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GM1 controlled lateral segregation of tyrosine kinase Lck predispose T-cells to cell-derived galectin-1-induced apoptosis

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ABSTRACT

One prominent immunoregulatory function of galectin-1 (Gal-1), a β-galactoside binding mammalian lectin, is induction of apoptosis in activated T-cells by a process depending on the activity of Src family tyrosine kinase, Lck. Although the requirement for Lck in Gal-1 induced T-cell death and the ability of Gal-1 to affect the membrane localization of extracellular Gal-1-binding proteins have been well documented, the consequence of the complex and related reorganization of extra- and intracellular signaling components upon Gal-1 treatment of T-cells has not yet been revealed. Therefore, we have analyzed the plasma membrane movement of Lck upon Gal-1 triggered signaling, and the significance of this event in Gal-1 induced T-cell death. Non-receptor tyrosine kinase, Lck primarily localized in the synapse of tumor cell-T-cell during 15 min of the established direct cell contact. Later, after 30 min, a lateral segregation of Lck from the cell synapse was observed. The migration of Lck to the opposite of the cell contact apparently depended on the expression and cell surface presentation of Gal-1 on the effector (tumor) cells and was accompanied by phosphorylation on the negative regulatory tyrosine residue, Tyr505. Receptor tyrosine phosphatase, CD45 played crucial role in this event since CD45 deficiency or inhibition of its phosphatase activity resulted in the failure of Lck membrane movement. Level of the Gal-1-binding glycolipid GM1 ganglioside also essentially regulated Lck localization. Segregation of Lck and Gal-1 induced apoptosis was diminished in T-cells with low GM1 expression compared to T-cells with high GM1. Our results show that spatial regulation of Lck by CD45 and GM1 ganglioside determines the outcome of apoptotic response to Gal-1 and this local regulation may occur only upon intimate effector (Gal-1 expressing) cell-T-cell attachment.

1. Introduction

T-cells and antigen presenting cells form immunological synapses, which are considered to assemble from specific membrane microdomains, rafts (Bromley et al., 2001; Dykstra et al., 2001), composed of T-cell receptor (TCR), co-receptors and adhesion molecules in a specific lipid environment, and scaffolded by the actin cytoskeleton (Burkhardt et al., 2008). The composition of membrane rafts varies during different differentiation and activation phases of T-cells, *e.g.* the amount of GM1 ganglioside is expressed at higher levels in effector cells, compared to resting T-cells (Tuosto et al., 2001). Regulation of GM1 expression is substantial, as alteration in lipid raft composition may lead to inappropriate T lymphocyte signaling and ultimately to development of pathological conditions (Jury et al., 2004). In resting T-cells, TCR and tyrosine kinases Lck and ZAP-70 are excluded from or weakly associated with rafts, however, they translocate into raft domains after TCR engagement (Montixi et al., 1998). Localization of activated Lck to rafts is important for the initial signaling which drives subsequent activation of signaling proteins, cytoskeletal changes, membrane remodeling and formation of stable signaling complexes at TCR activation sites (Salmond et al., 2009). Activity of Lck is regulated by phosphorylation of two conserved tyrosine residues, Tyr505 and Tyr394. Phosphorylation at Tyr505 results in a closed, enzymatically inactive conformation. When outside of the rafts, Lck is dephosphorylated by CD45 receptor tyrosine phosphatase at the Tyr-505, thereby it is potentiated for activation and autophosphorylation at the Tyr394 (Sieh et al., 1993; Rodgers and Rose, 1996). In turn, when the active Lck is recruited to adaptor Cbp/PAG in rafts,

Abbreviations: Gal-1, galectin-1; TCR, T-cell receptor; HeLa^{mock}, mock transfected HeLa cells; HeLa^{Gal-1}, Gal-1 transfected HeLa cells; β-CD, β-cyclodextrin; CTX-FITC, cholera toxin B subunit – fluorescein isothiocyanate.

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Csk kinase phosphorylates the negative regulatory Tyr505 of Lck (Kawabuchi et al., 2000) resulting in inactivation, hence terminating the activation process.

TCR clustering at the immune synapse is negatively regulated by particular members of the galectin family, β-galactoside binding lectins, which interact with cell surface glycoproteins and glycolipids and form lattice, thereby restricting glycoprotein movement in the cell membrane and inhibiting reorganization of signaling complexes (Grigorian et al., 2009). Accordingly, soluble Gal-1 binding to T-cells induces reorganization of Gal-1 binding receptors, and as a result CD45 and CD3 are co-localized on large islands of apoptotic blebs and CD7 and CD43 are co-localized in small patches (Pace et al., 1999). Specific membrane microdomain structure is crucial during Gal-1 induced T-cell apoptosis, since disruption of rafts results in failure of execution of Gal-1 signaling and induction of cell-death (Ion et al., 2006; Kovács-Sólyom et al., 2010). This apoptotic process is characterized by early tyrosine phosphorylation with involvement of tyrosine kinase, Lck, followed by ceramide production by acid sphingomyelinase, mitochondrial depolarization, caspase-9 and -3 activation (Ion et al., 2005, 2006).

Gal-1 is often upregulated in tumor cells and the surrounding stroma and it is supposed to contribute to tumor immune privilege (Rubinstein et al., 2004). We have recently shown that the Gal-1 produced by and bound to the surface of tumor cells induces cell death in Jurkat cells or PHA activated human peripheral blood T-cells in an *ex vivo* co-culture system (Kovács-Sólyom et al., 2010). This system resembles better the *in vivo* milieu than usage of soluble protein, as lymphocytes encounter Gal-1 attached to the cell surface of the producer cells or components of the extracellular matrix (He and Baum, 2004). In this work we analyze the plasma membrane movements of Lck, a major component of Gal-1 triggered apoptotic signaling. We show that the membrane localization of Lck is affected by CD45, GM1 ganglioside and intact raft organization. Most importantly, we provide a novel insight into those membrane proximal events which eventually lead to T-cell apoptosis.

2. Materials and methods

2.1. Cells

All cell lines were kept in an incubator with 5% CO_2 at 37 °C. Jurkat cell lines and the CD45 deficient Jurkat variant, J45.01 (European Collection of Cell Cultures) were cultured in RPMI-1640 (Gibco, Invitrogen) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and in the presence of 5% (Jurkat) or 10% (J45.01) heat inactivated fetal calf serum (FCS) (Gibco, Invitrogen). C32 human melanoma cells and mock transfected ($HeLa^{mock}$) or Gal-1 transgenic ($HeLa^{Gal\text{-}1}$) human cervix adenocarcinoma cells were cultured in MEM (Gibco, Invitrogen) supplemented with penicillin, streptomycin, L-glutamine and 5% (C32) or 10% (HeLa) FCS. Peripheral blood mononuclear (PBM) cells from healthy human donors were isolated through Ficoll gradient (GE Healthcare) and activated with 5 μg/ml phytohemagglutinin-M (PHA, Calbiochem) for 72 h in RPMI containing 10% FCS. Studies involving human blood samples were conducted in accordance with the guidelines of the Declaration of Helsinki and have been approved by the institutional ethics committee of the University of Szeged.

To separate cells based on their GM1 ganglioside expression Jurkat or human activated T-cells were washed and resuspended in DMEM at 10^7 cells/ml, and then 20 μg/ml cholera toxin B subunit (CTX)-biotin (Sigma) was added. After 15 min incubation on ice the cells were washed with DMEM and 50 μl BD IMag™ Streptavidin Particles Plus - DM (BD Bioscience) was added to 10^7 cells for 30 min at 4 °C. The cell concentration was set to 10^7 cells/ml and the tube was placed into the magnet (BD IMaq™ cell separation magnet) for 8 min. The negative fraction (supernatant) and the positive fraction (pellet) were separately cultured, and tested for the expression of GM1 by flow cytometry. Cells from negative fraction expressing low level of GM1 were designated as $J.GM1^{lo}$ and $GM1^{lo}$ T-cells for Jurkat and human activated T-cells, respectively. Activated T-cells with high GM1 expression were referred as $GM1^{hi}$ T-cells. $J.GM1^{lo}$ cells were maintained in RPMI 1640 with 5% FCS and tested for their GM1 expression before use by flow cytometry. Separated human T-cells were used immediately for apoptosis tests.

2.2. Flow cytometry

GM1 detection. The cells were washed in ice cold phosphate buffered saline (PBS) supplemented with 1% FCS and 0.1% sodium-azide (FACS-buffer) and resuspended at 2×10^6 cells/ml, and then CTX-FITC was added at a concentration of 10 μg/ml. After incubation for 30 min on ice the samples were washed with FACS-buffer, and measured on FACSCalibur flow cytometer using CellQuest™ software (Becton & Dickinson).

Detection of surface Gal-1. $HeLa^{mock}$ cells were treated with 50 μg/ml recombinant Gal-1 (rGal-1), then unbound lectin was removed with washing with FACS-buffer. Amount of surface Gal-1 on Gal-1-treated $HeLa^{mock}$ and transgenic $HeLa^{Gal\text{-}1}$ cells was analyzed by FITC conjugated anti-Gal-1 mAb (clone 2c1/6, produced in our laboratory, Kovács-Sólyom et al., 2010) and cytofluorimetry was carried out as described above.

2.3. Co-cultures of T-cells and Gal-1 producing tumor cells

C32 (10^4 cells/sample) or $HeLa^{mock}$ and $HeLa^{Gal\text{-}1}$ cells (5×10^3 cells/sample) were plated onto round cover slips (12 mm diameter, Menzel Gläser, Thermo Scientific) in 24-well plate. Jurkat, $J.GM1^{lo}$, J45.01 or $GM1^{lo}$ and $GM1^{hi}$ activated human T-cells ($2–5 \times 10^5$ cells/sample) were labeled with Hoechst 33342 (100 ng/ml for 30 min at 37 °C) and co-cultured with tumor cells for the indicated time points. In particular experiments Jurkat cells were incubated with the following inhibitors before added to tumor cells: β-cyclodextrin (β-CD) for raft disruption (10 mM, 30 min, Sigma) or PTP CD45 inhibitor (10 μM, 20 min, Santa Cruz Biotechnology). These inhibitors were present throughout the experiments.

For competing galectin binding to cell surface $HeLa^{mock}$ and $HeLa^{Gal\text{-}1}$ cells were incubated with 100 mM lactose (Sigma) for 30 min at 4 °C in cell culture medium, and then galectins were removed with extensive washing before co-culture. 50 μg/ml human recombinant Gal-1 was added to indicated samples of lactose washed $HeLa^{mock}$ cells for 30 min at 4 °C and then the unbound Gal-1 was removed with washing before co-culture.

2.4. Immunocytochemistry

T-cells and tumor cells were co-cultured for the indicated time points (ranging from 15 min to 1 h), then fixed in 4% paraformaldehyde for 4 min at room temperature, washed in PBS and the cover slips were saturated in FACS-buffer for 1 h prior immunostaining. The following antibodies were used: unlabeled or FITC conjugated mouse anti-Lck mAb (produced in our laboratory, Ion et al., 2005), PE conjugated mouse anti-LCK pTyr505 (BD™ Phosflow), 4G10 anti-phosphotyrosine mAb (Upstate Biotechnology, Millipore), unlabeled or FITC conjugated anti-Gal-1 mAb (clone 2c1/6, produced in our laboratory, Kovács-Sólyom et al., 2010). Cell surface staining was carried out in FACS-buffer. For intracellular staining the antibodies were added to the cells in FACS-buffer

containing 75 μg/ml L-(α)-lysophosphatidylcholine (LPC, Sigma), that rapidly permeabilize the cell membrane while retains cells' integrity (Chitu et al., 1999). Unlabeled primary antibodies were followed by anti-mouse IgG-NorthernLights-557 (R&D Systems). To visualize the cell-cell contact, F-actin was labeled with Rhodamine-Phalloidin (Invitrogen). The cover slips were mounted on slides with a drop of Fluoromount-G (SouthernBiotech). The samples were analyzed with Axioskop 2Mot (Carl Zeiss) fluorescence microscope using 40× objective magnification, or confocal images were taken with Olympus FV 1000 laser scanning confocal microscope, 60× objective and zoom function. The correct exposure was determined based on the intensity plots generated by FV10-ASW 2.0 Viewer software (Olympus) (Supplementary Fig. 1). Photos were taken from 10 randomly selected non-overlapping fields of the samples, and at least 100 cells/sample were analyzed. Localization of Lck in the cell-cell contact or in membrane domains opposite to cell-cell contact (sequestered) were determined from non-adjusted raw pictures and evaluated by the following equation: percentage of cells with the Lck sequestered or in cell contact = number of T-cells with Lck sequestered or in cell contact/number of T-cells in contact with tumor cells) × 100. To determine the relative effect of Gal-1, the percentages of cells with sequestered Lck in samples with $HeLa^{mock}$ were subtracted from results with $HeLa^{Gal\text{-}1}$. For presentation of the images, the contrast of the images was adjusted using Adobe Photoshop CS4 extended 11.0 (Supplementary Fig. 1).

2.5. Detection of apoptosis in co-culture system

After 16 h of co-culture the cells were fixed in 4% paraformaldehyde for 4 min at room temperature and labeled for phosphatidylserine exposure on the outer cell membrane as described previously (Kovács-Sólyom et al., 2010). The samples were analyzed with Axioskop 2Mot (Carl Zeiss) fluorescence microscope using AxioCam camera, AxioVision 3.1 software and 20 × objective magnification. The contrasts of the images were adjusted using Adobe Photoshop CS4 extended 11.0. At least 100 cells/sample were analyzed and the rate of apoptosis was determined as follows: % of apoptotic cells = (Annexin V positive cells/total cell number) × 100.

The active (cleaved) form of caspase-3 was detected after 24 h of T-cell/tumor cell co-culture. The cells were fixed in 4% paraformaldehyde for 25 min at room temperature and permeabilized with 0.1% Triton X-100 in FACS-buffer for 10 min, then saturated in FACS-buffer for 1 h. The samples were reacted with rabbit anti-caspase-3 (Cell Signaling Technology) and anti- rabbit Ig-FITC (Sigma). After washing the cover slips were mounted on slides with a drop of Fluoromount-G and analyzed with Axioskop 2Mot fluorescence microscope.

2.6. Statistics

Average and standard deviation were determined with Microsoft EXCEL software from the results of 3–5 independent samples, as indicated in figure legends. Statistical significance of the differences was determined using the Student's t test (set at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Cell-derived galectin-1 induces exclusion of Lck from cell synapse

We have recently shown that stimulation of T-cells with soluble recombinant Gal-1 induced remarkable tyrosine phosphorylation with the involvement of Lck non-receptor tyrosine kinase leading eventually to apoptosis (Ion et al., 2005, 2006). Lack of Lck in Jurkat derivative cell line, J.CaM1.6 rendered these T-cells resistant to Gal-1 induced cell death (Ion et al., 2006). Similarly, tumor cell-derived Gal-1 also induced T-cell death on an Lck dependent fashion (Kovács-Sólyom et al., 2010). The central role of Lck in Gal-1 triggered T-cell death motivated the analysis of early membrane remodeling, an event determining the final signaling outcome, with a special focus on Lck redistribution in T-cells upon Gal-1 stimulation, We applied a co-culture system using Gal-1 non-producing ($HeLa^{mock}$) or Gal-1 transgenic ($HeLa^{Gal\text{-}1}$) HeLa cell lines as effector cells (for characterization see Supplementary Fig. 2, and Kovács-Sólyom et al., 2010). As we showed earlier the ultimate requirement of $HeLa^{Gal\text{-}1}$ triggered T-cell apoptosis was the direct cell-cell contact (Kovács-Sólyom et al., 2010).

T-cells and HeLa cells formed intimate cell-cell interaction within 15 min of co-culture, and as a result, the membrane associated Gal-1 rapidly transferred from the surface of $HeLa^{Gal\text{-}1}$ to Jurkat cells (Fig. 1A right panel). Gal-1 was not detectable on Jurkat co-cultured with $HeLa^{mock}$ (Fig. 1A left panel). This result was in accordance to our previous finding (Kovács-Sólyom et al., 2010), and was reproducible when using another Gal-1 producing cell line, the C32 human melanoma (Supplementary Fig. 3). At early time point, 15 min of the cell-cell interaction, Gal-1 was mainly accumulated in cell contact (Fig. 1A) and then dispersed evenly on the cell surface of Jurkat (data not shown). Tight cell synapse between the tumor cell and T-cell, as detected by actin staining (Fig. 1B), generated different distribution of tyrosine kinase Lck depending on the absence or presence of Gal-1 in the effector cells: Lck clustered to the cell synapse of Jurkat and $HeLa^{mock}$ (Fig. 1B, upper panel) while it was sequestered to distal membrane site when Jurkat cells adhered to $HeLa^{Gal\text{-}1}$ (Fig. 1B, lower panel) after 60 min of co-culture. Localization of Lck to the opposite of the cell contact was significantly more frequent in cell-cell contact with $HeLa^{Gal\text{-}1}$ than $HeLa^{mock}$ cells (Fig. 1C and D), indicating the role of Gal-1 in this process. Plasma membrane distribution of Lck was uniform in untreated Jurkat cells (Supplementary Fig. 4). Lck appeared at the cell synaptic site when Jurkat cells were co-cultured with $HeLa^{mock}$ and kept this position for up to 60 min (Fig. 1E). In contrast, a gradual, time dependent accumulation of Lck distal from the cell synapse was observed when $HeLa^{Gal\text{-}1}$ and Jurkat cells established cell contact. (Fig. 1E).

Specific role of Gal-1 in Lck membrane movement from cell synapse was examined in various experimental designs. First, $HeLa^{mock}$ or lactose (competitor of galectins)-washed $HeLa^{mock}$ induced Lck sequestration only in minor percentage of T-cells. In contrast, $HeLa^{mock}$ cells covered with rGal-1induced high Lck sequestration (Fig. 1D lac $HeLa^{mock}$ +rGal-1). Second, removal of surface Gal-1 from $HeLa^{Gal\text{-}1}$ with lactose (Fig. 1D lac $HeLa^{Gal\text{-}1}$) diminished the Lck exclusion from cell synapse. Lck sequestration was higher when induced by rGal-1 treated $HeLa^{mock}$ than by transgenic $HeLa^{Gal\text{-}1}$ since surface Gal-1 amount was higher on $HeLa^{mock}$ +rGal-1 than on $HeLa^{Gal\text{-}1}$ (Supplementary Fig. 2B and C). To rule out the effect of other galectins, all cell-surface β-galactoside binding lectins were removed from $HeLa^{mock}$ (Fig. 1D lac $HeLa^{mock}$) surface with lactose, however, it had no effect compared to non-treated $HeLa^{mock}$ indicating that the directed Lck membrane movement was not due to other types of galectins (Fig. 1D $HeLa^{mock}$ *vs.* lac $HeLa^{mock}$). Moreover, analysis of gene expressions of Gal-1, Gal-3 (Supplementary Fig. 5) and other galectins, such as Gal-7 and -8 (data not shown) showed that $HeLa^{mock}$ did not express none of these galectins while $HeLa^{Gal\text{-}1}$ expressed only Gal-1.

The Gal-1 induced Lck sequestration was also detected when Jurkat cells were co-cultured with C32 melanoma (Supplementary Fig. 6), showing that other Gal-1 secreting cells are also capable to trigger this process. To ensure that the altered glycosylation in malignant T-cells (Ju et al., 2011) did not affect the examined cell

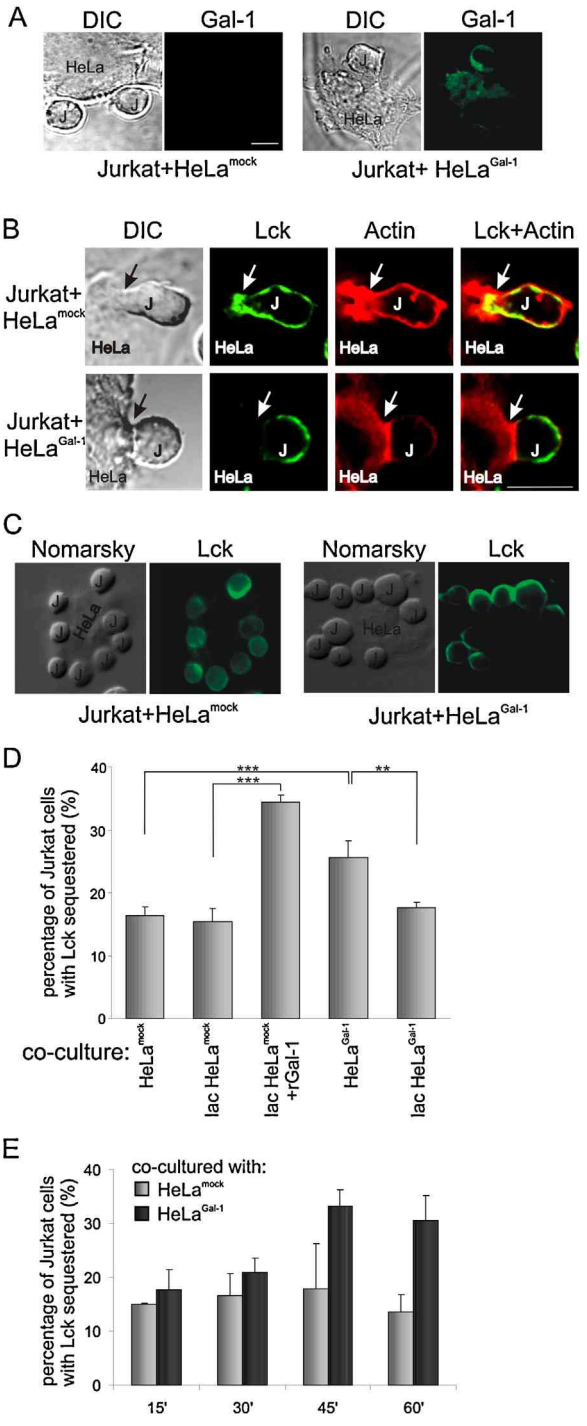

Fig. 1. Cell-derived Gal-1 induced sequestration of Lck from cell synapse in Jurkat cells. Jurkat (J) cells were co-cultured with Gal-1 negative ($HeLa^{mock}$) or Gal-1 producing tumor cells ($HeLa^{Gal-1}$) for different time points (15 min for A, 1 h for B–D, 15–60 min for E), and then the cells were fixed. (A) Cell surface bound Gal-1 was detected with anti-Gal-1-FITC in confocal microscope. (B and C) In permeabilized cells Lck and F-actin were labeled with FITC conjugated anti-Lck (green, B and C) and rhodamine–phalloidin (red, B) respectively, and detected with confocal (B) or fluorescence microscopy (C). Arrows indicate the cell synapse. (D) Jurkat cells were in co-culture with $HeLa^{mock}$, lactose pre-treated $HeLa^{mock}$ (lac $Hela^{mock}$), lactose pre-treated $HeLa^{mock}$ with recombinant Gal-1 bound (lac $Hela^{mock}$ + rGal-1), $HeLa^{Gal-1}$ or lactose pre-treated $HeLa^{Gal-1}$ (lac $Hela^{Gal-1}$) for 1 h. The cells were then fixed, permeabilized and labeled with mouse anti-Lck-FITC and analyzed in fluorescence microscope. The percentage of Jurkat cells with sequestered Lck was calculated as described in Section 2.4. The average and standard deviation was calculated from 3 to 4 samples. Student's *t*-test, $^{**}p < 0.01$, $^{***}p < 0.001$. (E) The percentage of Jurkat cells with sequestered Lck was determined for each time points (15–60 min) of co-culture and results from 2 independent experiments were presented. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

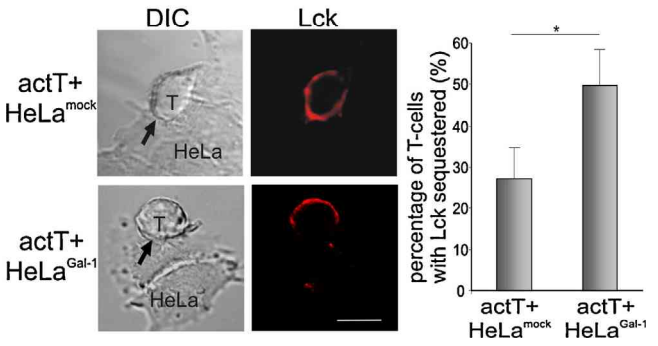

Fig. 2. Cell-derived Gal-1 induced sequestration of Lck from cell synapse in PHA-activated human T-cells. PBMC were activated with PHA for 3 days (actT) and then co-cultured with $HeLa^{mock}$ or $HeLa^{Gal-1}$ for 1 h. The cells were then fixed, permeabilized and labeled with mouse anti-Lck and anti-mouse IgG-NL557 (red) and analyzed with confocal microscope. Arrows indicate the cell synapse. Scale bar: 10 μm. The percentage of T-cells with sequestered Lck was determined as described in Section 2.4. The average and standard deviation was calculated from 4 samples. Student's *t*-test, $^{*}p < 0.05$. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

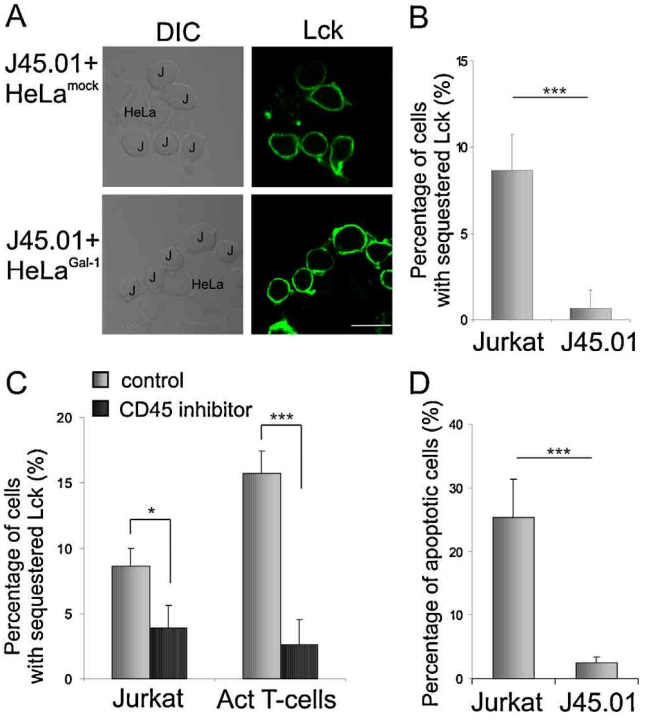

Fig. 3. Activity of CD45 phosphatase was required for Lck sequestration. (A) CD45 deficient J45.01 cells were co-cultured with $HeLa^{Gal-1}$ or $HeLa^{mock}$ for 1 h, then fixed and permeabilized. Lck was detected with mouse anti-Lck-FITC (green) by confocal microscopy. Scale bar: 10 μm. (B) Sequestration of Lck in Jurkat and the CD45 deficient J45.01 cells was compared in the co-culture with $HeLa^{Gal-1}$ or $HeLa^{mock}$ for 1 h. The average and standard deviation of the percentage of cells with sequestered Lck was calculated from 4 samples of two independent experiments, and the subtraction of the values obtained in co-culture with $HeLa^{Gal-1}$ and $HeLa^{mock}$ was presented, as described in Section 2.4. (C) Jurkat cells and PHA-activated T-cells were pre-treated with PTP CD45 inhibitor (10 μM for 20 min) and then co-cultured with $HeLa^{Gal-1}$ or $HeLa^{mock}$ for 1 h. Sequestration of Lck was determined from 4 samples of two independent experiments as described in previous point. (D) Jurkat and J45.01 cells were co-cultured with $HeLa^{Gal-1}$ or $HeLa^{mock}$ for 16 h and then reacted with Annexin V-AlexaFluor488. Apoptosis was detected by fluorescent microscopy, and calculated as described in Section 2.5. Student's *t*-test, $^{*}p < 0.05$, $^{***}p < 0.001$. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

response, we verified our findings using human PHA-activated T-cells (actT). Similarly to Jurkat cells, significantly higher percentage of sequestered Lck was detected in actT when co-cultured with HeLa^{Gal-1} as with HeLa^{mock} (Fig. 2). These results show that Gal-1 plays critical role in the spatial regulation of Lck upon encounter of tumor cells and T-cells.

3.2. Involvement of CD45

The receptor tyrosine phosphatase, CD45 has been implicated as the main regulator of Lck activity and importantly it has been identified as a major Gal-1 binding protein (Pace et al., 1999; Walzel et al., 1999; Fouillit et al., 2000; Fajka-Boja et al., 2002). Whether CD45 plays a role in Gal-1 induced Lck segregation has been examined. As Fig. 3 shows CD45 deficient variant of Jurkat, J45.01, readily attached to HeLa cells but did not respond with Lck sequestration, irrespectively of Gal-1 production by HeLa cells (Fig. 3A and B). This finding was further supported by using CD45 specific PTP inhibitor, since Lck segregation failed in the presence of the inhibitor either in Jurkat or actT-cells (Fig. 3C). Hence, both the presence and the phosphatase activity of CD45 were necessary in Gal-1 triggered Lck segregation. Effect of CD45 was not limited to Lck relocalization since J45.01 cells were unable to respond to HeLa^{Gal-1} with apoptosis (Fig. 3D). These findings supported that the early membrane movements are important in the downstream cell death process.

3.3. Kinetics of P-Tyr505 Lck exclusion from cell synapse

Kinase activity of Lck is negatively regulated by the tyrosine phosphorylation of its Tyr505 residue. Indeed, Lck species phosphorylated on this particular tyrosine were excluded from the synaptic zone in a time dependent manner when Jurkat cells were co-cultured with HeLa^{Gal-1} (Fig. 4A). The localization and kinetics of sequestration of inactive P-Tyr505 Lck coincided with that of the total Lck pool (Fig. 4B and Fig. 1E, respectively). As expected, the signaling cascade initiated by Lck resulted in accumulation of tyrosine phosphorylated proteins in the synaptic zone (Fig. 4C and D). It has to be noted that HeLa^{mock} did not trigger specific membrane localization of Lck, P-Tyr505 Lck or tyrosine phosphorylated proteins (Fig. 4B and D).

3.4. GM1 ganglioside affects Gal-1 induced Lck segregation and apoptosis

One of the major Gal-1-binding cell surface glycoconjugates is the GM1 ganglioside (Wang et al., 2009; Fajka-Boja et al., 2008). Moreover, GM1 is a regular lipid component of rafts, which are the platform of signaling complexes (Tani-ichi et al., 2005). We verified the importance of raft formation in Gal-1-induced apoptosis, since disrupting the integrity of membrane microdomains with β-cyclodextrin (β-CD), a cholesterol-chelator, inhibited the

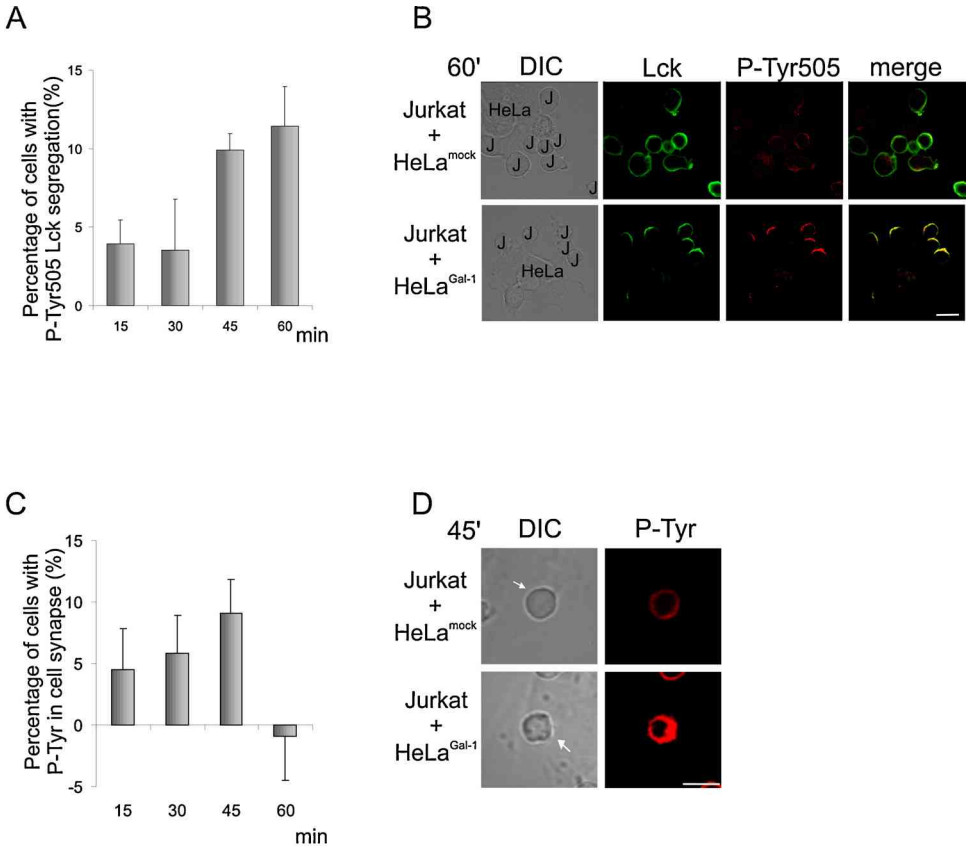

Fig. 4. Tyrosine phosphorylation in response to Gal-1. Jurkat cells were co-cultured with HeLa^{mock} or HeLa^{Gal-1} for different time points (15, 30, 45 or 60 min). (A and B) The cells were fixed and labeled with PE conjugated mouse anti-Lck P-Tyr505 (red) and FITC conjugated mouse anti-Lck (green), and analyzed with confocal microscopy. The average and standard deviation of the percentage of cells with sequestered Lck P-Tyr505 was calculated from two independent experiments, and the subtraction of the values obtained in co-culture with HeLa^{Gal-1} and HeLa^{mock} was presented, as described in Section 2.4. (C and D) After the co-culture the cells were fixed, permeabilized and the tyrosine phosphorylated proteins were detected using 4G10 anti-phosphotyrosine mAb and anti-mouse IgG-NL557 (red). The average and standard deviation of the percentage of cells with tyrosine phosphorylated proteins accumulated in cell synapse was calculated from 4 independent experiments, and the subtraction of the values obtained in co-culture with HeLa^{Gal-1} and HeLa^{mock} was presented, as described in Section 2.4. Arrows indicate the cell synapse after 45 min of co-culture (D). Scale bars: 10 μm. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

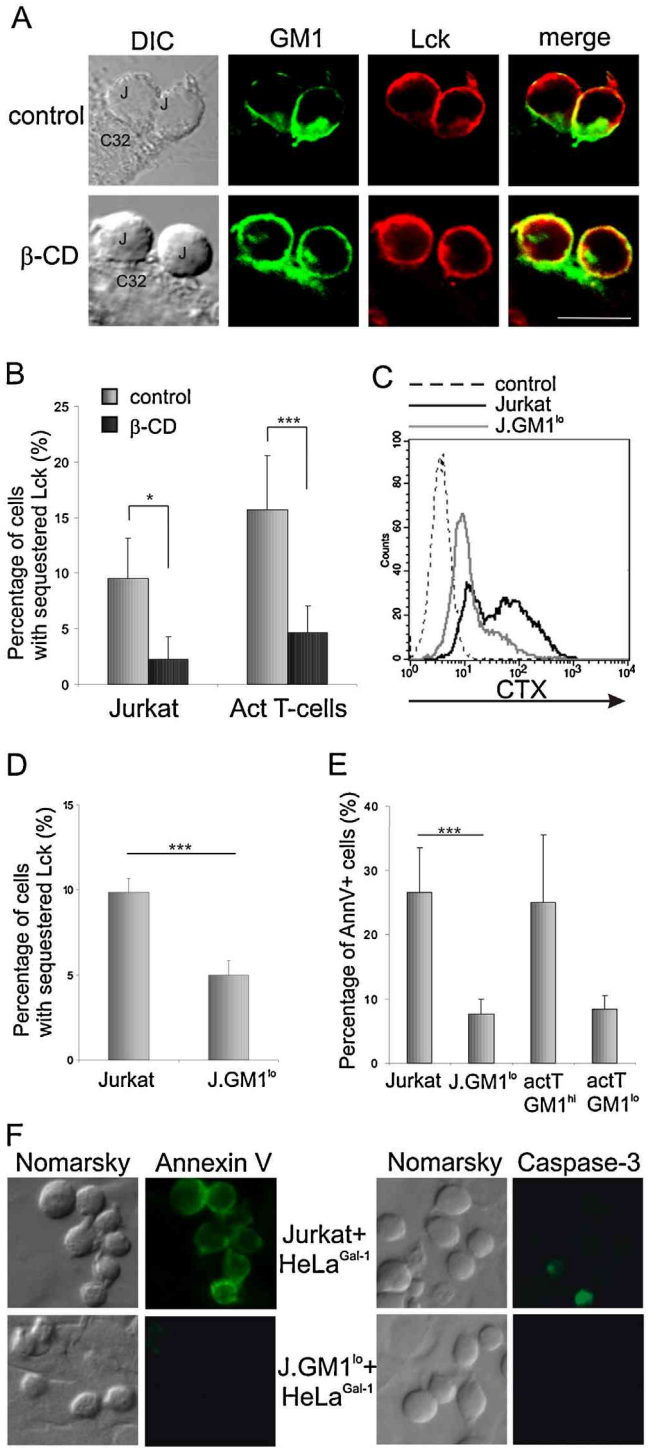

Fig. 5. Gal-1 induced apoptosis was dependent on GM1 expression of T-cells. (A) Jurkat cells were co-cultured with Gal-1 positive C32 cells for 1 h in the absence (control, upper row) or presence (lower row) of 10 mM β-CD. GM1 was detected by FITC conjugated CTX (green), Lck was labeled with mouse anti-Lck and anti-mouse IgG-NL557 (red), and then analyzed with confocal microscopy. Scale bar: 10 μm. (B) Jurkat cells or PHA-activated T-cells (actT) were co-cultured with HeLa^{Gal-1} or HeLa^{mock} for 1 h in the presence or absence of β-CD. The percentage of cells with sequestered Lck was calculated from 5 (Jurkat) or 4 (T-cells) samples as described in Section 2.4. (C) The expression of GM1 on the cell surface was tested by flow cytometry after CTX-FITC binding to Jurkat cells (black line) or J.GM1^{lo} (gray line), the control was left untreated (dotted line). (D) J.GM1^{lo} or Jurkat cells were co-cultured with HeLa^{Gal-1} or HeLa^{mock} for 1 h, and then the sequestration of Lck was analyzed from 5 independent samples as described in Section 2.4. (E) Jurkat, J.GM1^{lo}, the GM1^{lo} or GM1^{hi} subsets of activated human T-cells were co-cultured with HeLa^{Gal-1} or HeLa^{mock} for 16 h and then reacted with Annexin V-AlexaFluor488. Apoptosis was detected by fluorescent microscopy, and calculated as described in Section 2.5. Student's *t*-test, $^{*}p < 0.05$, $^{***}p < 0.001$. (F) Jurkat or J.GM1^{lo} were co-cultured with HeLa^{Gal-1} for 16 (left panel) or 24 h (right panel) and apoptosis was detected with Annexin V-AlexaFluor488 (left panel) or rabbit anti-active caspase-3 and anti-rabbit Ig-FITC (right panel), then analyzed with fluorescent microscope. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

apoptotic signal triggered by soluble Gal-1 in activated lymphocytes (Ion et al., 2006). Therefore we analyzed whether raft formation and interaction between Gal-1 and GM1 ganglioside regulated the Gal-1-triggered Lck membrane localization and eventually T-cell apoptosis. As presented on Fig. 5A, GM1 accumulated at the contact site between tumor and T-cells while Lck migrated to the opposite. In the presence of β-CD both accumulation of GM1 (Fig. 5A) and segregation of Lck (Fig. 5A and B) failed either in Jurkat or activated T-cells (Fig. 5B), indicating that intact raft organization was crucial for this process. Since level of GM1ganglioside varies depending on the differentiation and activation state of the cells or in pathological situation (Wang et al., 2009), the question remains to be answered whether these changes affect the outcome of a signaling event. We found that Jurkat and activated T-cells are heterogeneous for GM1 expression (Fig. 5C and Supplementary Fig. 7A). Jurkat and activated T-cells expressing low level of GM1 were enriched by magnetic bead selection (J.GM1^{lo}, Fig. 5C and actT GM1^{lo} Supplementary Fig. 7B) and co-cultured with HeLa cells. Segregation of Lck was significantly lower in J.GM1^{lo} than in wild type Jurkat (Fig. 5D). In parallel, sensitivity to Gal-1 induced apoptosis of GM1^{lo} Jurkat and act T-cells were compared to unselected Jurkat and GM1^{high} act T-cells, respectively. Reduction of cell surface level of GM1 resulted in diminution of apoptosis as detected with Annexin V-binding assay or caspase-3 activation in Jurkat (Fig. 5E and F) or activated T-cells (Fig. 5E).

4. Discussion

Apoptosis of T-lymphocytes is one of the hallmarks of the immunohomeostasis, including T-cell differentiation and down-regulation of effector functions. Among regulators of T-cell viability, Gal-1, a β-galactoside binding lectin has been emerged. Signaling pathway inducing death of activated T-cells and T-cell lines by Gal-1 has recently been revealed (Hahn et al., 2004; Ion et al., 2006; Brandt et al., 2008). One of the most detailed description has been published in our laboratory (Ion et al., 2005, 2006; Kovács-Sólyom et al., 2010; Blaskó et al., 2011), however even this failed to explain the earliest membrane events in context of the eventual apoptosis. Here we show that intimate cell contact between Gal-1 producing effector cells and activated T- or Jurkat target cells results in T-cell apoptosis which requires the early lateral segregation of non-receptor tyrosine kinase, Lck, a central component of Gal-1 induced cell death.

Previous report by Pace et al. suggested that reorganization of certain molecules within the membrane might be an important step during Gal-1 triggered T-cell apoptosis since Gal-1 binding induced spatial redistribution of its binding receptors into specific microdomains (Pace et al., 1999). In their model CD45 clustered with CD3 and CD7 co-localized in small membrane patches with CD43. The major limitation of this system has been the usage of soluble recombinant human Gal-1 in high concentration (Pace et al., 1999), a condition which does not occur physiologically. Therefore we applied cell-derived Gal-1 in co-culture system (Kovács-Sólyom et al., 2010) to ensure the natural presentation of Gal-1 and hence modeling the *in vivo* milieu. Moreover co-culture method avoided the disadvantages raising from using recombinant protein, such as requirement of reducing agent for maintaining functionality and affecting cell viability of Gal-1 (Stowell et al., 2007, 2008), and controversial effects depending on the concentration of Gal-1 during

the assays (Blaskó et al., 2011). It has to be noted that co-culture system has a disadvantage as well, namely that Gal-1 producing effector cells may express other galectins and unspecified factors which may affect the response of the target cells. Thus, the effects of the HeLa^{mock} (Gal-1 deficient), HeLa^{mock} covered with recombinant Gal-1 and Gal-1 transgenic counterpart, HeLa^{Gal-1} cells were compared. The results clearly confirmed the specific contribution of Gal-1 to Lck lateral movement and, as previously showed, to apoptosis (Kovács-Sólyom et al., 2010), since Gal-1 treated HeLa^{mock} and HeLa^{Gal-1} caused significantly higher Lck segregation and T-cell apoptosis than Gal-1 negative HeLa^{mock}. Moreover, the involvement of other galectin family members was excluded with lactose competition and real time PCR.

Encounter of T-cells with Gal-1 in a tumor cell-bound form induced a conducted rearrangement of membrane components, in which the cell-contact site determined the topological relations. After 30 min of cell-cell interaction Lck translocated to distal pole of the contact and p-Tyr505 Lck, the inactive form of the enzyme, strongly co-localized with the whole Lck pool. In contrast tyrosine phosphorylated proteins increasingly accumulated within the contact site up to 30 min of the cell-cell contact, then tyrosine phosphorylation signal declined. Receptor tyrosine phosphatase, CD45 played critical role in this process since specific inhibition or deficiency of CD45 prevented Lck sequestration and following apoptosis. As CD45 is one of the major regulators of Lck function by activating its kinase activity with dephosphorylating p-Tyr505 or maintaining Tyr 505 in dephosphorylated form (Sieh et al., 1993) it is conceivable that early activation of Lck within the cell contact zone is ensured by CD45. These results suggested the following serial of events: after interaction of effector Gal-1 presenting cells with T-cells, Lck promoted the tyrosine phosphorylation of signaling proteins accordingly our previous results (Ion et al., 2005, 2006; Kovács-Sólyom et al., 2010; Blaskó et al., 2011). After initiating the downstream processes, Lck left the signaling complex and was expelled to the distal region of T-cell membrane in an inactivated form. Sequestration of Lck from the cell synapse was sustained up to several h (data not shown), and the cells underwent apoptosis accompanied by phosphatidylserine externalization and caspase-3 activation. The role of CD45 is worth for some discussion. Early studies were controversial in judging this question. First studies suggested that CD45 was the receptor for Gal-1 and it was indispensible in Gal-1 induced apoptosis (Perillo et al., 1995). However, further studies failed to prove that CD45 is the apoptosis-mediating receptor for Gal-1 (Fajka-Boja et al., 2002; Pace et al., 2000). Finally the analysis of CD45 glycosylation clarified its role in Gal-1's cytotoxicity (Earl et al., 2010). It must be emphasized that all works, including our previous ones, used soluble Gal-1. The results presented here support the theory that in the case when Gal-1 acts as a cell- or extracellular matrix coupled protein, the pathways and signaling components leading to the eventual T-cell death may differ in some aspects from that induced by soluble, recombinant Gal-1.

Directed movement of membrane associated molecules largely depends on lipid components within the membrane. One of the determining ingredients is GM1-ganglioside which does not only play a role in raft generation but also regulates signal transduction. Activated T-cells show enhanced GM1 expression (Tani-ichi et al., 2005; Wang et al., 2009) suggesting an increase of the stimulatory pathways. Lowering the level of glycosphingolipids by using glucosylceramide synthase inhibitor attenuates TCR signaling, T-cell proliferation and IL-2 production (Zhu et al., 2011). Our recent results show that the integrity of membrane microdomains is essential during Gal-1 triggered T-cell apoptosis (Kovács-Sólyom et al., 2010). Accordingly, the GM1 composition of the cell membrane plays also a crucial role in response to Gal-1: Lck segregation is reduced and cells are less sensitive to Gal-1 induced apoptosis in T-cells with lower GM1 level compared to higher GM1 expressing T-cells. The importance of the GM1 level in T-cell membrane is also underscored by the observation that it is modified in pathological situations, such as in T-cells of diabetic NOD mice (Wu et al., 2011) or systemic lupus erythematosus (SLE) (Jury et al., 2004). Moreover, the altered GM1 composition along with reduced level of TCR ζ-chain (Liossis et al., 1998) and Lck (Jury et al., 2003) leads to abnormal TCR signaling and dysfunction of SLE T-cells. Importantly, GM1 is a binding partner for Gal-1 (Kopitz et al., 1998; Wang et al., 2009), hence upon binding to Gal-1 it may participate in regulation of membrane movements ensuring the collection of extra- and intracellular components of the membrane proximal signaling factors.

The current theory emphasize the regulatory role of lattice between galectins and cell surface glycans (Rabinovich et al., 2007; Garner and Baum, 2008; Grigorian et al., 2009; Ledeen et al., 2012). In parallel, our results show robust spatial remodeling of intracellular signaling elements in inner membrane leaflet upon encounter of T-cells with Gal-1 producing effector cells. When Gal-1 is presented by the producer/effector cell or the extracellular matrix, it defines a spatial distribution of membrane proteins in target T-cells, leading to sequestering of Lck apart from the cell-cell contact site, and switching the downstream signaling pathway from immune response to apoptosis.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.10.010.

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