration, ζ clustering duration was significantly ($p < 10^{-5}$) shorter in T/immature DC (46 ± 71 min) than in T/mature DC (144 ± 69 min) conjugates (Fig. 5D).
Because the model using T cell blast did not allow us to
dynamically follow TCR clustering, we performed movies to docu-
ment the dynamic of contact between T cell blasts and DCs. T cell
blasts interacting with TSST-1-pulsed mature DCs stopped migrat-
ing, rounded up, and exhibited long-lasting and rather static
contacts (supplemental movie 4). By contrast, the majority of contacts
with pulsed immature DCs resulted in a short stop before the T cell
continued moving and crawling around one or several DCs (sup-
plemental movie 5).

Together, these results show that T cell interactions with immu-
rate DCs induce less frequent and more transient TCR clustering,
and that DC maturation stabilizes the T/DC interactions, favors
cell clustering, and increases its duration.

**TCR down-modulation and IFN-γ production**

Therefore, DC maturation and superantigen concentration govern
the rate, intensity, and duration of TCR clustering. It has also been
demonstrated that immature or mature DCs induce different T cell
functional outcomes. We took advantage of these differences to
sort out whether a long-lasting and strong TCR clustering was
required for functional T cell responses, such as TCR down-mod-
ulation and IFN-γ production. DCs were loaded with increasing
amounts of TSST-1 and mixed with autologous TSST-1-amplified
blast T cells. After 6 h of incubation, both surface Vβ2 and intra-
cellular IFN-γ were labeled (Fig. 6A). Because the proportion of
IFN-γ Vβ2+ cells was similar in all conditions, they were gated
out from further analysis. TCR surface expression and percentage
of Vβ2+ IFN-γ-producing cells were plotted vs TSST-1 concen-
tration (Fig. 6, B and C, respectively). TCR down-modulation
increased with TSST-1 concentration, and the shapes of the curves
obtained with immature or mature DCs were similar. Without
TSST-1 and at low TSST-1 concentrations, mature DCs were
slightly more efficient than immature DCs at promoting TCR
down-modulation. At the plateau, differences remained faint
(−10%), although reproducible. Roughly mirroring the TCR
down-regulation, IFN-γ production in Vβ2+ T cells was TSST-
1-dose dependent and slightly higher in response to mature DCs at
low TSST-1 concentrations. The activation plateau corresponding
to almost all of the T cells synthesizing IFN-γ, was reached at
10−2 μg/ml both for immature and mature DCs.

To measure the extent of correlation between IFN-γ production
and TCR clustering in blast T cells, each of the six different con-
ditions (immature or mature DCs; 0, 10−2, or 1 μg/ml TSST-1; 1;
data from Figs. 2 and 6) was represented on a dot plot (data not
shown), and the Pearson coefficient was extracted from a linear
regression. It appeared that the linear correlation between the rate
of TCR clustering and the proportion of T cell blasts producing
IFN-γ was poor (r = 0.46; p = 0.35). In sharp contrast, the rate
of TCR clustering and the level of TCR Vβ2 enrichment at
the synapse were highly correlated, as witnessed by a Pearson co-
efficient close to 1 (r = 0.98; p = 4 × 10−6).

To rule out the possibility that different geometrical constraints
may explain the different superantigen concentration requirements

**FIGURE 5.** Interactions of Jurkat T cells with immature DCs result in more transient and weaker TCR clustering than with mature DCs. A and B, ζ-GFP-expressing Jurkat cells were mixed with mature DCs (A) or immature DCs (B) pulsed at 1 μg/ml SEE and imaged for up to 6 h by time-lapse videomicroscopy. Series of fixed images extracted from the movies at indicated time points are presented; phase contrast images are on the left, and the maximum intensity projections of GFP stacks are on the right. While arrows point to dense and stable ζ-GFP clusterings observed with mature DCs (A) or to weaker and more transient ζ-GFP clusterings seen with immature DCs (B, lower and upper panels). Open arrowheads on B show clear contacts between T and DC without detectable ζ-GFP clustering. Scale bar, 5 μm. C, Each T cell exhibiting a contact with immature DCs (□) or mature DCs (■) loaded with high (1 μg/ml; right panel) or intermediate (10−2 μg/ml; left panel) concentration of SEE was individually tracked to detect the first apparition of ζ-GFP clustering. Histograms show the percentage of cells presenting with ζ accumulation of any type (long-lasting or transient) after 10 min, 2 h, or 4 h of dynamic or static contacts, calculated from 84 cells analyzed from 20 independent movies. D, Duration of every visually detected clustering was measured. Distributions are significantly different (p = 1.8 × 10−6) between immature DCs (□; 36 measurements) and mature DCs (■; 23 measurements).

**FIGURE 6.** TCR surface down-modulation and optimal IFN-γ production induced by immature and mature DCs. A, Day 9 T cell blasts were mixed for 6 h with DCs pulsed with increasing TSST-1 concentrations and labeled for surface Vβ2 and intracellular IFN-γ. A logical gate based on forward light scatter/side light scatter parameters was used to exclude DCs; dot plots show T cell blasts activated by immature (upper row) or mature (lower row) DCs. B and C, Further analysis of surface TCR down-modulation (B) or IFN-γ production (C) vs TSST-1 concentration (□, immature DCs; ■, mature DCs) was performed by excluding Vβ2+ IFN-γ+ T cells, which proportion remained constant for every condition. TCR surface ex-
pression was normalized with respect to the mean fluorescence intensity measured on T cells interacting with immature DCs in the absence of
TSST-1. Data are mean ± SD from three independent experiments.
on coverslips rather than in round-bottom wells. In these conditions, optimal IFN-γ production was also obtained at $10^{-2} \mu g/ml$ (data not shown).

In conclusion, these results suggest that strong TCR clustering in the synapse is required for neither TCR down-modulation nor IFN-γ production.

**Activation of NFAT transcriptional activity**

As another functional readout, we sought to determine the role of TCR aggregation in the triggering of NFAT transcriptional activity. We measured this transcriptional activity at a single-cell level within a Jurkat-derived clone expressing an NFAT-driven YFP reporter. Because the maximum yield of YFP$^+$ cells after a strong stimulus (PMA plus ionomycin incubation) was $\sim 70\%$ (Fig. 7A), we excluded nonresponsive cells from the measurement by expressing results as percentage of the maximum (β). After 6 h, at $10^{-2} \mu g/ml$ SEE, there was a marked difference in the proportion of cells expressing YFP in response to immature or mature DCs. In contrast, at 1 μg/ml SEE, this difference diminished and immature DCs could induce NFAT activation in 75 vs 95% of T cells activated by SEE-pulsed mature DCs.

We concluded that the transient and weak TCR clustering observed with immature DCs is sufficient to trigger NFAT transcriptional activity.

**Discussion**

Using human DCs at different stages of activation as well as Jurkat T cells or autologous effector T cells, we demonstrate in this study that weak and transient TCR clustering is sufficient to promote TCR down-modulation, PKC-θ recruitment, NFAT transcriptional activity, and IFN-γ production. Our study shows that the classical bull’s-eye pattern originally described with B cells or lipid bilayers is not required for T cell activation.

In the current work, we use human monocyte-derived immature or mature DCs pulsed with superantigens from bacterial origin as APCs. Superantigens bind both the TCR and MHC class II molecules, leading to polyclonal T cell activation on the basis of Vβ expression. Their use allow the amplification of a large amount of T cells that homogeneously respond to a secondary stimulation by the same superantigen. Superantigen stimulation is thus different from a clonal activation by MHC-peptide complexes; however, it shares similarities. Interaction kinetics between the TCR and MHC-superantigen complexes are in the range of those measured between TCR and MHC-peptide complexes (30, 31). Immune synapses induced by superantigen-pulsed APCs have been described by us and others (27, 32, 33).

Whereas the mature synapse (including TCR and PKC-θ central colustering and CD43 exclusion) has been reported many times with B cells (6, 27) or artificial bilayers (2), synapse formation in an autologous human T/DC model had never been described so far. We found that CD43 exclusion can occur in the absence of TCR clustering (unpulsed mature DCs) and that optimal PKC-θ translocation can be observed at low intensities of TCR clustering (immature DCs; 1 μg/ml SEE). This striking decoupling in the patterning of proteins at the T/DC synapses shows that the classical bull’s-eye pattern originally described with B cells or lipid bilayers is not a canonical structure formed with all APC types.

One major result from our work is the differential surface molecular rearrangements observed at the interface between effector T cells and mature or immature DCs. Why would mature and immature DCs drive TCR clustering with different efficacies? One possibility is that TCR aggregation in cSMAC is uniquely controlled by the number of TCRs triggered by ligands presented by DCs. There is indeed a clear superantigen dose dependence of TCR clustering observed with mature DCs. However, we do not think it is the only explanation. Although TSST-1 loading was higher on mature DCs (as measured by a 7.5-fold better loading of biotinylated TSST-1; SD 2.5; n = 7; our unpublished results), levels of Vβ2 down-modulation in blast T cells at 40 min were similar with immature DCs and mature DCs, indicating that the number of triggered TCRs was the same. The establishment of a mature synapse may thus rely on intrinsic properties of the DCs, independent of the number of TCR ligands displayed at their surface. The fact that mature DCs, but not immature DCs, promote CD43 exclusion in the absence of superantigen (see Fig. 2C) supports this hypothesis. Furthermore, Qi et al. (34) reported that supramolecular organization of surface proteins at the synapse might be explained by a self-assembly process depending on biophysical and biochemical parameters. Some of these parameters, such as membrane stiffness, overall charge and size of molecules, which
depend on their glycosylation status (35), may vary along DC maturation and explain this differential capacity at inducing mature synapse formation.

In addition to the differences observed in the organization of proteins at the immature or mature DC/T interface, the overall features of the interactions were distinct. Although contacts between Jurkat cells and mature DCs were tight and accompanied by a strong $\xi$ clustering at the interface, contacts with immature DCs were more dynamic and often exhibited patterns such as repeated attachment/detachment from the DC or T cell crawling onto the DC. Moreover, $\xi$ clustering within one conjugate was weaker and more transient. These latter observations indicate that the cSMAC cannot properly form with immature DCs, therefore witnessing that formation of the mature synapse is impaired. A striking migratory behavior was observed in T cell blasts interacting with immature DCs as opposed to the more static contacts with mature DCs. Whether this static behavior is a cause or a consequence of SMACs formation remains to be established.

A matter of controversy for a few years has been whether the immune synapse controls T cell activation. Or should we prefer the following question: which aspects of T cell activation does the immune synapse control and to what extent? Although one report attributes to SMACs a role in regulation of phosphoryrosine patterns by orchestrating CD45 distribution (13), kinetic arguments suggest that the mature synapse would not be involved in early TCR signaling (12, 36). Indirect evidence for an implication of the synapse in late and continuous TCR signaling comes from the study by Huppa et al. (16), who found that a high PI3K activity was associated to a ring distribution of ICAM-1 persistent for 10 h in murine B/CD4$^+$ blast pairs. The same authors stated that a minimum of 10 h of synapse maintenance was required for shaping a full effector potential, in terms of IL-2 secretion and proliferation. In addition, one paper using an artificial lipid bilayer system (2) reported that formation of a stable central cluster was a determinant event for T cell proliferation.

In the present study, we found a low correlation between mature synapse formation and IFN-\(\gamma\) production by blast T cells (Pearson $\rho = 0.46$). In other words, in response to immature DCs or mature DCs pulsed at intermediate superantigen concentration, almost all of the T cell blasts secreted IFN-$\gamma$, whereas the percentage of T cells displaying mature synapse remained weak ($<40\%$). This indicates that formation of a mature synapse is not an absolute prerequisite for triggering IFN-$\gamma$ synthesis. In this line, a report combining in vitro and in silico experiments, examined the function of the cSMAC in mice defective for CD2AP (14). Although T cells from these mice do not exhibit SMACs when interacting with APCs, their sensitivity to Ag is enhanced as witnessed by sustained $\xi$ phosphorylation, absence of TCR degradation, and stronger proliferative responses, as opposed to CD2AP$^{-/-}$ controls. In other systems, the necessity for a highly organized junction, characterized by the formation of SMACs, to induce functional responses has also been questioned (37, 38).

As observed in long-term recordings of living cells, the intensity and the duration of TCR clustering were determined by DC maturation stage. However, the weak and transient TCR clustering observed with immature DCs could promote substantial TCR engagement and NFAT activation, suggesting that signals transduced by transient TCR clustering can accumulate within the T cell and trigger functional responses. These results are in line with previous data showing that intermittent TCR signals, interrupted by the addition of Src kinases inhibitors, are integrated and lead to IFN-$\gamma$ production (39). They support a model of temporal and spatial signal summation. In this model, the duration of TCR clustering would be integrated in time and would, above a certain threshold, induce NFAT transcriptional activity.

Whether these in vitro results reflect an in vivo reality is still an open question. A recent study using intravital microscopy has reported the occurrence of three distinct phases during naive T cell priming by DCs in lymph nodes (40). Whereas contacts with DCs were stable and long-lasting during the second phase, T cells underwent multiple short encounters with DCs during the earliest phase. However, although this study (40) and others, performed on explanted lymph nodes (41–43), bring valuable data on the interaction kinetics in vivo, almost no information exists on the molecular dynamics at the T/DC junction. To our knowledge, only one paper so far has shown that CD43 is excluded from the contact zone between T cells and DCs (43).

The organization of transmembrane receptors into higher order arrays has been shown in many cells from bacteria to neurons. In T lymphocytes, higher order assemblies of the TCR occur in the contact zone formed with APCs. However, their functional consequences are still controversial. First, TCR oligomerization would counteract the low affinity of TCR for MHC-peptide complexes by enhancing the likelihood of re-binding shortly after dissociation, instead of diffusing away from the interaction zone (44). Second, it would favor TCR binding to endogenous MHC-peptide complexes, a phenomenon improving the efficiency of Ag recognition (45, 46). Third, it would allow the T cell to increase its specificity and sensitivity through a negative and/or positive cross talk among the receptors (47). This cooperative effect might be mediated by the local concentration of positive signaling effectors and exclusion of negative regulators (48), thereby creating an environment of critical importance for the initiation of signaling cascades. By following TCR accumulation at the T/DC immune synapse, we show that moderate and transient TCR clustering observed with immature DCs can lead to cytokine production or NFAT activation. One explanation stated above is that 6 h of transient and weak TCR clustering allow the accumulation of signals sufficient for early activation. A second, nonexclusive, explanation is that the composition of signaling complexes may differ in response to immature DCs or to mature DCs. Assembly of multiprotein complexes, such as immunosomes or tolerosomes, varying in their biochemical composition, may indeed determine the polarity of the outcome of the response: immunity or tolerance, respectively (49). It is tempting to speculate that differential features of TCR clustering promote the formation of immunosomes with mature DCs vs tolerosomes with immature DCs. This pivotal but unresolved issue is under investigation in our laboratory.

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References


