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ORIGINAL ARTICLES

Differential Distribution of Both IL-12Rβ Chains in the Plasma Membrane of Human T Cells

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Abstract IL-12 is a cytokine that stimulates the expression of CD26, a T cell– and raft-associated ectopeptidase. IL-12 also enhances the interaction between CD26 and CD45RO, which removes the phosphatase CD45RO from raft microdomains. Since Janus kinases are known CD45 substrates, our hypothesis was that this relocation of CD45RO in nonraft areas of the membrane could be important to switch off the signaling via cytokine receptors, e.g., the IL-12 receptor (IL-12R). Accordingly, both IL-12R and CD45RO should be equally positioned in the cell membrane upon IL-12R ligation. However, there were no data available on the membrane distribution of IL-12R on human T cells. Working with phytohemagglutinin (PHA) lymphoblasts, we tried to fill that gap. The high-affinity IL-12R is made of two chains: IL-12Rβ1 and IL-12Rβ2. Using flow cytometry, Western blot and confocal microscopy, we obtained data suggesting that IL-12Rβ1 is mainly associated to phospholipid-rich membrane areas, a location even enhanced upon IL-12 incubation of PHA blasts. Instead, IL-12Rβ2 is found more segregated into membrane rafts, which could explain why two IL-12-triggered events, T-cell proliferation and ERK1/2 activation, are both methyl-β-cyclodextrin-sensitive events. Ligation of IL-12R with IL-12 seems to induce a partial enrichment of IL-12Rβ2 in phospholipid-rich areas, where according to our data IL-12Rβ1 is already present. Therefore, although new data will be required, the present results support the initial hypothesis.

Keywords IL-12 · IL-12Rβ1 · IL-12Rβ2 · Rafts · MAPK · ERK1/2 · Proliferation

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Introduction

Membrane rafts are membrane microdomains enriched in cholesterol and sphingolipids which have gained attention as important sites for signal transduction in a wide variety of cells. Some receptors are either constitutively or inducibly associated to rafts, and some of them are receptors for cytokines (e.g., IL-2R or IL-15R) (Goebel et al. 2002), growth factors (e.g., EGF) (Liu et al. 2007) or biological response modifiers (e.g., prothymosin-α) (Salgado et al. 2005). The functional IL-12R is made of two glycoproteins, IL-12Rβ1 and IL-12Rβ2 (Presky et al. 1996); but there are no data about the membrane location of both chains in T cells or the role of membrane rafts in cell signaling through IL-12R. This is an interesting issue because IL-12 is a key immunoregulatory cytokine that plays a central role in cell-mediated immune responses (Trinchieri 2003). Among its functions, this cytokine enhances T-cell proliferation, induces the production of proinflamatory cytokines, promotes T_{H1}-cell differentiation, upregulates certain proteins (e.g., CD26) and controls their segregation (e.g., CD26 and CD45RO) in different membrane compartments from human T cells (Trinchieri 2003; Cordero et al. 1997; Salgado et al. 2003).

IL-12Rβ1 is a constitutively expressed protein, but it is also upregulated upon T-cell activation (Wu et al. 1997). In contrast, IL-12Rβ2 is not present on resting T cells and a T-cell receptor (TCR)–mediated stimulus is required in

order to express this protein (Rogge et al. 1999; Wu et al. 2000). In a murine system, IL-12Rβ1 is responsible for both low- and high-affinity binding sites, while IL-12Rβ2 only confers low affinity for IL-12 (Wu et al. 2000). Instead, both human IL-12Rβ1 and IL-12Rβ2 proteins bind IL-12 with low affinity, generating a high-avidity and fully functional receptor when coexpressed (Presky et al. 1996). IL-12R lacks kinase activity, which means that its association with Janus tyrosine kinases (JAKs), TYK2 (IL-12Rβ1-bound) and JAK2 (IL-12Rβ2-bound) is required to deliver an intracellular signal (Zou et al. 1997). Upon IL-12 binding, JAKs phosphorylate themselves and, subsequently, phosphorylate the IL-12Rβ2 cytoplasmic region, providing docking sites for the SH2 domains of STAT proteins (signal transducers and activators of transcription). STAT4 is the main transcription factor activated by IL-12, and its binding to IL-12Rβ2 is followed by TYK-2-dependent tyrosine phosphorylation and the nuclear translocation of STAT4 (Naeger et al. 1999; Sugimoto et al. 2003). STAT4 is additionally phosphorylated in serine by p38, a mitogen-activated protein kinase (MAPK). Double tyrosine/serine phosphorylation of STAT4 is important, together with STAT3 (Sugimoto et al. 2003), for IFNγ production and T_{H1} differentiation (Visconti et al. 2000; Morinobu et al. 2002).

As many other cytokine receptors, alternative signaling pathways are triggered through the IL-12R, like the PI3K/Akt kinase (Athié-M et al. 2000) or the MAPK system (Pignata et al. 1994; Visconti et al. 2000; Morinobu et al. 2002). The MAPK family of protein kinases (extracellular signal–regulated kinases [ERKs], c-Jun N-terminal kinases [JNKs] and p38 kinase) are regulated by different stimuli (Mor and Philips 2006). IL-12 induces the activation of 44-kDa MAPK (Pignata et al. 1994) in human phytohemagglutinin (PHA) lymphoblasts, and very recently, ERK1/2 activation has also been shown in human NK cells costimulated with IgG and IL-12 (Kondadasula et al. 2008). However, some authors disagree with these results and exclude ERKs as part of the signal-transduction machinery triggered by IL-12 (Athié-M et al. 2000; Visconti et al. 2000). Membrane rafts (Janes et al. 1999; Viola 2001; Drevot et al. 2002) and ERKs (Mor and Philips 2006) are important to control T-cell proliferation. In this sense, we report that IL-12 induces cell proliferation and ERK1/2 activation. Both events are dependent on membrane raft integrity, which is connected with our findings, suggesting that IL-12Rβ2, the main IL-12R chain capable of delivering a proliferative signal (Presky et al. 1996; Zou et al. 1997), is a partially raft-associated protein. However, upon IL-12 binding, the initial segregation of both chains changes slightly since IL-12Rβ2 tends to move out of those microdomains to heterodimerize with IL-12Rβ1 and form the high-affinity IL-12R.

Materials and Methods

Materials

PHA, carboxy-fluorescein diacetate succinimidyl ester (CFSE), penicillin/streptomycin solution, RPMI 1640, methyl-β-cyclodextrin (MβCD), phenylmethylsulfonylfluoride (PMSF), leupeptin, aprotinin and mouse anti-β-actin monoclonal antibody were all supplied by Sigma (St. Louis, MO). Recombinant human IL-12 was from Peprotech (London, UK). Ficoll Paque^{TM} Plus, ECL^{Plus} and the Western blotting detection kit were manufactured by GE Healthcare (Waukesha, WI); BCA was from Pierce Biotechnology (Rockford, IL); Super RX film was from Fujifilm (Tokyo, Japan); PVDF membrane was from Millipore (Bedford, MA); the alkaline phosphatase (AP) conjugate substrate kit was from Bio-Rad (Richmond, CA); and Immunofluore mounting medium was from ICN Biomedicals (Costa Mesa, CA). Rat anti-human IL-12Rβ1 and IL-12Rβ2 antibodies with their corresponding isotypes, biotinylated polyclonal goat anti-rat Ig, phycoerythrin (PE)-labeled anti-CD71 (clone M-A712), FITC-labeled anti-CD59 (clone p282) and FITC-conjugated anti-phospho-ERK1/2 were all from BD Biosciences (San Diego, CA). Streptavidin-PE was from Serotec (Raleigh, NC), and CTB Alexa488, Streptavidin-Alexa Fluor® 633 and DiI (Vybrant® CM-DiI/C18) were from Molecular Probes (Eugene, OR). For Western blot experiments, goat anti-human IL-12Rβ2 extracellular domain (R&D Systems, Minneapolis, MN) and anti-IL-12Rβ1 (scC-20; Santa Cruz Biotechnology, Santa Cruz, CA) as well as anti-phospho-ERK1/2 (Thr-202/Tyr-204, Phospho-ERK1/2 Pathway Sampler Kit; Cell Signaling, Beverly, MA) were used.

Cell Isolation and Culture

Citrate anticoagulated venous blood samples were donated by healthy adult volunteers, and buffy coats were obtained by Centro de Transfusiones de Galicia (Santiago, Spain) according to institutional guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated from these buffy coats by centrifugation on a Ficoll density gradient. PBMCs were harvested, pelleted, washed and cultured in complete medium (RPMI 1640 supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% [v/v] heat-inactivated FBS) in a humidified incubator under 5% CO_2 (Cordero et al. 1997).

Extracellular Protein Labeling for Flow Cytometry and Confocal Microscopy

Resting T cells lack IL-12Rβ2 and require TCR stimulation to express this protein (Watford et al. 2004). Therefore, in order

to detect both IL-12R chains by flow cytometry, human PBMCs were first activated for 3 days with PHA (10 μg/ml, PHA lymphoblasts). These cells were then washed, fixed with ice-cold 1% PFA-PBS (pH 7.4) at room temperature (RT, 5 min) and placed in PBS–1% BSA–0.05% sodium azide. Fc receptors were blocked (30 min, 4°C) with human IgG, and cells were incubated (1 h, 4°C) with primary antibody (rat anti-IL-12Rβ1, anti-IL-12Rβ2 or isotype-matched unspecific antibody) afterward. Lymphoblasts were washed twice with PBS-BSA, incubated (1 h, 4°C) with biotinylated goat anti-rat Ig, washed again and stained using PE-conjugated streptavidin (1 h, 4°C). Finally, cells were washed before sample acquisition in a FACScalibur flow cytometer (BD Biosciences). To detect surface GM1, CD71 and CD59 levels, AlexaFluor 488 cholera toxin B subunit (CTB AlexaFluor488), anti-CD71-PE, anti-CD59-FITC or proper isotypes were used. When necessary, membrane cholesterol depletion was carried out with MβCD (2.5 mM) for 15 min at 37°C. Non-ionic detergent-sensitive areas were removed with 0.25% (v/v) TX-100 (5 min on ice) before fixation. Data analysis was performed with WinMDI software (www.methods.info/software/flow/winmdi.html).

For confocal microscopy experiments, all incubations were performed at 37°C (1 h). Due to the high IL-12R expression variability between different donors, 3-day PHA lymphoblasts were generated as above and flow-cytometric experiments were first conducted to select only those cells with the highest IL-12Rβ2 levels. Alexa 633-conjugated streptavidin was used instead of PE-conjugated streptavidin for IL-12Rβ1 or IL-12Rβ2 indirect staining. In order to compare the membrane distribution of rafts (GM1-enriched domains) with the localization of every IL-12R chain, cells were costained with CTB AlexaFluor488. Instead, other PHA blasts were stained, before cell fixation and IL-12R labeling, with the PFA-resistant lipid probe DiI (Vybrant CM-DiI/C18) (Bacia et al. 2004) to reveal glycerophospholipid-rich membrane regions (nonrafts). After a final washing step, cells were attached to microscope slides and mounted using Immunofluore antifade medium. Slides were sealed and analyzed using a spectral confocal microscope (TCS-SP2; Leica Microsystems, Barcelona, Spain). Colocalization analysis was performed by means of confocal software.

CFSE-Based Proliferation Assays

Human PBMCs were resuspended in RPMI 1640 at 10×10^6 cell/ml and incubated with 5 μM CFSE for 8 min at RT in the dark. FBS was added to cells to stop the reaction. After washing with complete medium, PBMCs were seeded in a 24-well plate at 0.25×10^6 cells/ml, activated with 1.25 μg/ml PHA ± IL-12 (2 ng/ml) and culture medium supplemented or not with 0.5 mM MβCD. CFSE fluorescence decay was measured by flow cytometry after 5 days.

Intracellular Immunofluorescent Phospho-ERK Staining

To detect phospho-ERK, 3-day PHA blasts were resuspended in RPMI. After 10 min at 37°C, an aliquot of cells (T = 0 min) was collected and fixed in 4% PFA-PBS (10 min, RT). IL-12 (2 ng/ml) was then added for 5 min at 37°C to the remaining cells. Then, lymphoblasts were collected and either fixed with ice-cold 1% PFA-PBS (pH 7.4) at RT (5 min) or used in Western blot experiments. For intracellular phospho-ERK immunostaining, 1×10^6 cells were placed in each test tube, washed (PBS pH 7.4) and permeabilized (90% methanol, −20°C, 30 min on ice). Lymphoblasts were then washed twice with PBS–2% FBS–0.05% sodium azide and their FcR blocked with human IgG (30 min, 4°C). Staining was performed with AlexaFluor 488–conjugated antibodies, either anti-phospho-ERK1/2 or the corresponding isotype antibody, in the dark (1 h, RT). After washing, cells were placed in 1 ml of PBS-BSA.

TX-100-Resistant Microdomain (Raft) Isolation by Equilibrium Density Gradient Centrifugation

Unless otherwise indicated, all steps were performed at 4°C and according to Ilangumaran et al. (1996). PHA blasts (50×10^6) were sequentially washed with PBS (pH 7.4) and TKM buffer (50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA) to eliminate residual serum proteins. Cell lysis was induced upon incubation (20 min on ice) with TKM buffer supplemented with 0.5% TX-100 (v/v) and a protease inhibitor cocktail (1 mM PMSF, 10 μg/ml leupeptin and 5 μg/ml aprotinin). Cell extracts were then adjusted to 40% (w/v) sucrose, placed into SW55Ti tubes (L8-M; Beckman, Fullerton, CA) and overlaid with two solutions made of 36% (w/v) and 5% (w/v) sucrose in TKM buffer. After ultracentrifugation (200,000 × *g*, 18 h, 4°C), 11 fractions (numbered 1–11 from top to bottom, 450 μl/each) were harvested. Rafts (fractions 3–6) were pooled separately from nonrafts (fractions 10, 11), and all samples were stored afterward at −20°C. The quality of raft microdomain separation was assayed by either AP activity or CD71 protein detection (dot blot assays).

SDS-PAGE and Western Blotting

For these experiments, 3-day PHA blasts were stimulated or not for 5 min with IL-12 and treated as described in "Results." IL-12Rβ1 and IL-12Rβ2 proteins were detected in both pooled raft and nonraft fractions from sucrose gradients (see above) as well as postnuclear lysates. To obtain postnuclear lysates, PHA blasts were placed in RIPA buffer

(1× PBS [pH 7.4], 1% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS) supplemented with protease inhibitors for 60 min on ice. Samples were then centrifuged at 13,000 × *g* (4°C). The amount of protein was calculated (BCA) for both raft and nonraft fractions as well as postnuclear lysates, and 5× SDS sample buffer was added to get a final concentration of 200 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 3% (w/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue and 0.1% (w/v) pyronine. In order to detect phospho-ERK, PHA lymphoblasts were stimulated (see "Intracellular Immunofluorescent Phospho-ERK Staining," above) and aliquots of 1.5×10^6 lymphoblasts were lysed in an equal volume of 2× SDS sample buffer. All samples were boiled for 5 min and their DNA content was broken with a syringe.

Proteins (10–15 μg/lane) were separated on an SDS-PAGE gel, transferred to a PVDF membrane and blocked with either 10% (w/v) semifat dry milk in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween-20) or, to detect phospho-ERK, 1% (w/v) BSA-TBS-T. Blots were incubated with goat anti-human IL-12Rβ2, anti-IL-12Rβ1 or anti-phospho-ERK1/2 (Thr-202/Tyr-204) in blocking buffer and washed with TBS-T; and a suited horseradish peroxidase–conjugated secondary antibody was used. Visualization was performed by the ECL^{Plus} method. When necessary (e.g., β-actin measurements), antibodies were stripped off in 0.2 N NaOH (15 min, RT) and the membranes blocked before reprobing.

Results

Disruption of Membrane Rafts with MβCD Affects T-Lymphocyte Proliferation Enhancement Induced by IL-12 Costimulation

Cholesterol-rich plasma membrane microdomains (rafts) function as specialized signaling platforms in immune cells (Janes et al. 1999; Viola 2001; Drevot et al. 2002). Some cytokine and growth factor receptors are associated to these membrane rafts in a constitutive or inducible fashion (Marmor and Julius 2001; Matkó and Szöllösi 2002; Goebel et al. 2002; Liu et al. 2007). IL-12 is a cytokine with various effects on T lymphocytes (Trinchieri 2003). For example, this cytokine enhances T-cell proliferation induced by the mitogenic lectin PHA, but the effect is dependent on the PHA dose and the initial cell concentration; indeed, this cytokine only behaves as a mitogenic factor when suboptimal amounts of PHA (<2.5 μg/ml) are used (Canda-Sanchez et al. 2008). To determine whether membrane raft microdomains are required for the proliferative function of IL-12, human PBMCs were labeled with CFSE and activated (initial density 0.25×10^6 cells/ml) for 5 days with

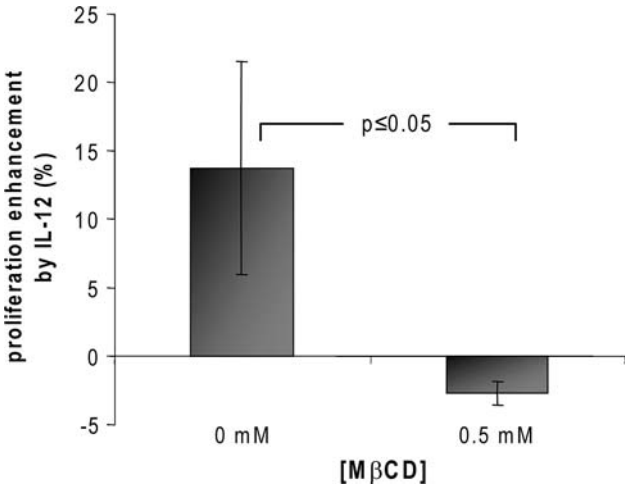

Fig. 1 Cholesterol depletion with MβCD leads to suppression of the mitogenic activity of IL-12. Human PBMCs were purified, seeded at 0.25×10^6 cells/ml and activated with PHA (1.25 μg/ml) ± IL-12 (2 ng/ml); and the culture medium was supplemented (or not) with 0.5 mM MβCD to reduce cholesterol levels within the plasma membrane. After 5 days, cells were harvested and CFSE fluorescence was measured by flow cytometry. Proliferation enhancement caused by IL-12 was expressed as a percentage (*y* axis) over the control (PHA alone). Data (mean ± SD) summarize the results from three independent experiments (three donors) where every culture condition was tested at least three times

PHA (1.25 μg/ml), with or without IL-12 (2 ng/ml). Cells were also treated (or not) with MβCD (0.5 mM), a molecule that disrupts raft structure after extracting cholesterol from the cell membrane (Ilangumaran and Hoessli 1998), and CFSE fluorescence was analyzed by flow cytometry after 5 days of culture. As expected, Fig. 1 shows that IL-12 strengthens cell proliferation induced by PHA; but our results also indicated that this enhancing effect of IL-12 was significantly attenuated when MβCD was present. Since IL-12Rβ2 (Presky et al. 1996; Heath et al. 2000; Jones et al. 2003) and its associated JAK-2 (Sugimoto et al. 2003) have been linked to the proliferative activity of IL-12, we hypothesized that at least one out of two IL-12R chains, the IL-12Rβ2 protein, could be an MβCD-sensitive, and therefore raft-associated, protein.

MβCD Significantly Reduces the Expression of IL-12Rβ2, but not IL-12Rβ1, in Human T Lymphoblasts

Although there is a growing amount of data regarding the cell membrane distribution of cytokine receptors, there is only one study on IL-12R (Kondadasula et al. 2008) and none undertaking that study in T cells with antibodies specific for both IL-12R chains: IL-12Rβ1 and IL-12Rβ2. In order to examine the localization of IL-12Rβ1 and IL-12Rβ2 in T cells, human PHA lymphoblasts (PBMCs activated for 3 days with 10 μg/ml PHA) were generated

using activation conditions previously set up by our group to get a good IL-12Rβ1/IL-12Rβ2 expression. After MβCD treatment, raft-associated proteins not only move from rafts to nonraft membrane regions but also are internalized, degraded or extracted from the membrane and released into the medium. Therefore, if a protein is found associated with membrane rafts, cholesterol depletion with MβCD will probably alter its expression on the cell surface (Ilangumaran and Hoessli 1998; Goebel et al. 2002; Salgado et al. 2003; Borroni et al. 2007). Initial dose–response assays with MβCD were carried out, and the expression of two raft and likely MβCD-sensitive markers, CD59 and GM1 (detected with CTB AlexaFluor488) (Ilangumaran and Hoessli 1998; Matkó and Szöllösi 2002; Rao et al. 2004), and a nonraft and probably MβCD-resistent protein, CD71 (Harder et al. 1998; Salgado et al. 2005; Babiychuk and Draeger 2006), was measured by flow cytometry. In those experiments it was observed that a single dose of MβCD (2.5 mM) eliminated cholesterol without damaging membrane integrity. However, both GM1 (not shown) and CD59 (Fig. 2a) displayed sensitivity to cholesterol depletion, while the nonraft marker CD71 (Fig. 2a) was resistant to the same concentration of MβCD. Bearing these results in mind, we next evaluated the effect of cholesterol depletion on the levels of both IL-12Rβ1 and IL-12Rβ2 proteins, the two chains of the IL-12R. As Fig. 2b shows, a 2.5 mM concentration of MβCD significantly downmodulated IL-12Rβ2 expression in human PHA lymphoblasts, while IL-12Rβ1 levels remained unaffected. Therefore, these results underline a differential recruitment of both IL-12R proteins in membrane rafts. Thus, the IL-12Rβ1 chain was closer to CD71-enriched and MβCD-insensitive areas of the membrane (nonrafts), while the β2 chain was more associated to CD59/GM1-enriched and MβCD-sensitive domains (rafts). Since ligand interaction could modify the membrane positioning of both proteins, we carried out the same experiments with PHA blasts preincubated for 5 min with IL-12. We found that receptor ligation made IL-12Rβ2 less sensitive to cholesterol extraction but did not affect the detection of IL-12Rβ1 (Fig. 2b).

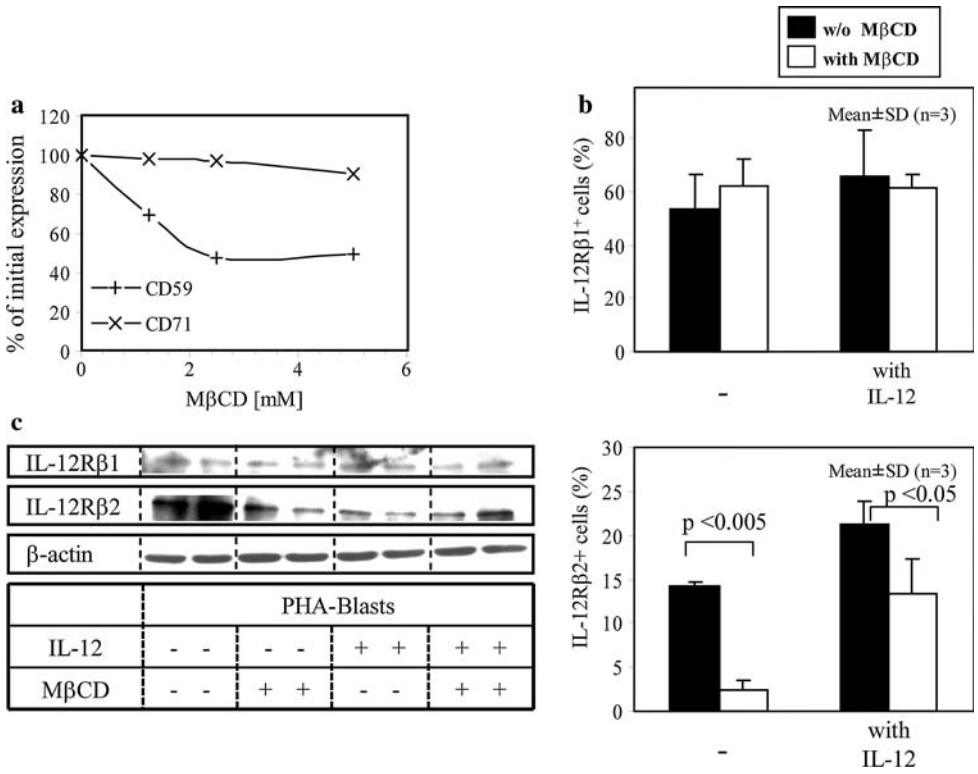

Fig. 2 Cholesterol extraction with MβCD differentially affects the expression of IL-12Rβ1 and IL-12Rβ2. **a** PBMCs activated for 3 days with 10 μg/ml PHA (PHA lymphoblasts) were treated with different concentrations of MβCD (15 min, 37°C). Expression of both CD59 (a raft marker) and CD71 (a nonraft marker) proteins was evaluated by flow cytometry with anti-CD59-FITC and anti-CD71-PE monoclonal antibodies, respectively. All data were relativized considering the highest value (without MβCD) as 100%. The experiment was repeated three times with similar results. **b**, **c** PHA lymphoblasts were incubated (or not) with 2 ng/ml IL-12 for 5 min and plasma membrane cholesterol was depleted (or not) with MβCD (2.5 mM, 15 min, 37°C). Reduction of IL-12Rβ1 and IL-12Rβ2 expression due to MβCD was measured by either flow cytometry (**b**, percentage of positive cells represents the mean for three independent experiments ± SD) or Western blotting (**c**, duplicated samples). In **c** constitutive β-actin expression was measured as a protein loading control

Similar results were obtained when PHA lymphoblasts were preincubated or not for 5 min with IL-12, treated or not with MβCD and postnuclear lysates separated by gel electrophoresis, transferred to a PVDF membrane and probed for both IL-12Rβ1 and IL-12Rβ2 proteins. As shown (Fig. 2c), IL-12Rβ2 was downmodulated following cholesterol depletion. Besides, IL-12 preincubation significantly reduced the amount of IL-12Rβ2 in the lysates, probably due to receptor internalization, but also made IL-12Rβ2 more resistant to the MβCD treatment (Fig. 1c). However, whatever the cell type (lymphoblasts preincubated or not with IL-12), no modification was observed for IL-12Rβ1 protein levels following cholesterol depletion with MβCD. Therefore, as a whole, our findings support the idea that IL-12Rβ2 could be repositioned from membrane rafts to nonrafts after ligand engagement. In contrast, the same data predict that IL-12Rβ1 accumulates within nonraft regions after receptor ligation.

TX-100 Differentially Reduces IL-12Rβ1 and IL-12Rβ2 Levels in Human T Lymphoblasts

Membrane rafts are microdomains highly resistant at low temperature to non-ionic detergents such as TX-100 (Ilangumaran and Hoessli 1998; Babiychuk and Draeger 2006; Salgado et al. 2003, 2005; Peng et al. 2007). To study the presence of certain proteins in membrane rafts, we first tested by flow cytometry the TX-100 sensitivity of CD71 (positive control, nonraft marker) and ganglioside GM1 (negative control, raft marker) (Fig. 3a). Therefore, PHA lymphoblasts were treated with a low TX-100 concentration (0.25% v/v, 5 min, 4°C) which was enough to fully remove CD71 (TX-100-sensitive), but not GM1 (TX-100-resistant), from cells (Peng et al. 2007; Salgado et al. 2005). Then, we examined the effect of TX-100 treatment on the expression of both IL-12Rβ1 and IL-12Rβ2 (Fig. 3b). IL-12Rβ1 expression was affected by lipid extraction with TX-100 in a similar way to the nonraft and TX-100-sensitive marker CD71. Moreover, the sensitivity of IL-12Rβ1 to TX-100 was further increased when PHA lymphoblasts were exposed to IL-12 for just 5 min prior to detergent addition, which could be explained considering an enrichment of IL-12Rβ1 out of membrane rafts caused by IL-12. However, IL-12Rβ2, in a similar way to GM1, was not downmodulated by TX-100 (Fig. 3b), probably indicating a strong association of IL-12Rβ2 with membrane rafts. We could not draw conclusions about the influence of IL-12 on the IL-12Rβ2 location based exclusively on these TX-100 experiments.

Considering our MβCD data, we had some indications that IL-12Rβ2 was associated to membrane rafts and that, once IL-12R engagement took place, the IL-12Rβ2 chain was rather associated to phospholipids-rich areas (nonrafts) together with the IL-12Rβ1 protein to form the high-affinity receptor. In order to give more support to those preliminary data, additional experiments were carried out. Membrane rafts have been traditionally evaluated through biochemical techniques based on the insolubility of these microdomains in non-ionic detergents at low temperatures and their density when submitted to a discontinuous sucrose gradient (Marmor and Julius 2001; Salgado et al. 2003, 2005; Babiychuk and Draeger 2006). Three-day PHA blasts were treated with a lysis buffer containing TX-100 and rafts (fractions 3–6) and nonrafts (fractions 9, 10) collected and pooled after ultracentrifugation. The localization of certain markers (AP, CD59 and CD71) (Salgado et al. 2003, 2005) was assessed to evaluate the quality of membrane raft purification, and Western blotting experiments were conducted to detect both IL-12Rβ1 and IL-12Rβ2 in those membrane fractions. IL-12β1 and IL-12Rβ2 were present in high-density (nonraft) and low-density (raft) fractions, respectively (Fig. 3c). However, we could not see a clear attenuation of the IL-12Rβ2 association with membrane rafts after IL-12 stimulation.

Quantitation of the Membrane Distribution of Both IL-12Rβ1 and IL-12Rβ2 Chains by Confocal Microscopy

It has been described that the use of detergents could present some limitations and generate artificial associations between surface proteins and membrane microdomains (Magee and Parmryd 2003). To exclude any possible detergent interference, we used confocal microscopy to assess the localization of both IL-12R chains on the plasma membrane of human T lymphoblasts. Two fluorescent molecules, CTB AlexaFluor488 and CM-DiI (Vybrant CM-DiI/C18), were selected to respectively identify raft and nonraft regions on cell membranes. As described above, CTB is a proven raft marker due to its specificity for GM1 (Rao et al. 2004; Salgado et al. 2005), while CM-DiI is a lipid probe which intercalates between phospholipids and labels nonraft membrane regions (Bacia et al. 2004). As expected, fluorescent signals from CTB AlexaFluor488 did not overlap with those from CM-DiI molecules in control experiments (Fig. 4a, c), so we next used IL-12Rβ1- and IL-12Rβ2-specific antibodies in combination with biotinylated goat anti-rat and Alexa633-conjugated streptavidin to visualize the location of both proteins. As Fig. 4b shows, a heterogeneous (patchy) distribution for IL-12Rβ1 and IL-12Rβ2 was revealed. Then, we investigated the fluorescent signals of both IL-12R chains relative to the fluorescence of either CTB AlexaFluor488 (rafts) or CM-DiI (nonrafts). Although a substantial overlap could be detected in a minor number of cases, in most cells no significant colocalization was observed between IL-12Rβ1

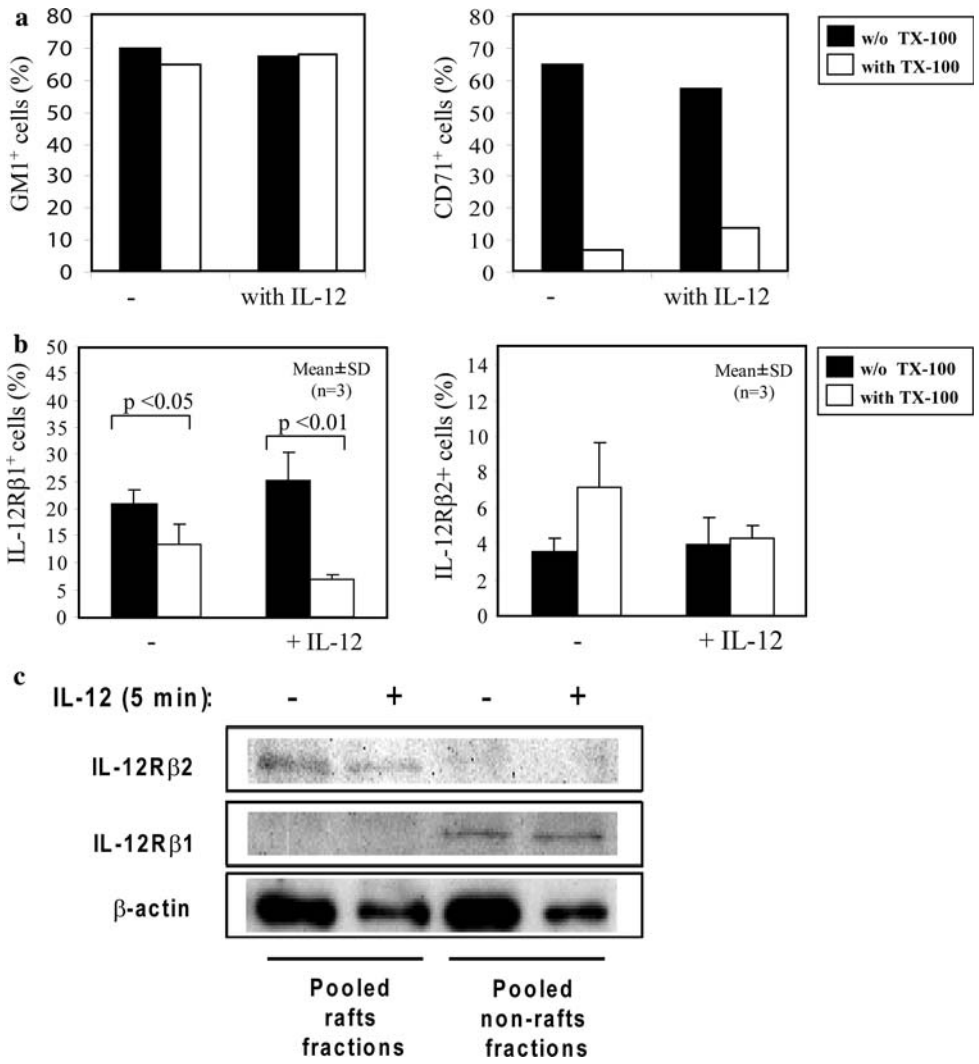

Fig. 3 IL-12Rβ1 and IL-12Rβ2 show differential sensitivity to the non-ionic detergent TX-100. **a**, **b** PHA lymphoblasts (10 µg/ml, 3 days) were harvested, washed, incubated with or without IL-12 (2 ng/ml, 5 min, 37°C) and exposed to PBS (pH 7.4) containing or not 0.1% (v/v) TX-100 for 5 min on ice. **a** Both ganglioside GM1 (a raft and TX-100-resistant marker; Salgado et al. 2005) and CD71 (a nonraft and TX-100 sensitive marker; Salgado et al. 2003) were detected by flow cytometry with CTB AlexaFluor488 and anti-CD71-PE, respectively. **b** IL-12Rβ1 and IL-12Rβ2 were measured by flow cytometry, and data from three independent experiments were expressed as mean ± SD. **c** Cell extracts from PHA lymphoblasts, incubated or not with IL-12, were subjected to discontinuous sucrose density gradient ultracentrifugation and 11 fractions, numbered from top to bottom, were collected. Raft (3–6) and nonraft (10, 11) fractions were pooled and AP activity and CD71 protein detected as purity controls (not shown). Then, proteins were separated by SDS-PAGE and IL-12Rβ1 and IL-12Rβ2 were detected with specific antibodies. β-actin expression was also measured as protein loading control. A typical experiment out of three carried out is shown

and CTB (Fig. 4b, c). IL-12 treatment of lymphoblasts for 5 min prior to cell labeling reduced even more the degree of colocalization between IL-12Rβ1 and CTB (Fig. 4b). On the contrary, IL-12Rβ1 showed an extensive colocalization with the nonraft marker CM-DiI, as we can conclude from the white regions in the cell membrane (arrows in Fig 4b). IL-12 slightly reinforced this colocalization between IL-12Rβ1 and CM-DiI; indeed, a larger and more polarized fluorescence overlap (white areas indicated by arrows in Fig. 4b) was observed after treating lymphoblasts for 5 min with this cytokine. On the other hand, the persistently low IL-12Rβ2 expression made it very difficult to evaluate the cell membrane distribution. In spite of this setback, we finally observed that IL-12Rβ2 colocalized mainly with the membrane raft marker CTB but barely with the nonraft marker CM-DiI (Fig. 4b). Upon IL-12 treatment of PHA blasts, this situation changed slightly and IL-12Rβ2 showed a small enrichment in DiI-positive areas of the cell membrane (Fig. 4b). Taken together, our results give more support to a model for human T lymphoblasts where IL-12Rβ2, a raft-associated protein, moves out from these membrane microdomains to

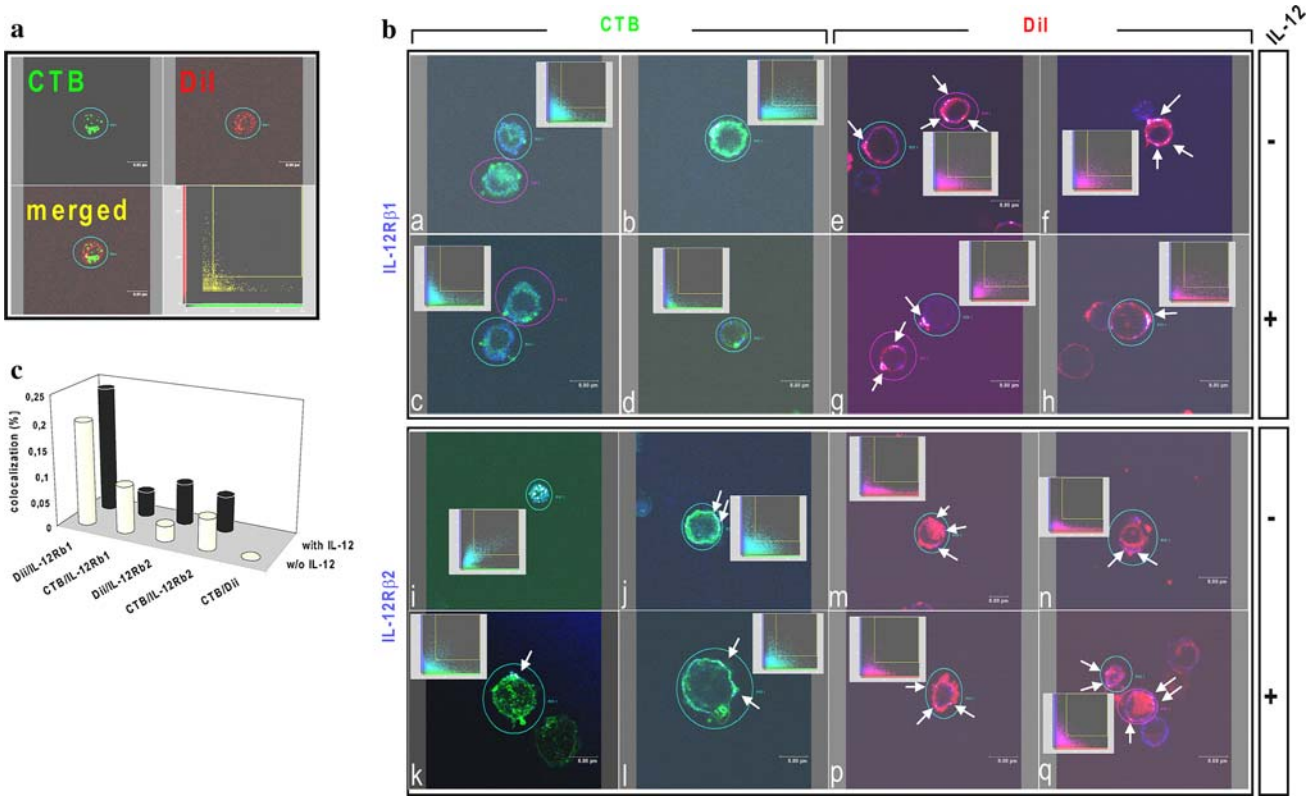

Fig. 4 IL-12Rβ2 and IL-12Rβ1 preferentially colocalize with raft and nonraft markers, respectively, on human PHA lymphoblasts. PHA lymphoblasts (10 μg/ml PHA, 3 days), treated or not with IL-12 (2 ng/ml, 5 min), were washed, fixed and stained. Ganglioside GM1 expression (raft marker) was revealed using CTB AlexaFluor488 (green). Nonrafts were detected with Vybrant CM-DiI/C18 (red), an unsaturated lipid probe selective for phospholipid-rich areas. The cell membrane distribution of either IL-12Rβ1 or IL-12Rβ2 proteins (blue) was compared with that of CTB or DiI. The overlapping of CTB and DiI fluorescence was evaluated as a negative control. For each pair of molecules between 84 and 235 cell sections were analyzed and the median value (percentage of colocalization) was calculated and plotted (**c**). Only representative cell sections (two for each condition) are shown in **a** and **b**. **a** Absence of colocalization between CTB and DiI markers in PHA blasts. **b** Colocalization analysis of IL-12Rβ1 and IL-12Rβ2 proteins with either CTB or DiI markers in IL-12-treated or untreated PHA lymphoblasts. For simplicity, only merged images with their respective cytofluorograms are shown. *White dots* and *arrows* indicate the presence of colocalizing molecules on the cell surface. Scale bar = 8 μm (refer color images in online)

a new membrane microenvironment (nonrafts), which is enriched in phospholipids (but impoverished in sphingolipids/cholesterol) and where IL-12Rβ1 is already present. Once both receptors find each other, the high-affinity IL-12R can arise (Fig. 6).

Chemical Disruption of Membrane Rafts with MβCD Attenuates the ERK1/2 Phosphorylation Induced by IL-12

According to our data, IL-12Rβ2 is a protein with affinity for membrane rafts. It is known that aggregation of these membrane microdomains with CTB plus anti-CTB antibody triggers signaling pathways similar to those induced by TCR ligation (e.g., ERK pathway activation) (Janes et al. 1999). Therefore, it was likely that IL-12 binding to IL-12Rβ2 could generate a similar response. Thus, despite some data that IL-12 does not trigger the Raf–MEK–ERK pathway (Athié-M et al. 2000; Visconti et al. 2000), we obtained data from different techniques indicating the use of this signaling route by IL-12 (Fig. 5) (Canda-Sanchez et al. 2008). Therefore, ERK1/2 phosphorylation was analyzed as a readout system to examine the role of these sphingolipids and cholesterol-rich microdomains in the IL-12-dependent activation of human T lymphoblasts. These cells were treated or not with 1 mM MβCD at 37°C for 15 min, a concentration which eliminates cholesterol without damaging membrane integrity. After that, cells were stimulated with IL-12, harvested at different times and permeabilized; and levels of phosphorylated ERK1/2 molecules were measured by flow cytometry. In parallel, postnuclear lysates from the same cells were analyzed by Western blot with anti-phospho-ERK1/2 antibodies. As Fig. 5 shows, upregulation of ERK1/2 phosphorylation was evident just after 5 min of incubation with IL-12. Nevertheless, ERK1/2 phosphorylation was not enhanced with

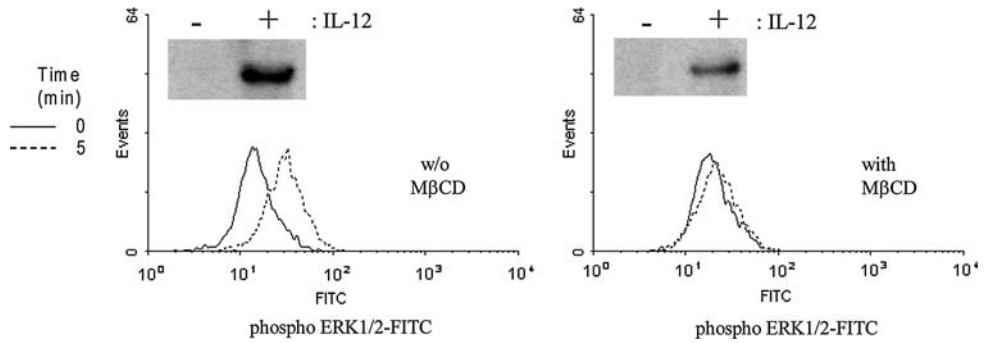

Fig. 5 ERK1/2 pathway activation is a raft-dependent event important for the mitogenic effect of IL-12. Human PHA blasts (10 μg/ml PHA, 3 days), treated or not with 1 mM MβCD (37°C, 15 min), were incubated for 5 min with 2 ng/ml IL-12. Cell samples were split in two and ERK1/2 phosphorylation was evaluated by either flow cytometry or Western blotting. Results are representative of three different experiments

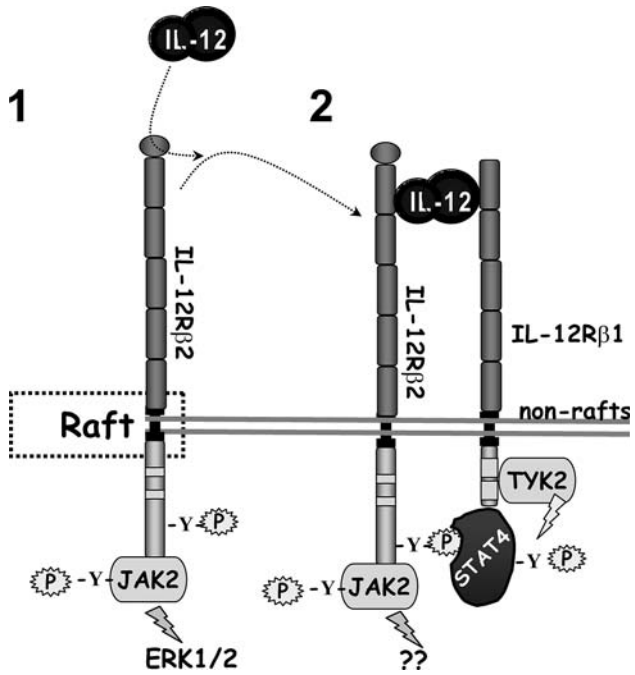

Fig. 6 Hypothetical model for the location of IL-12Rβ1 and IL-12Rβ2 chains in the plasma membrane of primary human T lymphoblasts. The figure shows our proposed model where IL-12Rβ2 is initially sequestered in membrane rafts to avoid accidental activation of IL-12R. Ligation of IL-12Rβ2 in rafts probably leads to ERK1/2 activation, which would reinforce ERK1/2 signaling (and T-cell proliferation) generated by the TCR/CD3 complex in these membrane microdomains. Then, IL-12Rβ2 is recruited to the phospholipid-rich bulk plasma membrane to give rise, with IL-12Rβ1, to the high-affinity IL-12R. This step could be important to bring STAT4 (bound to the cytoplasmic side of IL-12Rβ2) closer to TYK2 (the IL-12Rβ1-associated Janus kinase) and initiate its tyrosine phosphorylation

regard to background levels when cells were pretreated with MβCD (Fig. 5). Based on those data, we can conclude that the mechanism whereby IL-12 promotes ERK1/2 phosphorylation requires raft microdomain integrity and/or IL-12Rβ2 expression.

Discussion

Cell membranes are made of a complex mixture of different lipids, like sphingolipids, cholesterol and glycerolphospholipids, which do not distribute uniformly through the entire plasma membrane. Consequently, distinct liquid-ordered phase microdomains (membrane rafts), enriched in cholesterol and glycosphingolipids (e.g., GM1), arise in the plasma membrane of any cell (Magee and Parmryd 2003). Apart from lipids, proteins with glycosylphosphatidylinositol (e.g., CD59) or lipid (myristyl, palmitoyl, farnesyl; e.g., Src kinases) anchors can also be found in membrane rafts (Matkó and Szöllösi 2002), while many transmembrane proteins (e.g., CD71) are excluded or weakly associated with these important signaling platforms (Harder et al. 1998; Babiychuk and Draeger 2006). The present study focuses on the connection between membrane rafts and the IL-12R. This receptor is made of two transmembrane proteins, IL-12Rβ1 and IL-12Rβ2; and several lines of evidence indicate that both proteins are differentially segregated between diverse membrane microdomains from human T lymphoblasts. Thus, before IL-12 stimulation, IL-12Rβ1 seems to be more associated to glycerophospholipid-rich areas (nonrafts) of the plasma membrane, while IL-12Rβ2 is principally detected in raft domains. However, our results also suggest that once IL-12 binding takes place, IL-12Rβ2 partially translocates to cholesterol-impoverished zones to generate the high-affinity IL-12R together with IL-12Rβ1 (which is even more enriched in nonrafts upon IL-12 incubation) (Fig. 6).

Very recently, Kondadasula et al. (2008) provided the first data about the membrane distribution of the IL-12R. This report indicated that both FcRIIIa and IL-12R proteins stained diffusely around the cell membrane in resting human NK cells. Besides, signals delivered through either FcR or IL-12R alone did not affect the distribution of IL-12R. However, and according to this author, costimulation

via FcR plus IL-12R made both proteins rapidly colocalize within membrane rafts. The reason for the discrepancy between their observations and our data is unclear, but it could be related to the type of cells used (NK cells versus T lymphoblasts) or the fact that IL-12R colocalization with membrane rafts was mainly observed upon FcR/IL-12R costimulation in NK cells. On the contrary, in our experiments T lymphoblasts were generated first with PHA and then washed and activated with IL-12 alone. Since TCR signaling (e.g., anti-CD3 crosslinking) causes membrane raft coalescence (Janes et al. 1999), we do not know yet how signals through the TCR could influence the cell membrane distribution of IL-12R, especially in combination with IL-12 costimulation. In any case, the comparability of their data with our results is rather limited by the fact that they used a single antibody to detect the whole IL-12R, while in our experiments two antibodies (anti-IL-12Rβ1 and anti-IL-12Rβ2) were necessary to reveal the distribution of both IL-12R chains. Clearly, many questions remain to be answered and future studies, using other techniques—like fluorescence resonance energy transfer (FRET), single-particle tracking (SPT), electron microscopy—as well as other molecules—like JAK2, TYK2 and STAT4—will be important to confirm all the data above, reconcile these apparently conflicting findings and shed more light on the fine regulation of this important receptor.

On the other hand, it can be easily inferred that this initial segregation of both β1 and β2 chains in different membrane microenvironments may serve as a negative mechanism to control untimely signaling through IL-12R, something that other authors have also pointed out for other cytokine receptors (Goebel et al. 2002; Rao et al. 2004). It is also possible that the initial sequestration of IL-12Rβ2 in rafts would protect the protein from internalization, while its movement out of rafts to join the IL-12Rβ1 chain could rather favor its internalization and degradation (Goebel et al. 2002; Rao et al. 2004). However, another plausible explanation could be that membrane rafts play an important role in the process of T-cell activation (Viola 2001; Janes et al. 1999). Thus, IL-12 activates the ERK route (Fig. 5) (Canda-Sanchez et al. 2008; Pignata et al. 1994; Kondadasula et al. 2008) and makes human T lymphoblasts progress through the G_1 phase of the cell cycle via an IL-12Rβ2-dependent mechanism (Presky et al. 1996; Heath et al. 2000; Jones et al. 2003). The presence of IL-12Rβ2 in membrane rafts could be important for the phosphorylation of ERK1/2 and the mitogenic activity of IL-12. In this sense, these cholesterol-enriched microdomains are membrane platforms where important signaling molecules accumulate; some of them, like H-Ras (Niv et al. 2002), play a key role in ERK activation (Anderson 2006). Several findings support this idea: e.g., membrane cholesterol depletion with MβCD triggers the Ras-ERK1/2 pathway (Kabouridis et al. 2000; Wang et al. 2008), while phospho-ERK molecules localize in membrane rafts after a productive TCR signal (Morton et al. 2007). Besides, T-cell proliferation and ERK1/2 phosphorylation induced through the TCR (Xavier et al. 1998) are two events blocked after raft disruption with MβCD. Therefore, the description of an association between IL-12Rβ2 and membrane rafts adds to a growing body of data supporting the connection between these microdomains and the ERK1/2 pathway (Anderson 2006; Mor and Philips 2006). In agreement with this conclusion, Kondadasula et al. (2008) proposed that signals delivered through the Fc and IL-12 receptors act synergistically to activate ERK; besides, these signals were also blocked by raft disruption with MβCD. Moreover, IL-12 is a critical immunoregulatory cytokine that enhances T-cell proliferation (Trinchieri 2003) but only when there are very low levels of TCR occupancy (Canda-Sanchez et al. 2008). Thus, in a similar way as CD28 for naive T cells, IL-12 would provide, through IL-12Rβ2, a quantitative more than a qualitative signal, amplifying early TCR signaling (e.g., the MAPK cascade) initiated in membrane rafts from effector T cells (Viola 2001).

Finally, dipeptidyl peptidase IV (EC 3.4.14.5) is a transmembrane glycoprotein identical to the leukocyte surface antigen CD26 (Ohnuma et al. 2008). CD26 levels are enhanced on the surface of primary T cells upon TCR triggering, and IL-12 costimulation reinforces that expression (Cordero et al. 1997). The low–molecular weight isoform of the membrane tyrosine phosphatase CD45, CD45RO, interacts with CD26; and according to our working model, IL-12 promotes CD26 expression as part of a shuttling mechanism to gradually exclude CD45RO from rafts and control, at the same time, its tyrosine-phosphatase activity (Salgado et al. 2003). The IL-12R-associated TYK2 and JAK2 kinases are two CD45RO substrates (Irie-Sasaki et al. 2001); therefore, CD45RO could help to turn off the IL-12R as substrate and phosphatase get closer. Since after IL-12 ligation both IL-12Rβ2 and IL-12Rβ1 chains tend to be more enriched in nonraft microdomains (just where CD45RO is slowly moving toward because of the IL-12-dependent CD26 upregulation), these new results seem to give more support to our "negative feedback" hypothesis.

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