Engagement of CD99 Induces Up-Regulation of TCR and MHC Class I and II Molecules on the Surface of Human Thymocytes

Eun Young Choi, Weon Seo Park, Kyeong Cheon Jung, Soon Ha Kim, You Young Kim, Wang Jae Lee and Seong Hoe Park

*J Immunol* 1998; 161:749-754;
http://www.jimmunol.org/content/161/2/749

References
This article cites 40 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/161/2/749.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Engagement of CD99 Induces Up-Regulation of TCR and MHC Class I and II Molecules on the Surface of Human Thymocytes

Eun Young Choi,†§ Weon Seo Park,* Kyeong Cheon Jung,* Soon Ha Kim,* You Young Kim,†§ Wang Jae Lee,‡ Seong Hoe Park*†§

CD99 is a cell surface molecule involved in the aggregation of lymphocytes and apoptosis of immature cortical thymocytes. Despite its high level expression on immature cortical thymocytes, the functional roles of this molecule during thymic selection are only now being elucidated. Examination of the effects of CD99 engagement on the expression kinetics of the TCR and MHC class I and II molecules, which are involved primarily in thymic positive selection, revealed a marked up-regulation of these proteins on the surface of immature thymocytes. This increase was the result of accelerated mobilization of molecules stored in cytosolic compartments to the plasma membrane, rather than increased RNA and protein synthesis. Confocal microscopic analysis revealed the changes in subcellular distribution of these molecules. When CD99 was engaged, TCR and MHC class I and II molecules were concentrated at the plasma membrane, particularly at cell-cell contact sites. The TCRlow subpopulation of immature double positive thymocytes was much more responsive to CD99-mediated up-regulation than was the TCRhigh population. These findings suggest that CD99-dependent up-regulation may have possible implication in positive selection during thymocyte ontogeny. The Journal of Immunology, 1998, 161: 749–754.

Materials and Methods

Antibodies

Anti-human CD99 mAb (DN16) was obtained from hybridomas clones developed in this laboratory. The anti-CD99 mAb 12E7, which recognizes an epitope distinct from that of DN16, was a generous gift from P. N. Goodfellow (Smithkline Beecham, Essex, U.K.). The hybridoma clone that produces mAb 12E7 (reactive with human medullary thymocytes) was obtained from American Type Culture Collection (ATCC, Manassas, VA). Anti-TCR-FITC, anti-CD4-FITC, anti-CD8-PE, and an unconjugated form of anti-TCR Abs were purchased from Becton Dickinson (San Jose, CA). FITC-conjugated anti-MHC class I and II Abs and the unconjugated forms of anti-TCR Abs were purchased from Becton Dickinson (San Jose, CA).
of anti-MHC class I and II Abs were all purchased from Biosource (Camarillo, CA).

**Cell purification**

Human thymic tissues were obtained from donors undergoing cardiac surgery. Prior permission was granted from the parents of patients who required partial thymectomy for clear exposure of the heart. After incubation with A1G3 on ice for 30 min, TCR<sup>+</sup> DP thymocytes were purified by negative panning on plates coated with goat anti-mouse Ig Ab.

**Suspension culture**

Thymocytes (2 × 10<sup>6</sup> cells/ml) were cultured in 24-well tissue culture plates that contained RPMI 1640 medium supplemented with 10% FCS in the presence of DN16 (10 µg/ml) or 12E7 (10 µg/ml) and secondary Ab raised against mouse Ig (50 µg/ml).

**Northern blot analysis**

Total RNA was prepared from Ab-engaged thymocytes with use of the guanidine thiocyanate-cesium chloride method (13). RNAs were separated by electrophoresis through a 1.3% agarose gel containing 2.2 M formaldehyde and then transferred to nylon membrane filters. The filters were hybridized at 42°C overnight with the appropriate DNA probes. The final wash of the filters was at 55 to 65°C in 0.2 × SSC. The β region of TCR-β, HLA-B7, DRβ, and β-actin genes were used as probes for detection of TCR, MHC class I, MHC class II, and actin transcripts, respectively.

**Fluorescent staining of cell surface and intracellular molecules and flow cytometric analysis**

After engagement with Abs (see above under Suspension culture), thymocytes were washed three times with 1% FCS-1 × PBS, incubated on ice for 30 min with normal mouse serum to block the nonspecific Ab binding of cells, stained directly with FITC-conjugated mAbs, and analyzed on FACScan (Becton Dickinson). To gate the viable cells, thymocytes stained for surface expression of TCR were washed, suspended in 400 µl of 1% FCS-1 × PBS containing 10 µg/ml of propidium iodide (PI; Sigma, St. Louis, MO), and analyzed on flow cytometer. For cytoplasmatic staining of the TCR and MHC class I and II molecules, cells were first incubated with unconjugated TCR mAb, MHC class I mAb, or MHC class II mAb. The cells were then fixed with 1% paraformaldehyde in PBS at room temperature and permeabilized with 1% saponin (Sigma), which was included in all subsequent staining and washing steps. The fixed cells were washed, stained with FITC-conjugated mAbs, and were analyzed on a FACScan.

**Confocal analysis**

For immunofluorescence labeling, incubated cells were fixed in paraformaldehyde, permeabilized with 1% saponin, and incubated with FITC-conjugated Abs. Confocal analyses were performed with a 600 MRC equipped with an Argon/Krypton laser (Bio-Rad Labs, Hercules, CA). Green fluorescence was detected at λ > 515 nm after excitation at 488 nm.

**Results**

**CD99-mediated up-regulation of TCR and MHC class I molecules**

We examined the effect of CD99 engagement on the surface expression of TCR, MHC class I, CD4, and CD8 molecules, since these are considered to be the major molecules involved in thymic positive selection. The expression patterns of TCR and MHC class I molecules on untreated thymocytes vary greatly from low to high. When thymocytes were incubated with anti-CD99 Abs (DN16 or 12E7), the surface expression of TCR and MHC class I molecules increased dramatically, as compared with an anti-CD1a Ab-treated control group. Expression of CD4 and CD8 remained unchanged under both conditions (Fig. 1A). The up-regulation of TCR and MHC class I surface expression, when thymocytes were engaged with anti-CD99, occurred in a dose-dependent manner. A linear relation was evident between the degree of surface expression and the amount of Ab used within the 100 ng/ml to 10 µg/ml range. The maximal effect occurred at an anti-CD99 Ab concentration of 10 µg/ml (Fig. 2).

It was reported that incubation of thymocytes with anti-CD99 mAb 0662 induced cells to die (12) and that TCR expression was increased on apoptotic cells (14). Therefore, we asked if the increased surface expression of TCR molecules could be just a reflection of the apoptosis induced by CD99 engagement with DN16 mAb. To address this question, two-color flow cytometric analysis was done on the thymocytes treated with DN16 mAb for 1 h (Fig. 1B) in terms of TCR expression and cell viability. There was no identifiable difference in the percentage of dead cell population (R1) between DN16 mAb-treated and control Ab-treated cells. Gating on the PI-negative viable population (R2) clearly demonstrated that the TCR up-regulation was due to the specific effect of CD99 engagement rather than the apoptosis-related up-regulation of surface molecules. However, 1.3% of immature thymocytes remained unchanged in their TCR expression even after CD99 engagement (R3).

**Cellular mechanism for the up-regulation of TCR and MHC class I molecules**

The increased surface expression of TCR and MHC class I molecules was observed as early as 10 min after the CD99 engagement. To identify the cellular mechanism involved in the up-regulation of TCR and MHC class I surface expression, we tested whether de novo mRNA synthesis or subcellular distribution of already synthesized proteins was altered upon engagement of CD99. We found no evidence of quantitative changes in the mRNA synthesis of TCR or MHC class I mRNA after CD99 engagement, as compared with control Ab-treated samples (Fig. 3A). We next performed flow cytometric analysis to compare the kinetics of cytosolic and cell-surface expression of TCR and MHC class I protein complexes. As shown in Figure 3B, unengaged thymocytes expressed high levels of intracellular TCR molecules but were relatively low in their level of surface expression. With CD99...
engagement, the surface expression of TCR molecules increased markedly as the cytoplasmic concentration of TCR declined, suggesting that accelerated cellular transport might be a major mechanism for the up-regulation of TCR molecules on the cell surfaces.

This result indicates that, although 30% of unengaged immature thymocytes show no expression of TCR at their cell surface (in Fig. 1B), a vast majority of them have a considerable amount of TCR molecules in their cytosolic compartment. In fact, only a minimal fraction of immature thymocytes (R3 in Fig. 1B, 1.3%) constitutes cell population lacking internal TCR molecules, and this is a population in which TCRα-rearrangement was not completed yet.

The cellular mobilization pattern of MHC class I molecules was also examined with flow cytometric analysis, showing almost identical results to those of TCR molecules. To visualize directly the effect of CD99 engagement on the localization pattern of TCR and MHC class I molecules in thymocytes, confocal microscopic examination was performed. The TCR molecules were evenly distributed in both the plasma membrane and cytoplasm (Fig. 4A). The fluorescence intensity of TCR molecules at the plasma membrane increased with a concomitant decrease in intracellular fluorescence in response to anti-CD99 Ab treatment (Fig. 4B).
Engagement, almost all immature thymocytes had a considerable change in subcellular localization of MHC class I molecules when cells were engaged with anti-CD99 Ab was almost identical to that of TCR molecules, except that a more condensed localization of MHC class I molecules occurred at cell-cell contact sites (Fig. 4, C and D).

Cellular mechanism of CD99-dependent up-regulation of MHC class II molecules

We reported previously that MHC class II molecules are present on a significant fraction (10–30%) of fetal thymocytes throughout their developmental progression to mature T cells (15) and that there is actual T-T interaction during thymocyte development (3). Therefore, we examined the CD99-mediated up-regulation of MHC class II molecules on the surface of cultured thymocytes. MHC class II expression on unengaged thymocytes is on the average less than 30% but varies depending on the thymus sample. We found that CD99 engagement also induced the up-regulation of MHC class II molecules on thymocyte surfaces (Fig. 5A, left). However, the results of this experiment are partly in conflict with the data obtained for TCR and MHC class I molecules, in that the MHC class II-negative population, though small, still existed even after cells were incubated with maximal dose of anti-CD99 Ab.

As was the case in the TCR and MHC class I experiments, accelerated transport of previously synthesized molecules from cytosol to cell surface also appeared to be the major mechanism for CD99-mediated up-regulation of MHC class II molecules. The flow cytometric profile for the localization of surface and internal MHC class II molecules showed that, in the absence of CD99 engagement, almost all immature thymocytes had a considerable intracytoplasmic pool of MHC class II molecules, whereas expression was almost completely absent on their surfaces (Fig. 5A, dotted lines). As was the case with both TCR and MHC class I molecules, increased MHC class II surface expression after CD99 engagement was also associated with the concomitant reduction of intracellular staining (Fig. 5A, solid lines). Northern analysis showed no change in MHC class II mRNA expression upon engagement of thymocytes with anti-CD99 Ab (Fig. 5B). After CD99 engagement, accumulation of MHC class II molecules became evident on the cell surface and was concentrated at cell-cell contact regions as determined by confocal microscopy (Fig. 6, A and B). This analysis constitutes a direct visual demonstration that, upon CD99 engagement, MHC class II molecules migrate from an internal reservoir to the cell surface region where cell-cell contacts occur.

Immature TCRlow DP thymocytes are the major cell population that responds to CD99 engagement

Immature DP thymocytes are phenotypically heterogeneous with respect to the expression level of TCR (16–18) and can be subdivided into TCRlow and TCRhigh cell populations. Because the responses of TCRlow and TCRhigh cells to various stimuli can differ (19–21), we sought to determine what subpopulation of thymocytes showed up-regulated expression of TCR and MHC class I and II after binding of anti-CD99 mAb. For separate enrichment of the TCRlow and TCRhigh cells, DP thymocytes were applied to a panning plate after binding with A1G3 Ab, which was reported to recognize medullary thymocytes and to be associated with functional maturity of the thymocyte (22). Our results revealed that the A1G3 recognition Ag was expressed in most TCRhigh DP cells as well as in a mature medullary population of thymocytes (Fig. 7, dotted lines). Therefore, we conclude that A1G3 mAb is a powerful experimental tool that can distinguish between the two subpopulations of DP thymocytes.

CD99 engagement resulted in dramatic up-regulation of TCR and MHC class I molecules in a population of A1G3 cells (TCRlow cells), almost to the level of mature medullary thymocytes. In contrast, no significant changes in expressions of these molecules were evident in A1G3+ TCRhigh DP thymocytes (Fig. 7, solid lines). MHC class II molecules were expressed normally only on the surface of A1G3+ TCRlow thymocytes (Fig. 7, dotted lines), and this is also the major cell population in which CD99-mediated up-regulation of MHC class II molecules occurs (Fig. 7, solid lines). The TCRhigh cells were completely devoid of MHC class II
molecules on their surfaces, and the CD99-dependent changes in the localization of MHC class II molecules were negligible.

Discussion

In this paper, we report that the engagement of CD99 with anti-CD99 Ab generates molecular signals that lead to the up-regulated surface expression of TCR and MHC class I and II, while expression of CD4 and CD8 molecules is unchanged. The increased surface expression of these molecules was detected as soon as 10 min after CD99 engagement. It is well established that unassembled and partial complexes of TCR components are retained within ER, and pentamer (αβγδε) and fully assembled (αβγδεζε) TCR complexes exit the ER and transit through the Golgi system. The export of the TCR-CD3 complex from ER to Golgi is slow, requiring at least 3 h for the processing of half the complexes from the endonuclease H-sensitive to the endonuclease H-resistant form (reviewed in Ref. 23). Therefore, considering the time required for transport from the cytosolic compartment to the cell surface, it is not likely that up-regulated surface expression results from de novo synthesis and assembly of TCR molecules.

A similar hypothesis may also apply to the CD99-dependent surface expression of MHC class I and II molecules. MHC class I molecules assemble and achieve their native conformation within 2 to 15 min of synthesis (24). Thereafter, the half-time taken for MHC class I proteins to egress from the ER varies between 20 and 55 min, depending on the haplotype of the molecule (25). Newly synthesized MHC class II molecules accumulate in the MHC compartment after 4 h of synthesis (26), and MHC class II-containing vesicles concentrated in the MIIC move toward the plasma membrane within the 5-min period (27).

Experiments performed in this laboratory support the idea that the molecular signals generated by CD99 engagement induce actin polymerization, which leads to the mobilization of cell surface molecules. First, TCR up-regulation mediated by CD99 engagement was inhibited by treating thymocytes with inhibitors of actin polymerization (cytochalasin B and E; unpublished data). Second, the treatment of C3 exoenzyme, which inhibits Rho-mediated actin polymerization, completely abolished CD99-induced cell aggregation (unpublished data). The hypothesis that CD99 engagement induces actin polymerization is supported further by independent reports. For example, the transport of MHC class II molecules from lysosomal structures to the plasma membrane involves microtubules, as nocodazole and colcemid (microtubule blockers) inhibit the movement of MHC class II molecule-containing vesicles (27). In addition, the close interrelationship of actin- and microtubule-based systems has been demonstrated in organelle movement (28) and fusion of endosomal carrier vesicles (29). Finally, linker proteins (for example, p150GluCd) have been shown to reside between microtubules and actin filament (30, 31). Taken together, these data suggest that Ab engagement of CD99 can lead to either activation of cytoskeletal components or activation of linker proteins between microtubule- and actin-based cytoskeletal systems, thereby inducing accelerated mobilization of specific Ags to the cell surface. Our data generated from flow cytometric analysis and confocal microscopic examination lend firm support to the idea that up-regulated expression via CD99 engagement results from accelerated intracytoplasmic transport rather than de novo synthesis of these molecules. The unique distribution of TCR and MHC molecules, which are densely concentrated at cell-cell contact sites after CD99 engagement, is able to increase the number of proper contacts formed between these molecules.

The amount of class II in cells of the immune system is not static but can be up- or down-regulated often via intracellular transport activity (32–34) in response to a large number of external stimuli, including cytokines, cross-linking of surface Ags (for example, IgM, IgD, Lyb2, and B220), and mitogens (reviewed in Ref. 35). Human peripheral T cells in a resting state can transcribe mRNA for class II molecules but normally do not express this Ag at their surfaces in a detectable amount until after activation (36). Results from the present study imply that the same is true for the TCRlow stage of developing thymocytes, because surface expression of MHC class II molecules that are confined to the cytoplasmic pool must await the delivery of molecular signals generated by the engagement of CD99.

Instances of CD99-induced up-regulation of TCR and MHC class I and II molecules have certain characteristics in common. One is that induced surface expression of these Ags appears to be differentially regulated depending on the developmental stage of thymocytes: higher surface expression was observed on immature thymocytes (A1G3− cells) than on more mature ones (A1G3+ cells). The biologic relevance of CD99-induced up-regulation of TCR and MHC molecules at the cell surface can be discussed along with current representative hypothetical views on positive selection. In view of the differential avidity model for T cell selection, the increased surface levels of TCR and MHC class I and II after CD99 engagement might provide a basis for the increased avidity of TCR-MHC interaction on developing thymocytes (37). Therefore, immature thymocyte whose TCRs i) are expressed at low density, ii) encounter peptide-MHC complexes for which they have low affinity, or iii) encounter cells with peptide-MHC expressed at low density can be rescued from death by the up-regulation of these molecules through CD99 signaling. Lanzavecchia et al. have suggested a serial triggering model where the key parameter is the absolute numbers of TCR engaged (38, 39). Therefore, unlike cells that express low amounts of TCR, cells expressing higher numbers of TCRs rapidly reach the number of engaged TCRs required for selection. In this regard, a larger percentage of cells may be rescued from the lack of recognition when CD99 signaling is operative.

FIGURE 7. Response of A1G3-negative immature TCRlow thymocytes to CD99 stimulation. TCRlow and TCRhigh cells were separated with the panning method using A1G3 Ab. Each purified population of thymocytes was incubated in suspension cultures for 2 h in the presence (solid lines) or absence (dotted lines) of CD99 stimulation. After culture, cells were stained with mAbs for surface expression of TCR and MHC class I and II expression. Expression of these molecules on the total (unfractionated) thymocyte population is shown (top panel) for comparison with expression levels on TCRlow (A1G3− ) and TCRhigh (A1G3+ ) cells.
We reported previously that MHC class II molecules are present on a significant fraction of fetal thymocytes, particularly during the last trimester of the gestational period (15) and that there is an actual presence of T-T interaction during thymocyte development (3). Given that CD99 engagement increases the expression density of TCR and MHC class I and II molecules on the surface of thymocytes, it is not surprising that even more opportunities exist for substantial T-T interaction once the signal through CD99 is delivered. Using morphologic visualization, we observed that TCR and MHC class I and II molecules, after CD99 engagement, were concentrated at the plasma membrane near cell-cell contacting sites, which suggests the possibility of increased TCR-MHC interaction during T-T interaction. Therefore, the increased T-T interaction may also have additive effects on the selecting event, as compared with the situation where only TECs are present as the selecting cell. The observation and hypotheses outlined above emphasize the role of CD99 engagement in the generation of the increased opportunities for positive selection. Therefore, CD99 can be defined as a biologic enhancer during the thymic education process, increasing the number of thymocytes that are positively selected.

A role for CD99 in apoptosis has been reported recently (12). When combined with our observations, one may postulate that CD99 has a dual contradictory function: one is to create more opportunities for positive selection by increasing TCR-MHC avidity and the other is to induce apoptosis of immature thymocytes. We do not know at present why ligation of the same molecule, CD99, can result in such dramatically different responses. We propose that, in the course of thymocyte development, CD99 engagement may not be sufficient to induce differentiation of DP thymocytes, which requires coengagement of TCR and other coin- ducer molecules provided by thymocytes and TECs. Therefore, the decision regarding the fate of the thymocyte can be made only at the CD99-induced TCR$^\text{high}$ immature stage. Thymocytes positively selected after proper TCR-MHC interaction might lead to the increased synthesis of transcripts and proteins for the TCR molecule and then differentiate to single positive (SP) cells (40). Otherwise, the thymocyte should be removed by apoptosis.

In summary, we suggest that molecular signaling through CD99 engagement is a basic requirement for the survival, maintenance, and maturation of thymocytes and is involved in the regulation of its contradictory dual functions.

References