A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response

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ABSTRACT

The actual level of circulating estrogen (17β-estradiol, E2) has a serious impact on regulation of diverse immune cell functions, where their classical cytoplasmic receptors, ERα and ERβ, act as nuclear transcriptional regulators of multiple target genes. There is growing evidence, however, for rapid, “non-nuclear” regulatory effects of E2 on lymphocytes. Such effects are likely mediated by putative membrane-associated receptor(s) (mER), but the mechanistic details and the involved signaling pathways still remained largely unknown because of their complexity. Here, we show that in lymphocytes, mERs can signalize themselves, and upon ligation, they are able to coordinate translocation of other E2Rs to the PM. Our data firmly imply existence of a complex, dynamic network of at least seven ER forms in murine lymphocytes: cytoplasmic and membrane-linked forms of ERα, ERβ, or GPR30 and a mER that can receive extracellular E2 signals. The latter mERs are likely palmitoylated, as they are enriched in lipid-raft microdomains, and their E2 binding is also cholesterol dependent. The data also support that ligation of mERs can induce rapid regulatory signals to lymphocytes and then internalize and let the E2 liberate in lipid-raft microdomains, and their E2 binding is also cholesterol dependent. The data also support that ligation of mERs can induce rapid regulatory signals to lymphocytes and then internalize and let the E2 liberate in lipid-raft microdomains, and their E2 binding is also cholesterol dependent. This rapid E2R network can be considered as a tool to manage accommodation/fine-tuning of lymphocytes to rapidly changing hormone levels. J. Leukoc. Biol. 96: 857–872; 2014.

Abbreviations: CL=cell lysate,CTX-B= cholera toxin subunit B, DIC=differential interference contrast,E2=17β-estradiol,E2-BSA= O-carboxy-methyl)oxime-BSA, EEA-1= early endosome antigen-1, FRET=fluorescence resonance energy transfer, GPR30/GPER=G protein-coupled estrogen receptor family, HCG=human chorionic gonadotropin, KLH=keyhole limpet, LAMP-1=lysosomal-associated membrane protein 1, MβCD=methyl-β-cyclodextrin, mER=plasma membrane estrogen receptor, PFA=paraformaldehyde, PM=plasma membrane, PMSG=pregnant mare serum gonadotropin, ROI=region of interest, SHP-2=Src homology 2-containing tyrosine phosphatase 2, TD=T cell-dependent, TM=transmembrane, TMB=tetramethylbenzidine

Introduction

17β-Estradiol (E2), besides being one of the steroid sex hormones with an essential role in development of female reproductive organs and secondary sex characteristics, plays important physiological roles in many other areas of the body, such as the nervous, immune, vascular, muscular, skeletal, and endocrine systems [1]. Women typically experience more intense cellular and humoral immune response than men, making them more resistant to certain infections but therefore, also suffer from higher incidence of autoimmune diseases [2, 3]. These two statistically confirmed observations and the numerous in vivo and in vitro effects of sex steroids on the immune system [4] have focused the attention on the role of these hormones, especially of E2, in the direct regulation of immune cell development and function.

Significant impact of E2 on B cell lymphopoiesis in the bone marrow [5, 6] and development of CD4+CD8+ double-positive thymocytes in the thymus [6, 7] have been demonstrated earlier. E2 was also reported to modulate cytokine production and cytokine receptor expression of lymphocytes, as well as activation of effector lymphoid cells [8, 9]. In addition, we have shown recently by in vivo mouse experiments that ovariectomy largely reduced, whereas E2 readministration restored the antigen-specific IgG and IgM responses. E2 augmented only the TD but not the T-independent humoral immune response. Further in vitro data [10–12] convincingly, although somewhat controversially, demonstrated that E2 itself may induce rapid signals in lymphocytes, such as the Ca2+ signal, Erk and AKT phosphorylation, or NF-κB activation. These observations all underline the direct impact of E2 effects on the adaptive immune response.

Most controversies in interpretation of E2 effects on lymphocytes arise from the inaccurate and/or incomplete knowledge of the receptors mediating the diverse effects and the signaling pathways, resulting in the cellular responses. Signifi-
cant fluctuations in the extracellular E2 level may complicate the picture further [13, 14].

The two “classical” cytoplasmic ERs (ERα and ERβ) [15, 16] are polypeptides forming homo- and heterodimers and widely expressed in immune cells, including T and B lymphocytes [11, 12, 17–19]. ERα and ERβ are known to mediate the slow nuclear effects of E2 in lymphoid cells by regulating gene transcription of overlapping and distinct target genes [20]. Some reports suggest even their occasionally antagonistic action [21]. Most E2 effects are thus usually attributed to target-gene regulation through nuclear receptor binding; however, it has been increasingly appreciated that steroids, such as E2, also signals rapidly through “nongenomic” pathways [22–24]. The nature and function of the PM-linked receptor(s) mediating such rapid E2 signals in lymphocytes still remained highly unclear or controversial. Notably, our current view on the potential membrane E2R (mER) is almost entirely based on the use of the E2-BSA-FITC conjugate as a fluorescent and membrane-impermeable ligand, besides using the natural ligand [11, 12, 19].

To explain rapid effects of E2, membrane-associated forms of the ERαR as an original transcript or splice variant [25, 26] were hypothesized, but no solid evidence for this was reported so far in lymphoid cells. The picture is complicated further by a newly described GPR30/GPER as a possible mediator of such responses [27–30]. The functional differences between nuclear ERαR and ERβR and the membrane receptors are yet highly unclear, but some data suggest that immune cells may respond to E2 or related ligands with potentially disparate effects on T versus B cells as a result of a dynamic variance in the ER expression profile [31]. In addition, the level of ERα and ERβ was reported to change in T lymphocytes of healthy menopausal women after estrogen replacement therapy [32], supporting that immune cells can sense the changing E2 milieu.

In spite of this significant knowledge, many basic questions about E2 action on the adaptive immunity still remained unanswered or controversial. Among others, it is still not clear which receptor(s) and downstream signaling molecules mediate the rapid action of E2. Furthermore, it is also unclear how the different classical ERs and the GPR30 distribute within the cells and whether the actual serum E2 level can affect their localization and signaling.

Thus, one of the major aims of this study was to explore the expression and subcellular localization pattern of the various known kinds of E2Rs in mature murine lymphocyte subsets. This question was analyzed with specific mAb using flow cytometry, confocal microscopy, and Western blot techniques. As a protein database search resulted in only three hits for “estrogen-binding motif”-containing proteins (ERα, ERβ, and GPR30), our next aim was to identify whether any of them can serve as a membrane-linked ER, “mER”. To clarify this point, their PM and subcellular localization and their E2 dependence/dynamics was also analyzed in details by confocal microscopy.

Our data demonstrate existence of strongly lipid raft-dependent, membrane-linked E2R activity in T and B lymphocytes. We show that ERα, ERβ, and the GPR30 may all exist and function in a membrane-linked form, but the contribution of GPR30 in lymphocytes is less likely, as it is dominantly compartmented in the endoplasmic reticulum membrane. Changes in the E2 level were shown to induce a rapid redistribution of these subcellular ER pools in lymphocytes. Such an E2-dependent, dynamically redistributing E2R or network may, in part, account for the linkage between the slow nuclear and the rapid membrane-linked E2-signaling pathways in lymphocytes and fine-tune response of lymphocytes to activation signals, as demonstrated by the presented proliferation data.

MATERIALS AND METHODS

Reagents and antibodies

Estrogen dissolved in 96% ethanol (β-E2, 17β-estradiol), BSA, LPS from Escherichia coli, Con A, hemocyanin from Megathura crenulata (KLH), CFA, FITC, ionomycin, chloroquine diphosphate, latrunculin B, cytochalasin D, PMSG, HCG, E2-BSA (~30 mol steroid/mole BSA), and BSA-E2A-FITC was obtained from Sigma-Aldrich (St. Louis, MO, USA), and the BSA-estradiol conjugates were carefully purified before experiments using ultrafiltration [33]. MBCD was from CycloLab (Budapest, Hungary), Alexa555-, Alexa488-conjugated CTX-B, MitoTracker Orange chloromethyllethyltrimethylammonium, LysoTracker Red DND-99, BODIPY-Irredol A558/567, and Fluo-4 were obtained from Molecular Probes-Invitrogen (Carlsbad, CA, USA). DRAQ5 was purchased from BioStatus (Leicestershire, UK). Mouse ERα, ERβ, and GPR30-specific antibodies and rabbit anti-IgG as their isotype control were purchased from AbDcruz Biotechnology (sc-542; Heidelberg, Germany), Sigma-Aldrich (SAB4500814), Acris Antibody (SP4675; Herford, Germany), and Southern Biotechnology (Birmingham, AL, USA), respectively. Anti-mouse Thy-1-Alexa647 conjugate (G7.4, rat IgG2c), anti-mouse CD24 (ATCC TIB-125, M1, 16.9/11.1, rat IgG2b), anti-mouse CD19 unlabeled or Alexa647 conjugate (ATCC HB-305, I3, rat IgG2a), anti-mouse CD8 (53.6, rat IgG2a), anti-mouse CD4 unlabeled or biotin conjugated (ATCC TIB-207, GK1.5, rat IgG2b), anti-rat IgG2b (MAR4/4)-Alexa647 conjugate, anti-rat IgG2a (MAR1/8)-Alexa647 conjugate, and K9.361 mouse FcRIII/II-specific mouse IgG2a mAb [34] were purified from cell culture supernatants and then conjugated with the appropriate stain by the method of the manufacturer. Anti-mouse CD5/PE conjugated (17A2, rat IgG2b) and anti-mouse CD4/PerCP/Cy5.5 conjugated were purchased from BioLegend (London, UK). Streptavidin-PE was from BD Biosciences (La Jolla, CA, USA). Goat anti-rabbit IgG (H+L)-Alexa488 or Alexa555 and goat anti-rabbit IgG (H+L)-HRP, goat anti-mouse IgM (H+L)-HRP, and goat anti-mouse IgG (H+L)-HRP were purchased from Molecular Probes-Invitrogen and from Southern Biotechnology, respectively. Anti-LAMP1 (SAB3500285), anti-NF-κB p65 (sc-372), and anti-EEA1 (sc-6141) antibodies were obtained from Sigma-Aldrich and from Santa Cruz Biotechnology. Anti-IFN-γ (AF1894) was purchased from Cell Signaling Technology (Danvers, MA, USA). Thymidine [6-H]-37MBq was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA).

Mice and cell lines

Adult male and female C57BL/6/J wild-type mice (Charles River, Budapest, Hungary) were maintained under a 12 h light/dark cycle (lights on at 7 AM) at 23°C, and they were supplied with water and food ad libitum. The breeding and the experiments were carried out according to the rules by the European Union-conforming Hungarian Act of Animal Care and Experimentation [1998, XXVIII, Section 243/1998, modified by 40/2013. (II.14)]. Where not stated specifically, the male mice were used in the experiments. Using male mice during the experiments is a common method of estrogen research groups for better reproducibility and to eliminate hormonal fluctuations [35]. Cell lines used in the experiments were murine IP12-7 Th cell hybridoma [36] and the murine B lymphoma cell line A20 (ATCC TIB-208). Cell lines were cultured in RPMI 1640 (Invitrogen) as described earlier [37]. Activated splenocytes were cultured in estrogen-free conditions.
medium (phenol red-free DMEM, supplemented with 5% dextran-coated, charcoal-treated FCS, L-glutamine, Na-pyruvate, penicillin, streptomycin, and 2-β-mercaptoethanol). Before treatments, all cells were washed twice in estrogen-free medium.

**Estrus synchronization for monitoring ER pattern**

Fifty-six-day-old C57BL6/J female mice were i.p. injected with 100 μl, 5 UI PMSG (Sigma-Aldrich) i.p. in the morning, and 100 μl, 5 UI HCG (Sigma-Aldrich), 48 h later in the same time to induce superovulation, after which the mice’s cycles were synchronized [38]. Hormones were diluted in physiological salt solution. After this, three female and as a control, three male mice were processed per day for testing the ER expression pattern on different lymphocyte cell populations by flow cytometry through 5 days. Every day, 1-g fecal samples were collected from the common cage for determination of estrogen and progesterone levels and stored at −20°C in plastic sacs until assayed.

**Hormone extraction and E2 analysis from murine fecal samples**

For assaying progesterone and E2 metabolites, steroid content was extracted from feces. Feces (0.500 g) were mixed with 0.5 ml double-distilled water in thick-walled glass tubes suitable for centrifugation. After adding 4 ml 80% methanol, samples were shaken for 30 min with a multitupe vortex. Thereafter, samples were centrifuged (4°C, 2215 g, 30 min) and refrigerated immediately (~50°C, 30 min). From the upper methanol phase, 1 ml was drawn off for progesterone and estrogen determination by radioimmunoassay [39].

**Isolation of lymphocytes and purification of B and T cells**

All animals were killed by cervical dislocation, and spleens were removed. Cells were collected and washed in phenol red-free glucose-containing PBS. Red blood cells were lysed in ammonium chloride-Tris solution (pH 7.2). T and B cells were isolated by negative selection using MACS magnetic bead-based B cell isolation (130-090-862) or Pan T cell (130-095-130) isolation kits from Miltenyi Biotec GmbH (Bergisch-Gladbach, Germany), according to the manufacturer’s description. The purity of separated T cell (CD4+ and CD8+)* subsets was always checked by flow cytometry (using anti-CD3 labeling) and was found always >97%. B cell subsets [B-2 (>85%) and B-1 (<3%)] subpopulations [40] were checked for purity using anti-CD19 labeling and were also >97%.

**PM preparation and modification**

Spleen cells were incubated in hypo-osmotic buffer (10 mM HEPES, 42 mM KCl, 5 mM MgCl₂) containing a protease inhibitor cocktail (0.2 mM PMSF, 100 mM NaF, 5 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin) for 15 min on ice. The cell suspension was transferred onto a glass vessel homogenizer and pottered for 5 min on ice and then centrifuged for 5 min with 400 g at 4°C. To get rid of cell organelles, the supernatants were ultracentrifuged for 20 min with 120,000 g at 4°C (Optima TL Ultracentrifuge, Beckman Coulter, Brea, CA, USA; TLA 100.1 fixed-angle rotor). The pellet was resuspended in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 100 mM NaF, 250 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 5 μg/ml leupeptin, and 0.2 mM PMSF. After 60 min incubation on ice, cell membrane lysates were centrifuged for 15 min with 15,000 g at 4°C (B Braun Sigma 2K15 centrifuge), and the supernatants were used in subsequent experiments.

Cells were incubated with 10 mM MBCD for 10 min at 37°C to remove ~50% of PM cholesterol [41].

**Immunoblotting**

Postnuclear supernatants of detergent extracts or PM isolates were incubated with reducing SDS-PAGE sample buffer for 5 min at 95°C. The samples were subjected to electrophoresis through 12% SDS-PAGE gel, and the proteins were transferred onto nitrocellulose membranes (BioRad, Hercules, CA, USA), blocked with 5% BSA, then probed with anti-ER antibodies in blocking buffer, and developed by using HRP-conjugated anti-rabbit IgG secondary antibodies. The blots were developed by enhanced chemiluminescence detection (ECL system; Amersham International, Amersham, UK).

**Flow cytometry**

Forward- and side-scatter plots were used in every measurement to gate lymphocytes from spleen cell suspension. Based on labeling with CD11b, CD11c, and GR1 markers, the isolated splenocyte suspensions contained, in addition, 8% CD11b+ nonlymphocyte cells, from which the majority was CD11b+/GR1+ granulocytes (3–4%) and ~1% CD11b+/CD11c+ dendritic cells [42]. Where needed, specific fluorescent antibodies were used to analyze further T (anti-CD3) and B (anti-CD19) cell subsets by gating. To detect mERs, cells were incubated with E2-BSA-FITC or BSA-FITC as a control (1 mg/ml final concentration) for 15 min at 37°C, and then the unbound ligand was removed by washing samples [11]. A competitive binding assay was applied to prove the specificity of E2-BSA-FITC binding. Lymphocytes (10⁶/sample) were preincubated with 100 nM E2 or vehicle control (30 min on ice) and washed and then incubated with 1 mg/ml E2-BSA-FITC or 1 mg/ml BSA-FITC, as described previously. To measure internalization of mER, spleen cells (10⁶/sample) were labeled with 1 mg/ml E2-BSA-FITC (or 1 mg/ml BSA-FITC) for 15–90 min, as described previously. Before the flow cytometry, cells were resuspended (or not) in 5 μg/ml trypan blue to quench the extracellular fluorescence signal of FITC [43]. To investigate mER recycling, spleen cells were pretreated, first with 1 mg/ml E2-BSA (or 1 mg/ml BSA) for 15 min at 37°C and then washed twice to remove the unbound E2 conjugates. Samples were kept in a CO₂ incubator for the given times (between 0 and 75 min) to let the mER internalize and recycle. In some samples during this incubation, 5 μM latrunculin B or 20 μM cytochalasin D was used to inhibit the actin cytoskeletal rearrangement, thus the mER movement, or 0.2 mM chloroquine diphosphate to inhibit lysosomal degradation. Finally, cells were labeled with E2-BSA-FITC (or BSA-FITC) control as described above. To detect E2R, ERα, or GPR30 intracellularly, samples were fixed with 0.5% PFA for 10 min at room temperature, washed, and then permeabilized with 0.1% saponin + 1% BSA in PBS for 10 min at room temperature. Cells were then labeled with antibodies specific to ERα, ERβ, or GPR30 for 30 min at room temperature in 0.1% saponin + 1% BSA in PBS. After washing, the samples were incubated with goat anti-rabbit Alexa488 as a secondary antibody for 30 min at room temperature in the same buffer and then washed and analyzed. Cell-surface labeling was carried out in a similar manner in nonpermeabilized cells, but antibodies were diluted in PBS + 1% FCS.

Flow cytometric measurements were done using BD FACSCalibur or in the case of multicolor labeling (BD Biosciences). Data collections were performed with CellQuest Pro and DataInterpolating Variational Analysis (DIVA) software (BD Biosciences), respectively, and analyzed with FCS Express software (De Novo, Los Angeles, CA, USA).

**Confocal microscopy**

Colocalization of mERs and lipid-raft components was detected in the membrane of IP-127 T, A20 B cell lines, or T and B splenocytes. Cells were incubated with 1 mg/ml E2-BSA-FITC or 1 mg/ml BSA-FITC (as control) for 15 min at 37°C. After washing, cells were stained for GFP-anchored raftic membrane proteins. TH2 (con T cells) and CD24 (con B cells) were labeled with anti-Thy.1-Alexa555 staining as a lipid-raft marker (GM1/GM3 gangliosides), according to the manufacturer’s instructions. The colocalization was quantified by calculating Pearson correlation coefficients [44] from at least 500 ROIs/100–150 cells in each sample. This coefficient is used widely as a measure of a linear dependence between two variables, giving a value between +1 and −1. More than 0.2–0.3 refers to a strong correlation between the two molecules; 0.5 and higher correlation coefficients typically imply constitutively associated or cocompartmented molecules.
To measure the internalization of mER, splenocytes (5×10^5/sample) were labeled with 1 mg/ml E2-BSA-FITC or 1 mg/ml BSA-FITC for the given time between 15 and 60 min at 37°C and then washed and stained with CTX-B-Alexa555. After washing, the cells were fixed with 3% PFA for 20 min on ice. To examine the subcellular localization of the mER-E2-BSA-FITC complex after 30 min internalization, anti-EEA-1 antibody was used as a marker of early endosomes (in fixed permeabilized cells, 2 μg/ml, 30 min, room temperature). After 60 min internalization, LysoTracker 577/590 (live cells, 75 nM, 30 min, 37°C) served as a marker of lysosomes, BODIPY-brefeldin-A558/568 (in fixed and permeabilized cells, 100 nM, 30 min, 37°C) was used as a marker of the endoplasmic reticulum/Golgi complex, whereas mitochondria and cell nuclei were stained with MitoTracker 554/576 (live cells, 50 nM, 30 min, 37°C) and DRAQ5 (live cells, 2.5 μM, 10 min, room temperature), respectively. All of these markers were applied for cell labeling, as described in the manufacturer’s protocol. Cells were also stained for membrane gangliosides with Alexa555- or Alexa647-conjugated CTX-B, according to the manufacturer’s instructions. Before labeling the organelles, cells (10^5/sample) were incubated with 1 mg/ml E2-BSA-FITC for 30 min at 37°C.

To detect the influence of estrogen on the localization of classical ERs, splenocytes (2×10^6/sample) were pretreated with 1 mg/ml E2-BSA or 100 nM E2, respectively, as well as their controls for 60 min at 37°C. After washing, cells were stained with CTX-B-Alexa555 and DRAQ5 and then ER-specific antibodies used as described above.

For single-cell calcium imaging, suspensions of T cells, placed into wells of a Lab-Tek borosilicate-chambered coverglass microplate (Nunc, Rochester, NY, USA), were left to adhere at room temperature for 20 min. Then, cells were incubated with 10 μg/ml Fluo-4, plus 100 μg/ml pluronic acid (F127) at 37°C for 30 min. After washing, cells were treated with 100 nM E2-BSA. As a reference, some samples were also treated with 1 μg/ml ionic-mycin calcium ionophore. Changes in fluorescence intensity of individual cells were monitored (excitation: 488 nm) with a 10× objective in time-resolved acquisition mode (0.44 s/frame). To avoid out-of-focus intensity changes, data were normalized to DIC intensities.

For FRET analysis by donor photobleaching cell were labeled with 1 mg/ml E2-BSA-FITC (20 min on ice), washed, and then labeled with antibody specific to ERα and A555 conjugated anti-rabbit secondary antibody, as described above. The donor was bleached with maximal laser intensity at 488 nm. Changes in fluorescence intensity of individual cells were monitored (emission filter: 513±10 nm) with a 60× objective (8×zoom) in time-resolved acquisition mode. The photobleaching decay curves were fit to exponential function using GraphPad Prism 4.00 (GraphPad Software, La Jolla, CA, USA) to get phosphoestrogen time constants. The FRET efficiencies were calculated using the formula E = 1 – (τn/τa) [45].

Fluorescence microscopy was carried out on a Fluoview 500 laser-scanning confocal microscope (Olympus Europe, Hamburg, Germany), equipped with argon ion laser (488 nm) and two He-Ne lasers (with 543 and 632 nm excitation wavelengths, respectively). Typically, fluorescence and DIC images (512×512 pixels) were acquired using a 60× oil-immersion objective (NA: 1.29). Images were processed by ImageJ software (http://rsweb.nih.gov/j, National Institutes of Health, Bethesda, MD, USA) using the “Image Correlator Plus” analysis plug-in. The line-scan intensity distribution analysis (see Fig. 5) was performed using the Fluoview 5.0 software (Olympus Europe).

Measurement of cell activation and proliferation
Cell proliferation assays were performed in 96-well, round-bottom culture plates (Corning, Corning, NY, USA) in 200 μl final volume. B or T splenocytes (5×10^6) were cultured in estrogen-free medium. Con A (T cell cultures) and LPS (B cell cultures) were used at 1 μg/ml and 5 μg/ml, respectively. E2 (100 nM), 100 nM E2-BSA, or the appropriate controls were applied together with activators, or cells were pretreated in the hormone milieu (1 h, 37°C) and then washed before activation. Cell proliferation was measured by uptake of [3H] thymidine during the last 12 h of the 3 (T cell cultures) and 4 (B cell cultures) days by the Wallac 1490 liquid scintillation counter (Pharmacia, Turku, Finland).

In vitro assay for antibody production upon immunization with FITC-KLH
Fifty-six-day-old C57BL/6/J male mice were immunized with 200 μg FITC-KLH as a TD antigen. FITC-KLH (250 μl; diluted in physiological salt solution), mixed with 250 μl CFA, was administered, divided equally among footpads, the base of the tail, and the intraperitoneum. On the 10th postimmunization day, animals were killed. Lymph nodes and the spleen were removed for in vitro assay. Cells (10^6) were reactivated with 5 μg/ml FITC-KLH or 1 μg/ml FITC-LPS in estrogen-free medium in 96-well, round-bottom culture plates in 200 μl final volume. We used 100 nM E2, 100 nM E2-BSA, or the appropriate controls with activators. On the 5th day, fresh medium containing the same hormone milieu was added to the cell cultures, and supernatants were collected on the 3rd day for detection of antigen-specific antibodies by ELISA.

ELISA of mouse IgM and IgG
To determine the antigen-specific antibodies, the microtiter plates were coated overnight with 5 μg/ml FITC-KLH. Wells were then washed three times with PBS containing 0.05% Tween 20. Supernatant (50 μl) was added to the wells and incubated for 1 h at room temperature. After washing, 1:2000 dilution of HRP-conjugated anti-mouse IgG or IgM (Southern Biotechnology) was added and incubated for 1 h at room temperature. After washing, a TMB substrate was added, and after stopping the reaction, the optical density at 450 nm was measured by ELISA reader (Multiskan EX; Thermo Scientific, Waltham, MA, USA).

Statistical analysis
The “between two-group differences” were analyzed for significance using two-tailed, unpaired t-test with Welch’s correlation assuming equal variances) by GraphPad Prism 4.00 (GraphPad Software). Results were considered significant at *P < 0.05, and **P < 0.01. In the case of in vitro proliferation experiments and antibody ELISA, the statistical evaluation was based on triplicates of samples and expressed as mean ± sd. Three independent measurements were performed. In the case of ER expressional pattern measurements mean ± sem was represented from independent mice samples. The fluorescent micrographs shown in the figures are representative confocal microscopic images selected out of 100 cells/sample.

RESULTS
E2 estrogen has multiple, differentially expressed cytoplasmic and membrane receptors (mERs) in mature murine T and B lymphocytes
The rapid, non-nuclear effects of E2 on lymphocytes are supposed to be mediated by putative mERs, albeit the nature of these receptor(s) still remains highly controversial [10]. Among others, the membrane-bound form of ERα was implicated already [35, 46]. Here, we provide data that the available antibodies specific for the classical cytoplasmic ERα, ERβ, or the GPR30 forms of E2R cannot reach their receptors, unless the cell membrane is permeabilized (Fig. 1A).

On the other hand, mERs on lymphocytes were detected using a fluorescent, membrane-impermeable conjugate, βE2-BSA-FITC, commonly used to define E2 actions mediated by putative mERs [46]. E2-BSA bound specifically to the membrane surface of murine splenic lymphocytes (Fig. 1B). The binding was previously shown to occur in a saturatable fashion [10]. Supporting this, binding of E2-BSA could be blocked by addition of the 100 nM-free E2 (17β-estradiol) ligand (Fig. 1C), reflecting a competition for the same binding site at the surface of lymphocytes.
fluorescence of isotype control antibody, respectively. Also exist in a membrane-linked form in lymphocytes at least at a low E2 level. Likely the major candidate in mediating rapid E2 actions, at B cells are practically negative, suggesting that GPR30 is un-

GPR30 expression is low in resting T lymphocytes, and the results show that immunocytochemically labeled cells did not

mainly in the cytoplasm or nucleus. Our confocal microscopy results show that immunocytochemically labeled cells did not

Additionally, we found that mER is expressed differentially in splenic T and B lymphocytes. B cells have a two- to three-times higher level of such E2-binding sites than the T cells (Fig. 1D). In contrast, expression of classical ERα or ERβ or of the GPR30 did not show a significant difference between T and B cells (Fig. 1E). It is noteworthy that GPR30 expression is low in resting T lymphocytes, and the B cells are practically negative, suggesting that GPR30 is unlikely the major candidate in mediating rapid E2 actions, at least at a low E2 level.

ERα, ERβ, and GPR30 17β-estradiol-binding sites are accessible to their specific antibodies only intracellularly in mature murine splenic lymphocytes, as indicated by the lack of binding of these antibodies to intact cells (left), whereas significant ERα and ERβ and a somewhat lower GPR30 binding were found in permeabilized cells (right). Binding of cell-impermeant E2-BSA-FITC compared with its control, BSA-FITC, demonstrate existence of a cell-surface 17β-estradiol-binding site. Error bars represent SEM calculated from three independent experiments. (D) E2-BSA-FITC binding was more pronounced in B cells than T cells. Error bars represent SEM calculated from three independent experiments; two mice in each. (E) ERα and ERβ are expressed on T and B cells in a comparable extent, whereas GPR30 is detectable only on T cells. T and B cells were gated based on their CD3 and CD19 expression. **p < 0.01. RMF, Mean fluorescence of E2-BSA-FITC/mean fluorescence of BSA-FITC or mean fluorescence of ER-specific antibody/mean fluorescence of isotype control antibody, respectively.

Figure 1. Expression pattern of various ERs in murine lymphocytes. (A) Fluorescence flow cytometric histograms show that ERα, ERβ, and GPR30 are accessible to their specific antibodies only intracellularly in mature murine splenic lymphocytes, as indicated by the lack of binding of these antibodies to intact cells (left), whereas significant ERα and ERβ and a somewhat lower GPR30 binding were found in permeabilized cells (right). (B) Binding of cell-impermeant E2-BSA-FITC compared with its control, BSA-FITC, demonstrate existence of a cell-surface 17β-estradiol-binding site (mER) on intact splenocytes. (C) Competition of E2-BSA-FITC with E2 (100 nM) for mER demonstrates the specificity of an extracellular E2-binding site. Error bars represent SEM calculated from three independent experiments; two mice in each. (E) ERα, ERβ, and GPR30 17β-estradiol receptors may also exist in a membrane-linked form in lymphocytes. The classical ERs (ERα and ERβ) are thought to be localized mostly in the cytoplasm or nucleus. Our confocal microscopy results show that immunocytochemically labeled cells did not

ERα, ERβ, and GPR30 17β-estradiol-binding sites are accessible to their specific antibodies only intracellularly in mature murine splenic lymphocytes, as indicated by the lack of binding of these antibodies to intact cells (left), whereas significant ERα and ERβ and a somewhat lower GPR30 binding were found in permeabilized cells (right). Binding of cell-impermeant E2-BSA-FITC compared with its control, BSA-FITC, demonstrate existence of a cell-surface 17β-estradiol-binding site. Error bars represent SEM calculated from three independent experiments. (D) E2-BSA-FITC binding was more pronounced in B cells than T cells. Error bars represent SEM calculated from three independent experiments; two mice in each. (E) ERα and ERβ are expressed on T and B cells in a comparable extent, whereas GPR30 is detectable only on T cells. T and B cells were gated based on their CD3 and CD19 expression. **p < 0.01. RMF, Mean fluorescence of E2-BSA-FITC/mean fluorescence of BSA-FITC or mean fluorescence of ER-specific antibody/mean fluorescence of isotype control antibody, respectively.

Additionally, we found that mER is expressed differen-
tially in splenic T and B lymphocytes. B cells have a two- to three-times higher level of such E2-binding sites than the T cells (Fig. 1D). In contrast, expression of classical ERα or ERβ or of the GPR30 did not show a significant difference between T and B cells (Fig. 1E). It is noteworthy that GPR30 expression is low in resting T lymphocytes, and the B cells are practically negative, suggesting that GPR30 is unlikely the major candidate in mediating rapid E2 actions, at least at a low E2 level.

ERα, ERβ, and GPR30 17β-estradiol-binding sites are accessible to their specific antibodies only intracellularly in mature murine splenic lymphocytes, as indicated by the lack of binding of these antibodies to intact cells (left), whereas significant ERα and ERβ and a somewhat lower GPR30 binding were found in permeabilized cells (right). Binding of cell-impermeant E2-BSA-FITC compared with its control, BSA-FITC, demonstrate existence of a cell-surface 17β-estradiol-binding site. Error bars represent SEM calculated from three independent experiments. (D) E2-BSA-FITC binding was more pronounced in B cells than T cells. Error bars represent SEM calculated from three independent experiments; two mice in each. (E) ERα and ERβ are expressed on T and B cells in a comparable extent, whereas GPR30 is detectable only on T cells. T and B cells were gated based on their CD3 and CD19 expression. **p < 0.01. RMF, Mean fluorescence of E2-BSA-FITC/mean fluorescence of BSA-FITC or mean fluorescence of ER-specific antibody/mean fluorescence of isotype control antibody, respectively.

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Figure 2. ERα, ERβ, and GPR30 also exist in a PM-associated form. (A) In intact cells, these receptors are not accessible for antibody staining, as shown by the representative confocal image of anti-ERα-labeled splenocytes (red, Alexa555-CTX-B; blue, DRAQ5 nuclear staining). Intracellular staining of cells after a mild, 0.05% Brij98 treatment (B) or fixation and permeabilization (C) revealed a few membrane-localized clusters of ERs (white arrows, membrane-localized ERα; green, anti-ERα; blue, nuclear counterstain DRAQ5; red, Alexa555-CTX-B); meanwhile, the majority of classical ERs is localized in the cytoplasm or nuclei. Original scale bars, 5 µm. (D) Existence of ERα, ERβ, and GPR30 in murine splenocyte PM isolates is confirmed by Western blot. All three E2Rs were found in the membrane isolates (PM) and in the whole CL. The purity of our membrane isolate was validated by developing the blots for the whole CL. The purity of our membrane isolates (PM) and in the whole CL. The purity of our membrane isolate was validated by developing the blots for the whole CL. (E) TMpred hydrophobicity plot shows hydrophobicity scores and presumptive membrane-spanning regions of the ERα, ERβ, and GPR30 protein-coding sequences. The 0 value represents the PM bilayer.

The lymphocytes’ mERs are raftophilic, and the E2 binding is cholesterol sensitive

We have shown recently that E2 has a direct impact on B and T cells by inducing rapid, non-nuclear signaling effects, within several minutes of stimulation, presumably via mERs [10]. To understand this mechanism of action, among others, analyzing its binding features, E2 to the cell surface seems mandatory. As the membrane cholesterol was shown to limit “passive” estradiol uptake by liposomes or erythrocytes [47], next, we investigated how the outside E2-binding sites relate to the cholesterol-rich PM raft microdomains. The cell surface-bound fluorescent E2-BSA highly colocalized on primary T and B lymphocytes or on cell lines with markers of lipid rafts, such as GM1 gangliosides (marked by CTX-B), or T cell expressed Thy.1, and B cell expressed CD24 GPI-anchored membrane proteins (Fig. 3A–C). The calculated colocalization coefficients derived from confocal microscopic images indicate a substantial lipid-raft compartmentation of cell surface-bound E2-BSA. Moreover, depletion of membrane cholesterol by 10 mM MBCD largely suppressed binding of E2-BSA to the cells (by ~50%; Fig. 3D).

These data suggest that lipid-raft microdomains are essential targets for estrogen binding to the lymphocyte cell surface. To test whether the mER outside and the ERα on the cytoplasmic side of the membrane are located in the same microdomain, FRET measurement was done between cell surface-bound FITC-E2-BSA as donor and Alexa555-anti-ERα as acceptor on A20 B cells. The FRET efficiency obtained from donor photobleaching time constants (Fig. 3E; see Materials and Methods) was 8.7 ± 2.4%. This relatively low FRET efficiency can be interpreted as only a fraction of the labeled mER, and ERαRs are in close proximity, or alternatively, there is a larger (but still within 10 nm) distance between all of the receptor pairs. Both possible scenarios are consistent with a picture that the two receptors are localized in a common membrane microdomain on the extracellular and cytoplasmic face, respectively.

Cell-surface mERs display dynamic, ligand-dependent internalization and recycling

Earlier, we demonstrated the receptor-mediated fashion of E2-BSA-FITC binding to T and B lymphocytes. The binding at saturating conditions was relatively rapid: completed in ~15 min [10]. Further analyzing this process, here, we
found that the membrane-impermeable E2-BSA ligand, after binding to mERs, undergoes rapid internalization. Within ~15 min, the majority of the cell-bound E2-BSA-FITC became cytoplasmic, as shown by confocal microscopic images taken between 0 and 15 min after binding (Fig. 4A). The internalized mER did not show any significant colocalization with the classical ERs (data not shown); therefore, the direct ligand transfer between the various forms of ERs is highly unlikely.

Further microscopic analysis addressed the question of where the mER ligands traffic after internalization. The internalized mER did not colocalize with BrefeldinA, a marker of endoplasmic reticulum. No colocalization with mitochondrial (MitoTracker) or nuclear (DRAQ5) markers was observed either. The colocalization pattern of the internalized E2-BSA-FITC (Fig. 4B and C) reflects its trafficking to an early endosome (see high colocalization with EEA-1) and partly into the lysosome (see colocalization with LysoTracker). These data suggest that at least a part of the internalized E2-BSA ligand is targeted to lysosomes, where E2 likely could be released. Relocation of mER from endosomes to PM cannot be excluded either.

In splenic lymphocytes, within 30 min after E2-BSA-FITC addition, the fluorescence signal gradually increased in splenocytes, reaching a plateau (binding phase). Trypan blue quenching of the extracellular signal resulted in much lower fluorescence, suggesting that it is mostly originated from the inside of cells (Fig. 4D). After 30 min, however, the E2-BSA-FITC signal further began to rise in the unquenched and trypan blue-quenched samples, respectively. This might reflect existence of a yet-undescribed intracellular pool of mER. As expected, BSA-FITC control, under the same conditions,
Figure 4. mERs display rapid, dynamic subcellular translocations. (A) E2-BSA-FITC is internalized within 15 min after binding, as demonstrated by representative confocal images of spleen lymphocytes labeled with 1 mg/ml E2-BSA-FITC for the given times at 37°C (red, Alexa555-CTX-B; green, E2-BSA-FITC). Line-scan intensity distribution (lower) clearly shows segregation of the E2-BSA-FITC signal from the membrane label CTX-B (red). (B) Representative images show E2-BSA-FITC (green) subcellular localization after binding to splenocytes. The cells were counterstained with EEA-1 after 30 min incubation with E2-BSA-FITC. CTX-B (membrane), DRAQ5 (nuclei), LysoTracker (lysosomes), MitoTracker (mitochondria), and BODIPY-Brefeldin A558 (ER/Golgi) were used after 60 min E2-BSA-FITC incubation. Original scale bars, 5 μm. (C) Images were quantitatively analyzed for colocalization as well. Pearson coefficients of E2-BSA-FITC colocalization with these markers were calculated from two to three independent experiments, from 120 to 150 cells, and displayed as mean ± sd. (D) To monitor internalization of mER, E2-BSA-FITC was added (at zero time) to splenocytes, with or without 5 μg/ml trypan blue, a quencher of extracellular fluorescence signal, and the fluorescence was monitored at the indicated times using flow cytometry. BSA-FITC was used as control in this kinetic assay. Mean fluorescence intensity (MFI) is displayed as a mean of three independent experiments ± sd. (E) For direct monitoring of recycling of mER, splenocytes were pretreated, first with 1 mg/ml E2-BSA (15 min, 37°C) and then, washed to remove unbound E2 conjugates. Samples were then held in a CO2 incubator for the indicated time to let the mERs internalize and recycle. mER level was detected from time to time by addition of E2-BSA-FITC (1 mg/ml) or BSA-FITC (as control) to the cells (15 min, 37°C) before flow cytometry. The mER level in untreated, freshly isolated splenocytes was taken as 100%. (F) mER recycling is blocked significantly by inhibition of actin polymerization (20 μM cytochalasin D or 5 μM latrunculin B, respectively) in a same recycling experiment as shown in E. In contrast, chloroquine diphosphate (0.2 mM) as a lysosomotropic agent increased the cell-surface level of mER by inhibiting its lysosomal degradation. The mER level in untreated, freshly isolated splenocytes was taken as 100%.
showed only a very weak background signal without any time dependence (Fig. 4D).

Next, to investigate mER recycling more directly in splenic lymphocytes, cells were pretreated, first with nonfluorescent E2-BSA ligand (15 min, 37°C) to induce receptor internalization, and then, unbound E2 conjugates were removed by washing. Cells were then kept further in the CO₂ incubator for the indicated times to let the mERs be internalized and recycle or signaling and liberation to the intracellular pool. The “0-min level” describes the mER level in freshly isolated, untreated splenocytes (before induction internalization by E2-BSA). Then aliquots were taken from time to time, and the actual level of mER was monitored by staining with E2-BSA-FITC and flow cytometry. During the first 30 min, the E2-BSA-FITC signal decreased gradually by ~20%, consistent with internalization of mERs. However, at longer times after binding (60 and 90 min), the level of cell surface-bound E2-BSA-FITC signals was restored and even exceeded the baseline (by ~15%; Fig. 4E), suggesting a massive recycling of mERs, possibly from an existing intracellular pool.

This proposed mechanism of dynamic recycling of mER was confirmed further with selective blockers of lysosomal degradation (chloroquine diphosphate) or actin-dependent processes (inhibitors of actin polymerization: cytochalasin D and latrunculin B). E2 stimulation (for 60 min), itself or applied together with chloroquine diphosphate, significantly (>30%) increased the mER level. It is noteworthy that chloroquine diphosphate alone also increased it (~20%). In contrast, both blockers of actin polymerization largely decreased the mER level, even in the presence of E2 stimulation (Fig. 4F), suggesting that internalization and recycling are strongly actin-dependent processes in lymphocytes.

Expression and subcellular distribution of various ERs in lymphocytes dynamically changes with the fluctuating E2 level

Next, we asked the question of whether the E2, by binding to mERs, can influence the subcellular localization of ERα, ERβ, and GPR30 ERs. Therefore, we tested the effect of E2 (100 nM) and E2-BSA (1 mg/ml) ligands on PM association (using CTX-B as a membrane raft marker) of classical ER expressed by splenocytes by confocal microscopy. It should be emphasized that the E2-BSA ligand is used to define estrogen actions, mediated exclusively by mERs, whereas E2 may act via membrane and intracellular ERs. ERα highly colocalized with PM GM1 lipid-raft markers, independently of changes in the extracellular E2 level (Fig. 5A and B). In contrast, membrane raft localization of ERβ was slightly decreased, whereas that of GPR30 was increased significantly by E2 administration (Fig. 5C–F). It is noteworthy that whereas GPR30 showed an E2-dependent translocation to the PM, its majorly is localized in the endoplasmic reticulum membrane, and its PM association in lymphocytes is very low without E2 administration (Fig. 5G). Contrarily, E2-BSA, as a ligand of mERs, significantly increased the membrane localization of ERβ and GPR30 (Fig. 5D and F). This is demonstrated further by a confocal line-scan analysis of intensity distribution of the fluorescent labels (Fig. 5A, C, and E), directly showing redistribution of classical ERs to the juxtamembrane regions.

The analysis of binding the E2-BSA-FITC ligand in the splenocytes of adult male and female mice, functional mERs [10], was found in both genders. Females, however, showed a much higher level of bound E2-BSA with extremely high deviation than the males (lower level of bound E2-BSA, no deviation; Fig. 6A). As mERs displayed dynamic, ligand-dependent internalization and recycling (Fig. 4D–F), we assume that the mER expression level may be sensitive to the menstrual cycle. Therefore, next, we asked whether whether the classical ERs, ERα/β and GPR30, also exhibit this kind of dynamic control. To investigate their expression pattern, we synchronized the estrous cycle of mice with PMSG and HCG injection. Gonadal steroid metabolite quantification in feces has already been validated in the rat and mouse by several investigators [48]. As the murine estrous cycle generally lasts 4–5 days, and the fecal E2 content is only slightly delayed but follows these hormonal changes [49], a 1-g fecal sample was collected from the common cage for determining estrogen and progesterone levels during 5 days after simultaneous ovulation of the mice (Fig. 6B). Simultaneously, the ER pattern was monitored temporally in T and B lymphocytes by flow cytometry (Fig. 6C). The fecal samples around the 4th day showed an E2 peak (a new estrus; Fig. 6B), which indicates that the hormonal treatment/synchronization was successful [50]. The temporal expression patterns of ERα and ERβ but not of GPR30 correlated well with circulating the E2 level during the 5 days of the cycle. T and B cells have the highest receptor expression levels between the 2nd and 4th days (Fig. 6C). The rising E2 level seemed to promote the mER expression on T and B cells.

mERs and cytoplasmic ERs mediate differential effects on lymphocytes’ proliferation and antibody response

The presented, representative data on the calcium response of lymphocytes, consistent with earlier data on other signals [10, 12], confirmed that the E2-BSA ligand can induce rapid signaling (Fig. 7A). As we still do not know much about the relationship between the slow, nuclear and the rapid, membrane-linked E2-signaling pathways, we investigated here how E2 and E2-BSA influence the in vitro activation of splenocytes. Our results show that estrogen alone did not induce cell proliferation (Fig. 7B), but it had an impact on T and B cell proliferation induced by polyclonal mitogen activators Con A and LPS, respectively. Interestingly, E2 decreased (Fig. 7B), whereas E2-BSA enhanced, activation-induced T and B cell proliferation (Fig. 7C).

Our previous results [10] provided the first evidence that estrogen can enhance the TD immune response. In female mice, E2 readministration after ovariectomy increased the number of hapten-specific IgM- and IgG-producing cells in response to FITC-KLH as a TD antigen. Therefore, next, we investigated whether E2 and E2-BSA ligands have an influence on antibody production in vitro. Isolated spleen and lymph node cells of FITC-KLH-immunized male mice were cultured in
the presence of FITC-KLH and 100 nM E2 or E2-BSA (see Materials and Methods). Supernatants from cultures without FITC-KLH or with FITC-LPS as controls did not show any difference in their antigen-specific IgM and IgG levels (Fig. 7D), whereas in cells cultured with FITC-KLH, E2-BSA, as a mER ligand, enhanced the antigen-specific IgG production, and E2 had no effect on it (Fig. 7D). These data suggest that the positive regulatory effects of E2 were presumably mediated by mERs.

**DISCUSSION**

Generally, steroid hormone receptors function as transcriptional factors by inducing nuclear pathways, resulting, finally, in regulation of the target gene(s) [51–53]. There is growing evidence, however, that steroid sex hormones, including 17β-estradiol, may induce rapid, non-nuclear pathways, as well. Whereas the function of classical ERs (ERα, ERβ) or of non-classical GPR30/GPER is relatively well characterized in many cell types of the endocrine and nervous system [31, 52, 54], in lymphocytes, it is still very poorly understood and controversial [10–12, 35], although it is widely accepted that estrogens may have a serious impact on immunoregulation and also on autoimmune diseases [2, 3]. The complexity of E2 effects may arise partly from the existence of the two classical receptors [55, 56] and their several splice variants, as well as their different regulation [57–59]. Concerning the nature of mERs, post-transla-
tionally modified forms of ERα and ERβ were proposed as possible candidates [24, 60–62].

In accordance with findings of other groups on other cell types, our data also clearly show that the specific antibodies available against the known ERs do not access the respective receptors in intact mature murine T or B lymphocytes [11, 35]. As the membrane-impermeant E2-BSA could elicit rapid signaling on the lymphocytes [10, 12, 19], we can assume existence of functional mER. Pierdominici et al. [35] have shown, for example, expression of mERα46 as a shorter ERα (46 kDa) isoform in human PBLs. We could also detect ERα (but only the full-length, ~66-kDa variant), ERβ, and GPR30 in the isolated PM fraction of mouse splenocytes. These data firmly suggest that any of the three intracellular ERs may also function in a PM-linked form in human and murine lymphocytes, as described already in other cell types [62–66]. Based on hydrophathy analysis, the full-length ERα (66 kDa) and ERβ (43 kDa) are unlikely to function as TMRs binding E2 extracellularly. In contrast, the weakly expressed GPR30, typical seven-span TMR, may be transferred to the PM (by vesicular transport) and thus, cannot be excluded as a candidate of the extracellular E2-binding site. The lack of its extracellular immune-labeling might be a result of the inaccessibility of its epitope to the available GPR30-specific antibody. Of course, contribution of another yet-undefined cell-surface protein to E2 binding is also conceivable.

The presence of ERα and ERβ at the PM raises several new functional possibilities: 1) This could promote ligand binding of the intracellular receptors by recruiting them into close proximity to the site of E2 entrance [62]. 2) As ERs do not have intrinsic kinase activity, the linkage with another adaptor and signaling molecule is essential (e.g., G-proteins, heat shock protein 90, caveolin-1, matrix metalloproteinases, tyrosine kinases Shc and c-Src, proline-glutamine acid- and leucine-rich protein-1), in which they may form an estrogen-specific signalosome and mediate rapid signals [67, 68]. 3) This signalosome might have a coupling to the extracellular E2 binding site (mER), as supported by our FRET data.

Our data on lymphocytes, expressing only caveolin-1-negative lipid rafts in their PM, show that the mER-bound E2-BSA-FITC ligand and the supposedly reversibly palmitoylated form of classical ERs [24, 60–62, 64, 65] are both enriched in lipid-raft membrane microdomains. In addition, binding of the membrane-impermeant E2-BSA ligand to T or B lymphocytes, simulating physiological E2 binding to mER, was substantially dependent on lipid-raft integrity. In accordance with our results, ERα and ERβ were reported to be associated with lipid-raft and/or caveolar fractions, and similar lipid-raft control on
signaling function [66, 69–71] and even on the degradation of ERα has been proposed recently in other cell types [63, 65, 66]. This lipid-raft dependence might also be of further interest, in light of studies showing that in lymphocytes of patients with autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis), the level of PM raft lipids (cholesterol and sphingolipids) is elevated significantly [72].

Perhaps the most intriguing finding of the present work is the demonstration of the E2-dependent, dynamic subcellular translocation of the various ER forms. Similar selective, estradiol-depen-
dent ERβ but not ERα translocation was observed in hippocampal cell lines and primary neurons [73], proposing different scaffolding proteins for the two ERs. Such a possibility cannot be excluded in lymphocytes either.

Effect of the circulating hormone level on immune cells is still far from being understood. It was shown that the menstrual and pregnancy estrogen levels positively correlate with the CD4+ forhead box P3+ regulatory T cell level [13, 14] and B cell survival [13, 74]. Here, we found that mERs, internalized rapidly after estradiol binding, are quickly restored, even at a higher level at cell surface, referring to a dynamic equilibrium with a yet-undefined intracellular mER pool. As the mER-ligand complex is targeted to endosomes/lysosomes, we can assume that this process is unlikely accompanied with ligand transfer to the intracellular ERs but rather, with degradation. It is noteworthy that the mER level was found much higher and more diverse in female mice than in males. This may reflect that the mER level at the cell surface correlates somehow with the circulating hormone level. This supposition was confirmed by in vitro data, showing a positive-feedback mechanism, where E2 signals could rapidly increase the mER level. Our cycle-synchronized mouse model also shows a strong correlation between the daily changes of E2 (but not of progesterone) levels and the expression of ERα and ERβ in T and B lymphocytes. These result together suggest that lymphocytes could dynamically respond to the hormonal fluctuations, presumably as a result of rapid signaling through mERs. Comparative expression studies with regard to the classical receptors were done in human samples [5, 35, 75] but not in mice, especially not with respect to the mER. Although the specific role of each classical ER and mER still remained poorly described in lymphoid cells, their temporal expression pattern is likely an important factor in determining their immunoregulatory role.

To understand better the E2-mediated cellular regulation, attention should be paid to the mER function as well. 17β-Estradiol and E2-BSA were both shown to alter the activity of certain key signaling molecules, such as ERK1/2, Akt, and NFκB in cell lines and primary lymphocytes alike [2, 10]. However, the exact regulatory role of the distinct ER types in cell activation still remained largely unexplored. Consistent with data published on other cell types [35, 76], here, we show that E2-BSA, through mER, can increase cell proliferation but not E2. These results suggest that mER primary mediates positive

Figure 8. Schematic model for regulation and fine-tuning lymphocyte function by estrogens. E2 predominantly enters the cells through the lipid-raft (cholesterol-, sphingomyelin-, and ganglioside-rich) microdomains of the PM, as the mER is also enriched here; moreover, its ligand binding is cholesterol dependent. In the nuclear pathway, ERα and ERβ function as transcriptional factors and induce protein synthesis. Besides, this mER provides the opportunity for dynamic cellular response to the hormonal fluctuations by inducing rapid signal transduction pathways. As a result of the multiple forms and various compartments of ERs, resulting in a complex ER network, and a possible cooperation of the two pathways, a fine regulation of lymphocyte function could be achieved. It is noteworthy that the mER-mediated signals can also promote translocation of the classical E2Rs to the PM, a mechanism that may make uptake of the membrane localized E2 by the receptor easier. On the other hand mER directs the bound E2 from the surface toward lysosomal degradation. The E2-dependent ER translocations may also act as a positive feedback, hence amplifying the hormonal effects. PC, phosphatidylcholine; PE, phosphatidyethanolamine; PS, phosphatidylserine.
activation signals to the cells, but together with the classical receptors, the effect is more balanced. Our earlier estrogen-replacement experiments with ovariectomized mice also show that E2 could positively influence the TD immune responses [10]. Our in vitro examination on cells from TD antigen-immunized mice also showed an elevated antigen-specific IgG level upon addition of E2-BSA but not of E2. As shown here, the mER by itself conveys positive signals to the cells, whereas if all receptors are concerned, the different effects are summed and may change the final outcome of the cellular responses. We note that besides lymphocyte proliferation or antibody production, E2 may also affect function of other important innate cellular components of the immune system, such as macrophages [77] or dendritic cells [78]; thus, exploring the network of ERs on these cells remained a challenge in the near future.

In conclusion, as shown in a summarizing model of dynamic ER networks in lymphocytes (Fig. 8), we provide here evidence for a complex, dynamic E2 estradiol receptor network consisting of seven different cytoplasmic or membrane-bound (modified) forms of three receptor gene families: ERα, ERβ and GPER/GPR30. These receptors can translocate to the nucleus and act as transcription regulators or translocate to a PM compartment, preferentially to lipid rafts in lymphocytes, and mediate rapid E2 signals. Our results suggest existence of a spatiotemporal coordination of ERs and GPER/GPR30 by E2 level and mER activity. This means that the actual expression level and localization of the various ER forms may define a concentration-dependent regulatory mechanism [79, 80]. As the mER is the first encounter point for E2 on cells, we can assume that it will be saturated first, when the amount of circulating E2 level is low; therefore, rapid, non-nuclear signals will dominate at such conditions. In contrast, at high circulating E2 levels, the nuclear signals will dominate. This way, depending on the circumstances, the same ligand presumably could induce different signals—either positive or negative—to the cells. The nuclear pathway seems to regulate complex, steady cell functions (cell activation/proliferation, antibody or cytokine production), whereas the rapid signals are mediated presumably by mERs [10, 67, 68, 79, 80] and likely provide the cells with a chance to adapt to hormone-level fluctuations, i.e., promote a fine regulation. The exploration of further, missing functional details and signaling pathways of the complex E2R network proposed here may help us to understand more deeply the mechanisms making estrogen a risk factor in some autoimmune diseases and can also help to design safe strategies for using oral contraceptives and hormonal-replacement therapies without malfunctions in the immune system.

**AUTHORSHIP**
A.E.S. designed and performed research and drafted the manuscript. É.K., K.S., and E.K. performed some experiments. E.A.T. performed flow cytometric cell separations. M.K. helped in quantitate hormone levels from sera and feces samples. J.M. and G.L. designed experiments and drafted the manuscript.

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A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response

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