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Cutting Edge: TCR Engagement and Triggering in the Absence of Large-Scale Molecular Segregation at the T Cell-APC Contact Site

Rossana Zaru,* Thomas O. Cameron,† Lawrence J. Stern,† Sabina Müller,* and Salvatore Valitutti2*

We investigated the functional role of large-scale molecular segregation at the T cell-APC contact site during T lymphocyte Ag recognition. Inhibition of CD2-CD58 interaction markedly affected segregation of CD2 and CD2AP from CD45. Under these conditions, Ag-induced calcium mobilization, PKCθ clustering at the immunological synapse, and IFN-γ production also were inhibited. However, early TCR signaling and T cell polarization toward APCs were unaffected. Our results indicate that the “raison d’être” of a large-scale segregation of surface molecules and intracellular enzymes and adapters, in Ag-stimulated T cells, is to reinforce the assembly of the signal transduction cascade rather than favor TCR engagement and triggering. The Journal of Immunology, 2002, 168: 4287–4291.

A peculiar characteristic of T cell Ag recognition is that TCR engagement by peptide-MHC complexes occurs at the intracellular space between opposing T lymphocytes and APCs, in parallel with the engagement of several accessory molecules. Although the specificity of Ag recognition is determined by TCR interaction with its ligand, the outcome of T cell-APC cognate interaction depends on the integration of signals from TCR with signals from accessory molecules (1).

Recent studies have provided evidence for the existence of a specialized signaling domain at the T cell-APC contact site. This domain named supramolecular activation cluster or immunological synapse (IS), is characterized by large-scale molecular segregation of TCRs, accessory molecules, and intracellular signaling components (1–3).

Molecular segregation is commonly considered to be more a consequence of productive TCR engagement than a prerequisite for TCR-peptide-MHC interaction (4). Indeed, large-scale molecular segregation occurs several minutes after T cell-APC conjugation, a time period that is not compatible with a role of IS in initiating TCR engagement and signaling (2, 3). Conversely, it has been recently shown, in resting T cells interacting with dendritic cells, that IS formation may occur in the absence of antigenic peptide and MHC molecules, suggesting that reorganization of accessory molecules and of signaling components may predispose T cells to Ag recognition (5).

An unresolved question concerns the role of molecular segregation at the T cell-APC contact site in T cell biological response. Although it is well established that the formation of a stable molecular array at the cell-cell contact site correlates with T cell activation (3), the precise function of this reorganization is still elusive (4, 6).

In the present work, we addressed the question of the “raison d’être” of a large-scale molecular segregation in Ag-stimulated T lymphocytes. The ideal approach to address this question was to manipulate molecular segregation without directly affecting TCR triggering. This was accomplished by stimulating T cells under conditions in which CD2-CD58 interaction was impaired.

It has been proposed that CD2-CD58 interaction may exert a crucial role in molecular segregation at the T cell-APC contact site (1). Due to the relatively small size of CD2 and its ligand CD58, their interaction may promote the formation of areas of tight adhesion between T cells and APCs where TCR engagement may be facilitated and from which large inhibitory molecules such as CD45 may be excluded (1, 7).

CD2 may also play an important role in the supply of intracellular signaling components to the IS. Several transducing enzymes and adapter proteins have been shown to interact with the intracellular portion of CD2 (8). Among these is CD2AP, which contains in its sequence multiple protein-protein interaction domains and has been shown to be fundamental in cross-talk between CD2 and T cell tubulin cytoskeleton (9). We stimulated T cells in the absence of CD2-CD58 interaction. Our results show that CD2 binding is mandatory for large-scale molecular segregation at the T cell-APC contact site and for full T cell activation. Surprisingly, under these conditions, the earliest steps of TCR-mediated signal transduction are unaffected.

Materials and Methods

T cell clones and APCs

Two DRB1*0101-restricted T cell clones (639p5.1.2 and SDMV 3.5) specific for the measles virus fusion protein peptide PS (F34–268) and a DRB1*1104-restricted T cell clone (KS140) specific for the tetanus toxin

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3 Abbreviations used in this paper: IS, immunological synapse; ERK, extracellular signal-regulated kinase; PKC, protein kinase C.

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peptide TT<sub>830–843</sub> were used. DR1-matched EBV-transformed B cells were used as APCs. T cell clones and EBV-B cell lines were generated and maintained as described previously (10).

**IFN-γ production, TCR down-regulation, and intracellular Ca<sup>2+</sup> concentration measurement**

IFN-γ production, TCR down-regulation, and intracellular Ca<sup>2+</sup> concentration were measured as previously described (10). In some experiments, T cells were conjugated at a 1:1 ratio with polystyrene latex microspheres (Polysciences, Warrington, PA, coated or not with 0.5 μg/ml anti-CD2 mAbs; BD PharMingen, Mountain View, CA) coated with 500 μg/ml avidin (Sigma-Aldrich, St. Louis, MO) in presence or in absence of either biotinylated DRB1*0101 P5 or DRB1*0101 TIR (TI680–696). Human MHC class II-peptide complexes were prepared as described elsewhere (11).

**Intracellular staining**

T cells were conjugated with EBV-B cells as previously described (7). In some experiments, EBV-B cells were treated for 30 min before conjugation with 10 μg/ml anti-CD58 mAbs (either Ts1/9, American Type Culture Collection (ATCC), Rockville, MD, or 1C3, BD PharMingen). Cells were fixed and permeabilized as described previously (7) and stained with anti-phosphotyrosine mAbs (Santa Cruz Biotechnology, Santa Cruz, CA) and either anti-CD2 (either HB222, ATCC, or RPA-2.10, BD PharMingen) or anti-CD45 mAbs (either 10G10 or 9.4; ATCC), or anti-CD2AP mAbs (9), or anti-CD2AP mAbs (green). Three-dimensional reconstruction of the images was performed using the Imaris software (Bitplane, Zurich Switzerland).

**Measurement of intracellular phosphotyrosine by FACS analysis**

The phosphotyrosine fluorescence in T cells was analyzed on a FACScan as described elsewhere (12).

**Extracellular signal-regulated kinase (ERK) phosphorylation analysis**

T cells were conjugated with EBV-B cells at a 3:1 ratio. Western blot analysis of ERK phosphorylation was performed using anti-phosphorylated-ERK1 and ERK2 mAbs (Sigma-Aldrich); membranes were stripped and reprobed with anti-ERK2 mAbs (Santa Cruz Biotechnology).

**Results and Discussion**

Reduced production of IFN-γ in the absence of CD2-CD58 interaction

To test the role of CD2 in costimulating T cell activation, we initially investigated whether the block of CD2-CD58 interaction would inhibit the activation of T cell biological responses. We measured IFN-γ production in T cells interacting with APCs previously treated with anti-CD58 Abs. Treatment of APCs with anti-

**FIGURE 1.** IFN-γ production is decreased in the absence of CD2 engagement. A, T cells were conjugated with peptide-pulsed APCs for 5 h. Treatment of APCs with anti-human MHC class I mAbs did not affect IFN-γ production (data not shown). B, T cells were conjugated for 5 h with polystyrene beads coated with either avidin alone or with biotinylated peptide-MHC complexes HLA-DR1-P5 or with the control peptide-MHC complex HLA-DR1-TIR in the presence or in the absence of anti-CD2 mAbs. Similar results were obtained with two T clones (6396 p5.1.2 and SDMV 3.5). Data in this and following figures are from one representative experiment of three.

**FIGURE 2.** CD2-CD58 interaction is required for IS organization. T cells were conjugated either for 5 min (A, B, and D) or for 30 (C) min at 37°C with peptide-pulsed APCs. A, APC (red) were either untreated (a–c) or treated with anti-CD58 mAbs (d–f). Cells were stained with anti-phosphotyrosine mAbs (blue) and anti-CD2 mAbs (green). B, APCs (red) were either unpulsed (a) or pulsed with the specific peptide (b–d). Cells were stained with anti-phosphotyrosine mAbs (blue) and anti-CD2AP Abs (green). C, APCs (red) were either untreated (a–c) or treated with anti-CD58 mAbs (d–f). Cells were stained with anti-CD2 mAbs (blue) and anti-CD2AP Abs (green). D, APCs (red) were either untreated (a and b) or treated with anti-CD58 mAbs (c and d). Cells were stained with anti-phosphotyrosine mAbs (blue) and anti-CD45 mAbs (green). Three-dimensional reconstruction of a series of z-sections is shown. In the presence of anti-CD58 mAb, molecular segregation was not observed as late as 30 min after conjugate formation (C and data not shown).

CD58 mAbs before conjugation with T cells significantly reduced IFN-γ production in T cell-APC conjugates (Fig. IA).

In addition, in T cells stimulated with peptide-MHC complexes coated on polystyrene beads, IFN-γ production was sharply enhanced by coimmobilization of anti-CD2 Abs (Fig. 1B). Taken together, these results are in agreement with previous reports showing that CD2 interaction with its ligand enhances T cell responses both in mouse and human T lymphocytes (13, 14).

**CD2-CD58 interaction is required for IS organization**

We have previously shown that CD2 rapidly accumulates at the T cell-APC contact site where it colocalizes with phosphorytrosine staining. TCRs progressively diffuse from the entire T cell surface into the phosphorylation area, whereas the phosphatase CD45 is...
To test the role of CD2-CD58 interaction in promoting molecular segregation, we studied IS structure in T cells interacting with APCs previously treated with anti-CD58 mAbs. As shown in Fig. 2A and Table I, Ag-induced recruitment of CD2 to the T cell-APC contact area was completely blocked in the absence of CD2-CD58 interaction. This indicates that CD2 recruitment to the IS requires both TCR signaling and CD2 engagement with its ligand.

We next tested whether treatment with anti-CD58 Abs would affect CD2AP dynamics in Ag-stimulated T cells. In resting conditions, CD2AP appears to be distributed throughout the T cell and APC cytosol (Fig. 2Ba). Interestingly, in T cells conjugated with cognate APCs, CD2AP swiftly moves toward the IS and colocalizes with phosphotyrosine and CD2 (Fig. 2, B, b–d, and C, a–c).

Treatment with anti-CD58 mAbs abolished CD2AP enrichment at the T cell-APC contact site, indicating that CD2AP recruitment to the IS is dependent on CD2 engagement and polarization toward the APC (Fig. 2C, d–f).

Finally, as shown in Fig. 2D and Table I, in T cells interacting with anti-CD58-loaded APCs, the exclusion of CD45 from the TCR signaling area was strongly reduced. Taken together with data from Shaw and coworkers (9), the above results indicate that signals derived from TCR and CD2 engagement cooperate to deliver CD2AP to the signaling area and to facilitate CD45 exclusion.

Table I. Measurement of the distribution of CD2, CD2AP, CD45, PKCØ, tubulin cytoskeleton, and Golgi system at the T cell-APC contact site in T cells conjugated with peptide-pulsed APCs either untreated or treated with anti-CD58 mAbs

<table>
<thead>
<tr>
<th>Type of Treatment</th>
<th>% Untreated</th>
<th>n</th>
<th>% Anti-CD58</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>CD2 enrichment</td>
<td>89</td>
<td>120</td>
<td>4</td>
<td>120</td>
</tr>
<tr>
<td>CD2AP enrichment</td>
<td>85</td>
<td>102</td>
<td>29</td>
<td>117</td>
</tr>
<tr>
<td>CD45 exclusion</td>
<td>77</td>
<td>127</td>
<td>11</td>
<td>126</td>
</tr>
<tr>
<td>PKCØ enrichment</td>
<td>86</td>
<td>121</td>
<td>18</td>
<td>136</td>
</tr>
<tr>
<td>Tubulin polarization</td>
<td>81</td>
<td>129</td>
<td>82</td>
<td>132</td>
</tr>
<tr>
<td>IFN-γ polarization</td>
<td>72</td>
<td>134</td>
<td>77</td>
<td>139</td>
</tr>
</tbody>
</table>

*For each Ab, the number of T cell-APC conjugates exhibiting tyrosine phosphorylation (except for IFN-γ staining) were randomly selected from three independent experiments and scored for CD2, CD2AP, and PKCØ enrichment at the contact site, CD45 exclusion, tubulin cytoskeleton, and Golgi system polarization. Segregation was scored by visual inspection in a double-blind study (7); only cells exhibiting a clear pattern were scored as positive. n, Number of conjugates analyzed. %, Percentage of conjugates exhibiting a given pattern of staining for n = 100%. Similar results were obtained with three T cell clones (6396p5.1.2, SDMV 3.5, and KS 140). Treatment of APCs with an anti-human MHC class I mAb before conjugation did not affect CD2 recruitment and CD45 exclusion from the IS (data not shown). Conjugation of T cells with unpulsed APCs resulted neither in CD2, CD2AP, and PKCØ enrichment, nor in CD45 exclusion, nor in tubulin cytoskeleton and Golgi system polarization toward the APCs (data not shown and Refs. 7 and 17).
These results are in apparent contrast with data using planar lipid bilayers, which suggest that the coincident engagement of LFA-1 by ICAM-1 and of TCR by peptide-MHC is sufficient for molecular segregation (2) in the absence of CD2 engagement.

This discrepancy might be due to the fact that different stimulation systems were used in the two studies. The function of ICAM-1 in the planar bilayers may differ from the more physiological context of an APC due to interaction with cytoskeleton, membrane microdomains, or other membrane molecules. This may facilitate molecular segregation in the absence of CD2 ligand.

**TCR triggering does not require CD2 accessory function**

Figure 2 depicts an unexpected result concerning phosphotyrosine staining, which appeared to be similar in conditions in which CD2-CD58 interaction was impeded. This observation suggested that molecular segregation of CD2 and CD45 is not required for TCR engagement and early signaling. We therefore tested this possibility by examining TCR signal transduction in Ag-stimulated T cells. T cells were conjugated with APCs either treated or not with anti-CD58 Abs. TCR down-regulation, phosphotyrosine staining, and calcium mobilization were evaluated by FACS analysis and ERK phosphorylation was assayed by Western blot analysis. Strikingly, as shown in Fig. 3, A and C, Ab-mediated block of CD2-CD58 interaction did not affect phosphotyrosine staining or ERK phosphorylation. Accordingly, TCR down-regulation, a process controlled by early protein tyrosine kinase activation (15), was also unaffected (Fig. 3B).

However, under these conditions, calcium mobilization was significantly reduced (Fig. 3D). These results indicate that whereas productive TCR engagement and triggering may occur in the absence of CD2 accessory function, signal transmission to calcium/protein kinase C (PKC) pathways is augmented by CD2 engagement. This prompted us to investigate whether block of CD2-CD58 interaction may also perturb PKC\(\theta\) enrichment at the T cell-APC contact site (16). In unstimulated T cells, PKC\(\theta\) is diffusely distributed throughout the cytosol (data not shown and Ref. 16). Following conjugation with the cognate APCs, T cells exhibited a massive enrichment of PKC\(\theta\) at the signaling area (Fig. 3, E and F, and Table I). In T cells in which CD2 binding to CD58 was impeded, PKC\(\theta\) translocation to the cell cortex and its recruitment to the signaling area were markedly reduced (Fig. 3, E and F, and Table I).

The molecular mechanisms responsible for this inhibition are still not clear. We propose that the block of CD2 signaling and of CD2/CD2AP recruitment may generate a defect in the formation of a molecular scaffold required for rearrangement of membrane molecules and docking of cytosolic components to the signaling area which is required for signal transmission to the calcium/PKC pathways.

These results, along with those shown in Figs. 1 and 2, indicate that molecular segregation at the T cell-APC contact site is dispensable for the TCR triggering. However, segregation contributes to full assembly of TCR signaling cascade and augments T cell biological response.

**T cell polarization toward APC is not affected by inhibition of CD2-CD58 interaction**

We next asked whether in the absence of molecular segregation T cells exhibit a defect in polarization of their secretory machinery. As shown in Fig. 4A and Table I, inhibition of CD2 binding did not affect the polarization of T cell tubulin cytoskeleton toward the APCs, as detected by T cell microtubule organizing center orientation toward the cell-cell contact site (17). Accordingly, even though in T cells stained with anti-IFN-\(\gamma\) Abs a reduction of staining in the absence of CD2 binding could be appreciated, the percentage of cells exhibiting polarization of the Golgi system toward the APCs was unaffected (Fig. 4B and Table I).

Taken together, the above results indicate that in the absence of CD2 and CD2AP polarization and of CD45 exclusion, T cells can still polarize their tubulin cytoskeleton and Golgi system toward the TCR signaling area. Thus, T cell polarization appears to be a biological function easier to trigger than IL production itself; it does not require CD2 accessory function and large-scale molecular segregation. This may allow cytotoxic T cells to rapidly polarize their lytic machinery toward target cells displaying little or no CD58.

In conclusion, these results provide new insights to the understanding of the role of large-scale molecular segregation in T cell activation. We propose that T cells form random conjugates with APCs, mediated by the engagement of adhesion molecules. This is followed by an initial examination of the APC surface looking for antigenic peptides. If a few are encountered, productive TCR engagement triggers protein tyrosine kinase and ERK activation, TCR down-regulation, and cytoskeleton polarization. These initial processes combine with the engagement and signaling of different accessory molecules at the cell-cell contact site and together coordinate large-scale molecular reorganization to enable a more careful inspection of the APCs. In this model, large-scale molecular segregation could serve as a quality control function. Molecular segregation concentrates various actors in the membrane and the fine balance of signal mediators pass final judgment on whether to trigger the full response or not. TCR and accessory molecules would therefore cooperate to allow the signal flow to pass through several checkpoints dispersed along the signaling pathways.

This mechanism may have been developed to allow T lymphocytes to adapt their biological responses to the context in which the Ag is presented.

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