Cell. Mol. Life Sci. (2010) 67:1661–1674
DOI 10.1007/s00018-010-0270-5

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Estrogen augments the T cell-dependent but not the T-independent immune response

Mónika Ádori · Endre Kiss · Zsuzsanna Barad · Klaudia Barabás · Edda Kiszely · Andrea Schneider · Erna Sziksz · István M. Ábrahám · János Matkó · Gabriella Sármay

Received: 25 July 2009 / Revised: 6 January 2010 / Accepted: 13 January 2010 / Published online: 7 February 2010

Abstract Estrogen plays a critical regulatory role in the development and maintenance of immunity. Its role in the regulation of antibody synthesis in vivo is still not completely clear. Here, we have compared the effect of estrogen on T cell-dependent (TD) and T cell-independent type 2 (TI-2) antibody responses. The results provide the first evidence that estrogen enhances the TD but not the TI-2 response. Ovariectomy significantly decreased, while estrogen re-administration increased the number of hapten-specific IgM- and IgG-producing cells in response to TD antigen. In vitro experiments also show that estrogen may have a direct impact on B and T cells by inducing rapid signaling events, such as Erk and AKT phosphorylation, cell-specific Ca^{2+} signal, and NFκB activation. These non-transcriptional effects are mediated by classical estrogen receptors and partly by an as yet unidentified plasma membrane estrogen receptor. Such receptor- mediated rapid signals may modulate the in vivo T cell-dependent immune response.

Keywords B cells · Antibody production · TD and TI-2 antigen · Estrogen receptors · Cell activation · Signal transduction

Abbreviations

AEC	3-Amino-9-ethylcarbazole
$[Ca^{2+}]_i$	Intracellular free Ca^{2+}
CLSM	Confocal laser scanning microscopy
DCC-FCS	Dextran-coated charcoal-treated foetal calf serum
αE2	17α-Estradiol
βE2	17β-Estradiol
eNOS	Endothelial isoform of NO synthase
ERα	Nuclear estrogen receptor α
ERβ	Nuclear estrogen receptor β
HRT	Hormone replacement therapy
KLH	Keyhole limpet hemocyanin
OVX	Ovariectomised
SHAM	Sham-operated
SLE	Systhemic lupus erythematosus
TD	T cell dependent
TI-2	T cell independent type 2

Introduction

It is well known that the gonadal sex hormone, estrogen, influences the differentiation, maturation, and emigration of lymphocytes, which are all essential for an adequate immune response [1]. Moreover, preferential susceptibility

M. Ádori and E. Kiss contributed equally.

M. Ádori · Z. Barad · E. Kiszely · A. Schneider · E. Sziksz · J. Matkó · G. Sármay (✉)
Department of Immunology, Eötvös Loránd University, Budapest, Hungary
e-mail: sarmayg@elte.hu; sar7197@mail.iif.hu

Z. Barad · I. M. Ábrahám (✉)
Department of Physiology, Centre for Neuroendocrinology, University of Otago, Dunedin, New Zealand
e-mail: istvan.abraham@otago.ac.nz

K. Barabás
Proteomics Laboratory, Institute of Biology, Eötvös Loránd University, Budapest, Hungary

E. Kiss · J. Matkó · G. Sármay
Immunology Research Group of the Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, Hungary

of females to autoimmune diseases in both humans and animals [2] also suggests a critical role of estrogen in regulation of immune responses. Animal and in vitro studies shed light on the complexity of estrogen effect on B and T cell development and survival. Estrogen suppresses both B cell and T cell development, induces thymic atrophy, and reduces all developing T cell populations, while it potentiates B cell survival in response to antigen [3–5]. In clinics, hormone replacement therapy (HRT) with conjugated estrogen was shown to decrease T cell numbers, while B cell numbers were unaffected or upregulated, suggesting a stimulating effect of estrogen on B cells [6]. Indeed, HRT increases the risk of developing systemic lupus erythematosus (SLE) and the occurrence of flares in diagnosed SLE, the B cell-dependent autoimmune disease [7]. These findings were corroborated by studies in mice where autoimmune models have clearly shown that estrogen treatment accelerates the onset of disease and increases mortality in autoimmune-prone NZB/NZW F1 and MRL/lpr mouse strains [8, 9].

Previous data suggest that estrogen enhances the accumulation of Th1 CD4+ T cells in response to antigen [10]. Furthermore, it was reported that estrogen inhibits Th1 proinflammatory cytokine production (IL-12, IFNγ, and TNFα), while it stimulates Th2 anti-inflammatory cytokine production (IL-4, IL-10, and TGFβ) [11]. Thus estrogen may suppress Th1-dependent while potentiating Th2-dependent diseases. However, little if any attention has been given to distinguishing the role of estrogen in T cell-dependent and T cell-independent humoral immune responses.

Estrogen receptors (ERs) were shown to be present in cells of the innate and adaptive immune system [12, 13]. ERα is expressed in CD4+ and CD8+ T cells, B cells, NK cells, and macrophages [5, 10, 14–19]. The presence of ERβ has been reported in DN T cells in the lpr/lpr mouse, T cells, B cells, monocytes, and macrophages [5, 15, 19]. Both ERα and ERβ are required for complete downregulation of B-lymphopoiesis, while only ERα is needed to upregulate immunoglobulin production in both bone marrow and spleen [20].

Generally, estrogen can regulate the function of B and T cells in two different ways: either via direct DNA-binding and transcriptional activity of ligated ERs (classical effect) or via activation of intracellular Ca^{2+} rise and signaling pathways [12, 21]. We define the latter action of estrogen as non-classical action, because this downstream process indirectly acts on gene transcription via signaling pathways and it is exerted rapidly. Although estrogen was shown to alter the activity of signaling molecules such as ERK1/2, Akt, and NFκB in many cell lines [22–24], the estrogen-induced non-classical action in primary B and T cells still remained largely unexplored.

In the present study, we examined the effect of estrogen on T cell-dependent and T cell-independent B cell responses in vivo using selective immunization protocols, and found that only the TD response is upregulated by estrogen. To understand the effects of estrogen on lymphocytes, we focused on the estrogen-induced non-classical rapid effects. Investigating Akt, Erk1/2, NFκB activation, cytokine (IFNγ) gene activation, and intracellular Ca^{2+} levels as indicators, we characterized the estrogen-induced signaling in vitro in primary mouse splenic B and T lymphocytes, as well as in B and T cell lines. We found that 17-β-estradiol (βE2) can initiate rapid signals in lymphocytes that may modulate the antigen-induced activation. The rapid signals of estrogen may be differentially mediated by putative membrane-associated receptors (mER) on B and T lymphocytes. The majority of mERs was found to be not identical to the classical ERs.

Materials and methods

Reagents and antibodies

Estrogen, 17β-estradiol (βE2; Sigma), dissolved in absolute ethanol was diluted in culture medium (ethanol concentration, $\leq$0.01%). Phenol red-free RPMI-1640-modified medium (supplemented with L-Glutamine, Na-pyruvate, penicillin, streptomycin, dextran-coated charcoal-treated foetal calf serum (DCC-FCS), and mercaptoethanol) and Keyhole limpet haemocyanine (KLH) were from Sigma. FITC-conjugated dextran was purchased from Fluka BioChemika (USA). The goat anti-mouse IgM $(Fab')_2$ fragment was kindly provided by Dr. J. Haimovich (Tel Aviv University, Tel Aviv, Israel). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM and IgG were purchased from Southern Biotechnology Associates (UK). Affinity-purified rabbit polyclonal IgG antibody against pSer473 of Akt was obtained from Santa Cruz Biotechnology (CA, USA). Phospho-ERK1/2 rabbit polyclonal antibody specific for pThr202/pTyr204 and pThr183/pThr185 residues of ERK1 and ERK2, respectively, and anti-SHP-2 was purchased from Cell Signaling Technology (MA, USA). Goat anti-rabbit IgG HRP-conjugated antibody was purchased from DakoCytomation (Denmark). Affinity-purified anti-mouse CD3 (145-2c11, hamster IgG), was a generous gift from Gloria Laszlo (Eötvös Loránd University, Budapest, Hungary). Mouse ERα-specific antibody was from Santa Cruz (sc-542) and ERβ-specific antibodies were purchased from Santa Cruz (sc-8974) and Zymed (Z8P). 17β-,17α-b-BSA (βE2-BSA, αE2-BSA), and 17β-,17α-E2-BSA-FITC (βE2-BSA-FITC, αE2-BSA-FITC), and BSA-FITC (Sigma-Aldrich Hungary) were carefully purified before experiments using ultrafiltration [25].

Mice and cell lines

Adult female C57BL6/J wild-type mice (Charles River, Hungary) were maintained under a 12 h light/dark cycle (lights on at 0700 hours) 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 EU-conforming Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243/1998). Cell lines used in the experiments were murine IP12-7 T cell hybridoma of helper phenotype and the murine B lymphoma cell line A20 (ATCC TIB208). Cell lines were cultured in supplemented RPMI-1640 as described earlier [26]. Before treatments, all cell lines were cultured in phenol red-free RPMI-1640 with 5% charcoal-stripped foetal bovine serum (Invitrogen, Carlsbad, CA, USA) for 24 h.

Ovariectomy and immunization of mice

Experiment 1

Two groups of 42- to 56-day-old C57BL6/J female mice were bilaterally ovariectomised (OVX group; $n = 12$) or sham-operated (SHAM group; $n = 12$) under Avertin anaesthesia. Two weeks later, animals were immunized with 200 μg of T cell-dependent (TD) (KLH-FITC) or -independent (TI) (dextran-FITC) antigen. As TD antigen, KLH-FITC (in 50 μl PBS) mixed with 250 μl Complete Freund's adjuvant (CFA) was administered subcutaneously (s.c.) into the tail base. Dextran-FITC (in PBS) was administered intraperitoneally (i.p.). The control mice for KLH-FITC or dextran-FITC injection received CFA and PBS, respectively. On the sixth post-immunization day, all animals were euthanized with an overdose of Avertin, and the spleen was removed for ELISPOT assay.

Experiment 2

A group of 42- to 56-day-old C57BL6/J female mice were OVX under avertin Anaesthesia (OVX group; $n = 24$). On the 2nd, 7th, 12th, and 17th post-operation days, the mice were administered with 1 μg 17β-estradiol (βE2; Sigma; in 0.1 ml ethyloleate vehicle, s.c., OVX + E2 group, $n = 6$) or vehicle alone (OVX group, $n = 6$). Previous studies have shown that estrogen administered in this manner effectively reduce the OVX-induced luteinizing hormone (LH) level and mimic the estrogen pattern of the oestrus cycle in mice. Two weeks later, six estrogen- and six vehicle-treated mice were immunized with i.p. injection of TI or s.c. injection of TD antigen. All mice were euthanized with an overdose of Avertin after the sixth day of immunization and underwent the same experimental procedure as described above (Experiment 1).

ELISPOT assay

The numbers of IgM- and IgG-secreting spleen cells were evaluated by ELISPOT. Briefly, 96-well nitrocellulose plates (Millipore Multiscreen HA plate) were coated with 10 ml sterile PBS containing 10 μg/ml of FITC-BSA, and 5×10^6 or 10^6 freshly isolated splenocytes were added to the wells in triplicates. After overnight incubation at 37°C, plates were washed and HRP-conjugated goat anti-mouse IgM and IgG were added. Finally, after washing, plates were developed with 3-Amino-9-ethylcarbazole (AEC) substrate (Sigma-Aldrich Hungary). Each well was examined for the appearance of red spots. The plaques were counted with ImmunoScan ELISPOT reader (Cellular Immunology) and the results were evaluated with ImmunoScan software (C.T.L. Europe, Aalen, Germany).

Isolation of lymphocytes and purification of B and T cells

All animals were euthanized by an overdose of Avertin and spleens were removed. Cells were collected and washed in phenol red-free GKN. Red blood cells were lysed in 5 ml of ammonium chloride-Tris solution (pH 7.2) for 1 min. B cells were enriched by cytolytic elimination of T cells, using affinity-purified rat anti-mouse Thy-1.2 monoclonal antibody (ATCC cell line, 30-H12) and baby rabbit serum as a complement source. T cells were isolated by negative selection using MACS magnetic bead-based separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's description.

Stimulation of B and T cells and preparation of cell lysates

Cells (5×10^6) were treated in vitro with 1-100 nM 17β-estradiol, and 6 μg $(Fab')_2$ fragment of μ-chain-specific goat antibodies, respectively, at 37°C for the indicated time. T cells were activated by 15 μg/ml anti-CD3. Cells were pelleted, then immediately frozen in liquid nitrogen and solubilized in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 100 mM NaF, 250 mM NaCl, 10 mM EDTA, 2 mM sodium-*o* -vanadate, 10 mM sodium pyrophosphate, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 5 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride. After 30 min incubation on ice, cell lysates were centrifuged at 15,000*g* for 15 min at 4°C, and the supernatants were used in subsequent experiments.

Immunoblotting

Post-nuclear supernatants of detergent extract obtained from 5×10^6 untreated, or 17β-estradiol-treated B or T

cells, were incubated with reducing SDS-PAGE sample buffer for 5 min at 95°C. The samples were subjected to electrophoresis through 10% SDS-PAGE gel, and the proteins were transferred onto nitro-cellulose membranes (BioRad), then probed with different antibodies and developed by using HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies. The blots were developed by enhanced chemiluminescence detection (ECL system; Amersham International, Amersham, UK).

Measuring NFκB activity from nuclear extracts

B cells were separated by negative selection using EasySep Mouse B cell Enrichment Kit (StemCell Technologies, USA) according to the manufacturer's instructions. B cells were treated with either anti-IgM, βE2, or the carrier vehicles for 60 min. The activation was stopped by addition of ice-cold PBS. Nuclear extracts of the samples were prepared as follows. Samples of 2×10^7 cells were resuspended in buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5% NP-40, 0.5 mM DTT; 0.5 mM PMSF) and kept on ice for 20 min. The mixture was overlaid on sucrose cushion (30.8% sucrose (w/v) in Buffer A) and centrifuged at 4°C, 8,000*g* for 10 min. Supernatants were completely removed, and the pellet was taken up in Buffer C (20 mM HEPES, pH 7.9; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 25% glycerol; 0.5 mM DTT; 0.5 mM PMSF). Following homogenization, the samples were kept on ice for 30 min, and then centrifuged at 4°C, 15,000*g* for 10 min. The supernatants were collected and equal volume of Buffer D (20 mM HEPES, pH 7.9; 0.2 mM EDTA; 20% glycerol; 0.5 mM DTT; 0.5 mM PMSF) was added. The samples were then subjected to SDS-polyacrilamid gel electrophoresis and blotted into nitrocellulose membranes. The nuclear translocation of the NFκB proteins was checked by probing the membrane with different Rel protein specific antibodies (Rel-A/p65; Santa Cruz Biotechnology; p100, Cell Signaling Technology).

Immunfluorescent labeling, flow cytometry, confocal microscopy

Spleen cells of C57Bl6 mice in RPMI-1640 medium were attached to the surface of poly-lysine-coated coverslips for 1 h at 37°C. The coverslips were washed and the attached splenocytes were fixed with 4% paraformaldehide (PFA) for 20 min at room temperature. The cells were permeabilized with 0.1% Tween in PBS for 15 min then endogenous biotin blocking kit (Molecular Probes E-21390) was applied for 30 min. The blocking was completed by 15 min incubation in 5% BSA in PBS. The attached cells were labeled with antibodies specific to mouse ERα (sc-542; Santa Cruz) or ERβ (Z8P; Zymed), respectively, for 30 min in 5% BSA-PBS. The coverslips were washed 3 times and further incubated with biotinylated B220 or Thy1.2 antibodies (kind gift of Dr. Alexander McLellan, Department of Microbiology and Immunology, University of Otago) for 30 min. After further washings, the coversplips were incubated with secondary antibodies anti-rabbit Cy5 (711-176-52; Jackson Immunoresearch) for ERα and ERβ and with streptavidin-Alexa 488 (S11223; Invitrogen) for B220 and Thy1.2. After the final washes the coverslips were mounted and analysed by Olympus BX51 Epifluorescence Microscope using Olympus Cell P imaging software (SoftImageing, Germany).

To detect cell membrane estrogen receptors, cells (10^6) were incubated with βE2-BSA-FITC, αE2-BSA-FITC, or BSA-FITC, as a control, in PBS, at 1 mg/ml final concentration for 15 min at 37°C. For colocalization analysis, cells were also labeled with cholera toxin B Alexa 555 and rat anti-mouse Thy-1 Alexa Fluor 647 conjugates or with anti-B220 PE/Cy5 conjugate and subsequently fixed with 3% paraformaldehyde. For NF-κB staining, cells were fixed and permeabilized with ice-cold mixture (1:1) of acetone and methanol for 10 min, then pelleted and rehydrated in PBS containing 1% BSA for 10 min. Then, cells were incubated with rabbit anti-p65 NF-κB (clone C-20; Santa Cruz Biotechnology) for 45 min at room temperature. After washing, cells were stained with goat anti-rabbit IgG (H + L) Alexa Fluor 488 conjugate and with the DNA-specific dye DRAQ5 at room temperature for 45 min. Flow cytometric measurements were done in a BD FACSCalibur instrument (Becton-Dickinson, La Jolla, CA, USA). Data collection and analysis were performed with CellQuest Pro software (Becton-Dickinson). Fluorescence microscopy was carried out on an Olympus FLUOView 500 laser-scanning confocal microscope (Hamburg, Germany) equipped with an air-cooled argon ion laser (488 nm) and two He–Ne lasers (with 543 and 632 nm emission wavelength. Fluorescence and DIC images (512×512 pixels) were acquired using a $\times 60$ oil-immersion objective. Images were processed by IMAGE J software (Wayne Rasband, NIH, Bethesda) available together with color colocalization analysis plugins at the website of Wright Cell Imaging Facility, Toronto, ON.

Single cell calcium imaging

Suspensions of T and B cells (at $5 \times 10^6/cm^3$ density) were placed into wells of a Lab-Tek borosilicate chambered coverglass microplate (NUNC, Rochester, NY, USA) and were left to adhere at RT for 20 min. Then, cells were incubated with 10 μg/ml Fluo-4 (Invitrogen/Molecular Probes, Carlsbad, CA, USA), plus 100 μg/ml Pluronic acid

(F-127), at 37°C for 30 min. After washing, cells were treated with 100 nM βE2 or βE2-BSA. As a reference, some samples were also treated with 1 μg/ml ionomycin (Sigma, St. Louis, MO, USA). Changes in fluorescence intensity of individual cells were monitored in Olympus FluoView 500 laser-scanning confocal microscope (excitation: 488 nm) with a ×10 objective, in time-resolved acquisition mode (0.44 s/frame). During data analysis, mean fluorescence intensities obtained from single cell recordings were normalized to DIC intensities (to avoid out of focus intensity alteration effects).

Mouse IFN-gamma ELISA

An amount of 100 μl spleen cell suspension (5×10^6 cells/ml) from C57BL6/J female mice were cultured in phenol red-free RPMI-1640-modified medium (supplemented with L-Glutamine, Na-pyruvate, penicillin, streptomycin, 10% DCC-FCS, and mercaptoethanol) with 1, 10, or 100 nM 17-β estradiol and/or 15 μg/ml ConA for 72 h. IFNγ level was measured in the cell culture supernatant using the Quantikine ELISA kit according to the manufacturer's protocol (R&D Systems, Minneapolis, USA). Briefly, 50 μl of supernatants and 50 μl of assay diluents were added to the wells. Plates were incubated at room temperature for 2 h, washed, and then anti-mouse IFN-γ conjugate was added to the wells. After 2 h incubation at room temperature, the plates were washed again and 100 μl substrate solution was added for 30 min. Finally, the reaction was stopped and the optical density was measured at 450 nm with wavelength correction at 620 nm by ELISA reader (Thermo Electron, Multiscan Ex). Protein amount was calculated by the formula obtained from standards.

Statistical analysis

In the case of in vivo experiments, the results were expressed as mean ± SD of triplicate samples from multiple groups of mice. Two-way ANOVA followed by post-hoc Neuman–Keults test was performed to test the significance of differences.

In the case of in vitro signaling experiments, such as WB, the statistical evaluation was based on densitometric evaluations of the blots from at least triplicates of independent samples and expressed as normalized mean ± SD. The control samples were taken as 100%. (The SD over the independent WB developments was typically in the range of 10–20%.)

The fluorescent (and DIC) micrographs shown in the figures are representative confocal microscopic images of 150–200 cells/sample.

Results

Ovariectomy alters the T-dependent (TD) but not the T-independent type 2 (TI-2) immune response

To examine the effect of gonadal steroids on TD and TI-2 immune response, we evaluated IgM and IgG synthesis following immunization with KLH-FITC and dextran-FITC of SHAM operated (intact) and OVX mice. Hapten-specific antibody production was observed in both OVX and intact animals. However, in OVX mice, the FITC-specific IgM and IgG production was considerably lower than in the SHAM-operated mice (Fig. 1a, b). In contrast, when mice were immunized with TI-2 antigen, FITC-conjugated dextran, the FITC specific IgM and IgG titers were similar in sera of OVX- and SHAM-operated control animals (Fig. 1c, d).

In order to reveal whether the decreased serum Ig level is a consequence of the lower number of antibody producing B cells in OVX animals, ELISPOT assays were performed. The number of hapten-specific IgM- and IgG-producing plasma cells in OVX mice immunized with TD antigen was significantly ($p < 0.001$) lower compared to the control SHAM operated animals (Fig. 2a, b), indicating that the TD response is upregulated by the gonadal hormones.

In the second set of experiments, when mice were immunized with TI-2 antigen dextran-FITC, no significant difference was found in the number of IgM- and IgG-producing cells of the OVX and control groups, respectively (Fig. 2c, d). This suggests that the reduction of antibody response after ovariectomy is T cell dependent.

Effect of βE2 replacement on antibody production in OVX mice

To investigate whether the attenuated TD response after ovariectomy is mediated by the lack of estrogen, replacement experiments were also performed in OVX mice. The replacement of estrogen in the gonadectomized animals significantly ($p < 0.001$) increased the number of FITC-specific IgM- and IgG-producing cells in the spleen of immunized OVX mice as detected by ELISPOT assay (Fig. 2e, f). Since ovariectomy had no effect on TI-2 response (Fig. 2c, d), these data also suggest that estrogen enhances the antibody response to T-dependent but not to T-independent antigen.

How does βE2 act on lymphocytes?

Estrogen may exert its effect on the humoral immune response via binding to intracellular/nuclear and also to

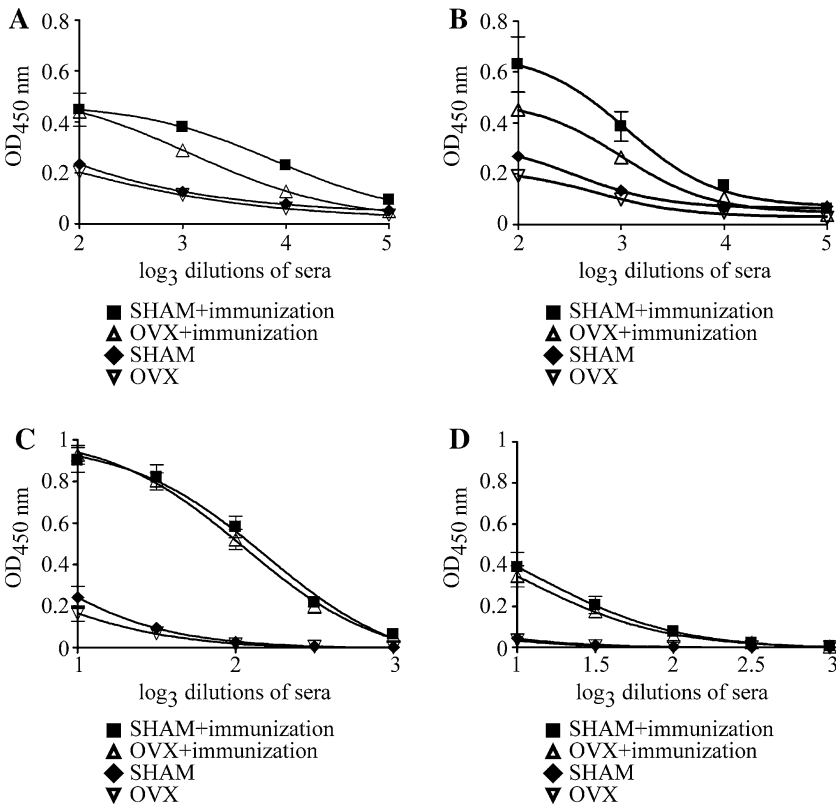


Fig. 1 Effect of ovariectomy on IgG and IgM production by mouse splenocytes following T cell-dependent or -independent immune responses showing the IgM (**a**) and IgG (**b**) contents of the sera following immunization with TD antigen (**a**,**b**, respectively) or TI-2 antigen (**c**,**d**, respectively). The results are means ± SD. Values from triplicate samples

putative membrane receptors on lymphocytes. Both cytoplasmic and membrane receptors were implicated in the rapid non-classical estrogen effects of many cell types [27, 28]. Therefore, we next examined the expression of the classical cytoplasmic ERs and the putative membrane receptor in separated primary murine T and B cells, as well as in T (IP12-7) and B (A20) cell lines by flow cytometry and confocal laser scanning microscopy (CLSM). All these cells expressed both ERα and ERβ, but ERβ expression was significantly lower (Fig. 3a, b, upper panel). However, western blot analysis has clearly shown that ERα and ERβ are equally expressed in both B and T cells (Fig. 3b, lower left panel). ERα was localized in the cytoplasm as well as in the nuclei of B and T cells. In addition, βE2 induced the translocation of ERα to the nucleus, indicating its functional integrity (Fig. 3b, lower right panel).

In order to detect whether βE2 has cell membrane binding sites in lymphocytes, a fluorescent, cell-impermeant covalent conjugate, βE2-BSA-FITC, and as control, αE2-BSA-FITC, were used. βE2-BSA is commonly used to define E2 actions mediated by estrogen-binding receptors located in the plasma membrane of cells [29]. As shown in Fig. 4a–g, both splenic B and T cells bound βE2-BSA-FITC, indicating the existence of membrane binding sites/receptors for βE2. The background (BSA-FITC binding), as a control, was found to be negligible (Fig. 4j–k). Similarly to the primary mouse lymphocytes, the T (IP12-7; Fig. 4h) and B (A20; Fig. 4i) cell lines also selectively bound βE2-BSA.

Concentration-dependence of β- and α-E2-BSA-FITC binding to both T and B lymphocytes (Fig. 4j, k) clearly demonstrates its specificity and receptor-mediated fashion. Time-dependence data, at 1 mg/ml βE2-BSA-FITC, (data not shown), indicated a relatively rapid binding (saturation within 10–15 min). The BSA conjugate of the functionally inactive isomer, 17-α-estradiol did also bind to the surface of both T and B cells, indicating a selective binding of E2 to a putative (yet unidentified) membrane receptor.

βE2 induces oscillating Ca^{2+}-signals in T cells but not in B cells

Changes in intracellular Ca^{2+} level may play a crucial role in estrogen-induced non-classical actions. Earlier mERs were implicated in mediating a rapid rise of intracellular free $[Ca^{2+}]_i$ in T cells and in MCF7 tumor cell line [30, 31]. Therefore, we tested the effect of βE2 and βE2-BSA on $[Ca^{2+}]_i$ in both B and T lymphocytes. Both βE2 and βE2-BSA induced a slow, oscillating, and sustained $[Ca^{2+}]_i$ response in splenic T cells, while B cells did not respond (Fig. 5). The machinery mediating $[Ca^{2+}]_i$ changes, however, was functionally intact in B cells, as indicated by the calcium response to anti-IgM-stimulation (data not shown). Responses of murine T and B cell lines

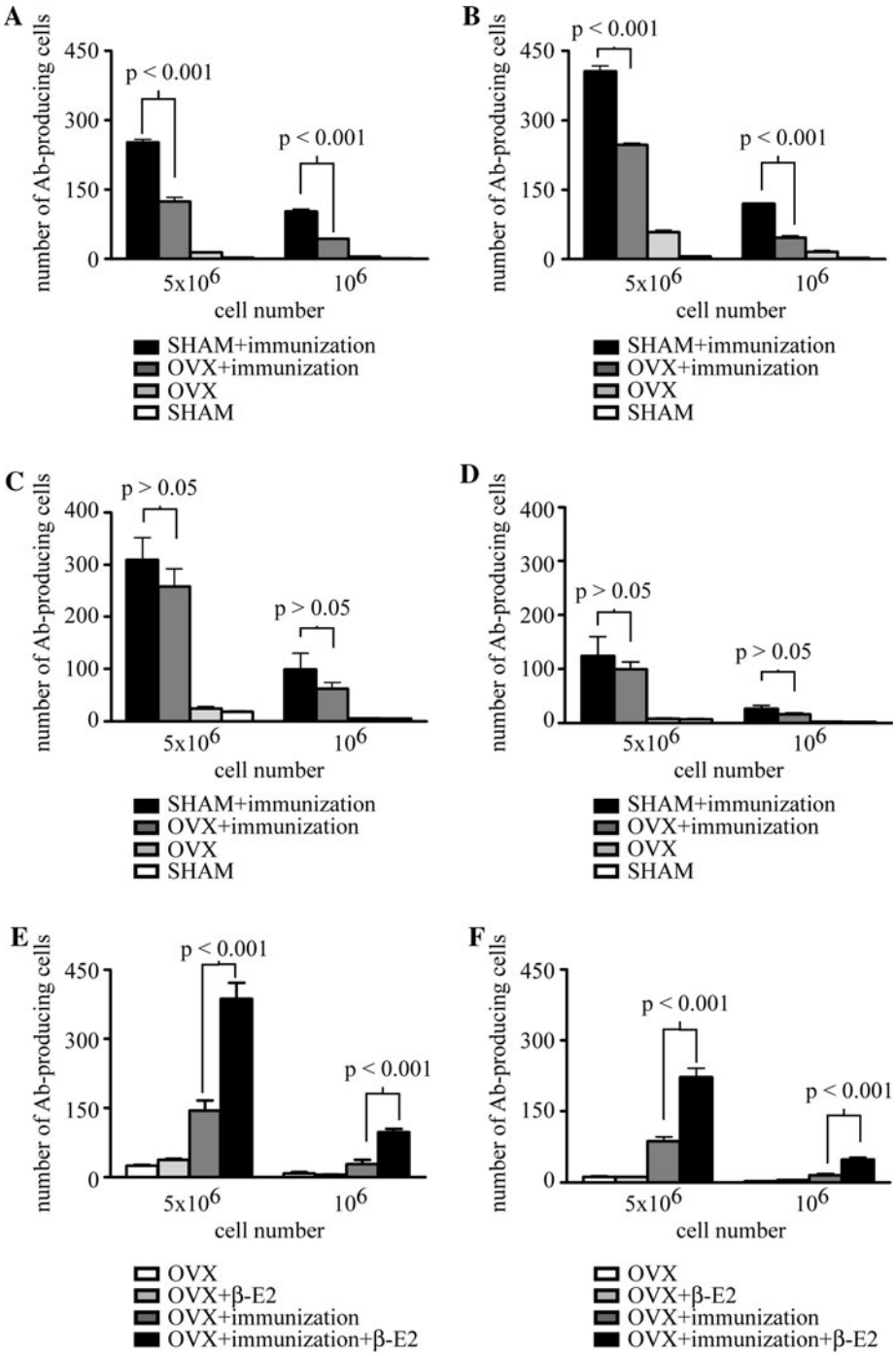
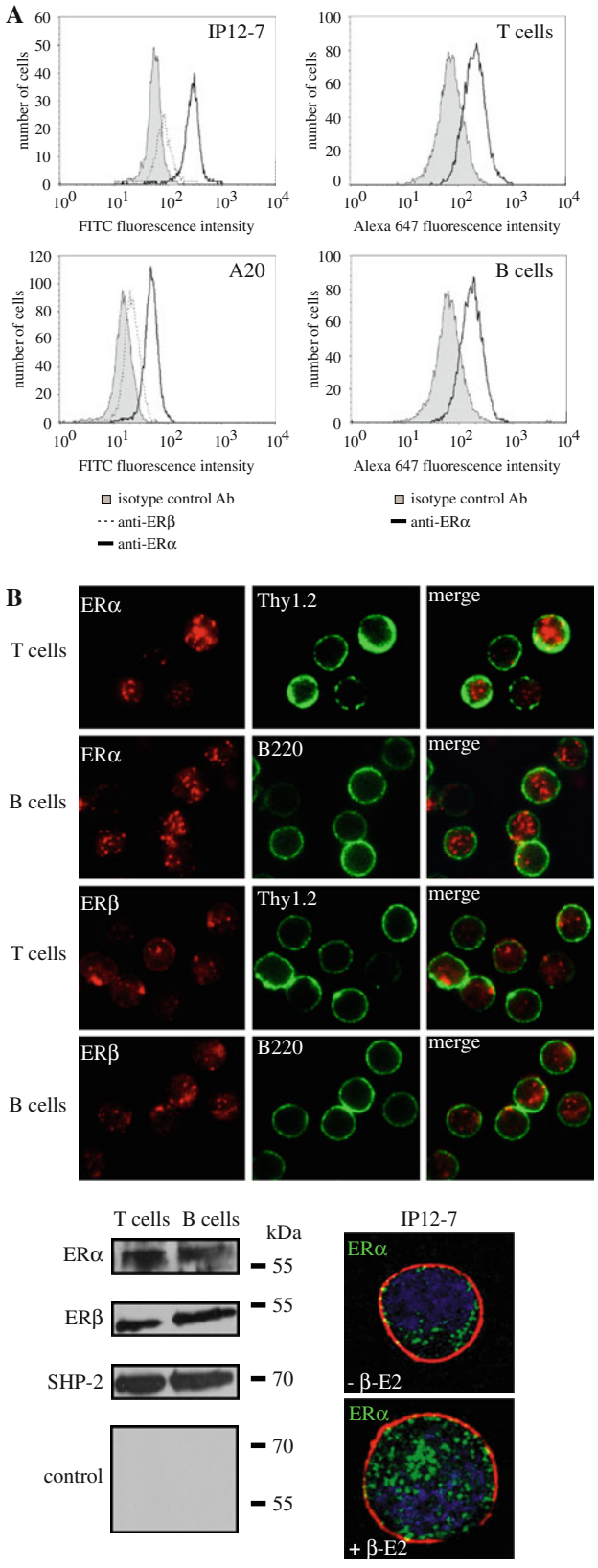


Fig. 2 Analysis of hapten specific IgG- and IgM-producing cells as a response to TD and TI-2 antigens in mice: effect of ovariectomy and estrogen replacement. The number of IgM- (**a**) and IgG- (**b**) producing cells are shown as a response to immunization with TD antigen in OVX- and SHAM-operated control mice. The same cell numbers in response to TI-2 antigen are shown in **c**,**d**, respectively. The numbers of IgM- and IgG-producing cells (**e**,**f**, respectively) are displayed in OVX mice immunized with TD antigen under estrogen replacement conditions. The results are means ± SD of triplicate samples from multiple groups of mice. Significance analysis was performed as described in "Materials and methods"

(IP12-7 and A20, respectively) further confirmed the data obtained with primary lymphocytes. Note that the 17-α-estradiol-BSA conjugate could not induce any significant Ca^{2+} response in either B or in T cells (data not shown).

Estrogen induces rapid Akt and ERK activation in B and T cells

The non-classical estrogen action was shown to modulate the activation of kinase cascades; therefore, it may play role in estrogen effect on T cell-dependent B cell response. Here, in an in vitro model system, derived from animals used in the in vivo experiments, we investigated the activation of kinases Akt and ERK in cell lysates of separated murine B and T cells treated in vitro with various doses of βE2 for different time periods. In B cells, βE2 at 1 nM dose induced a time-dependent phosphorylation of both ERK1/2 and Akt, while higher doses ($\geq$10 nM) were less efficient (Fig. 6a, left). In contrast, βE2 induced only a slight phosphorylation of Akt and Erk in T cells (Fig. 6a, right).



◀ **Fig. 3** Classical estrogen receptors, ERα and ERβ, are expressed in murine B and T lymphocytes. ERα and ERβ receptors are expressed in both primary murine lymphocytes (**a** *right panels*) and in T and B cell lines (**a** *left panels*) as assessed by flow cytometry (**a**) microscopy (**b** *upper panel*). Representative confocal microscopic images show expression and mainly cytoplasmic localization of both ERα and ERβ in both B and T splenic lymphocytes (**b** *upper panels*). Upon βE2 treatment, significant nuclear translocation of ERα is observed (**b** *lower right*) (*red* cholera toxin B membrane staining; *green* intracellular staining with anti-ERα; *blue* nuclear counterstaining). The presence of ERα and ERβ proteins in T and B cell lysates was also analyzed by western blots (**b** *lower left*). Anti-SHP-2 was used as loading control, and, to see the specificity of the signal, only the secondary antibody (goat anti-rabbit IgG-HRPO) was added (**b** *bottom left*)

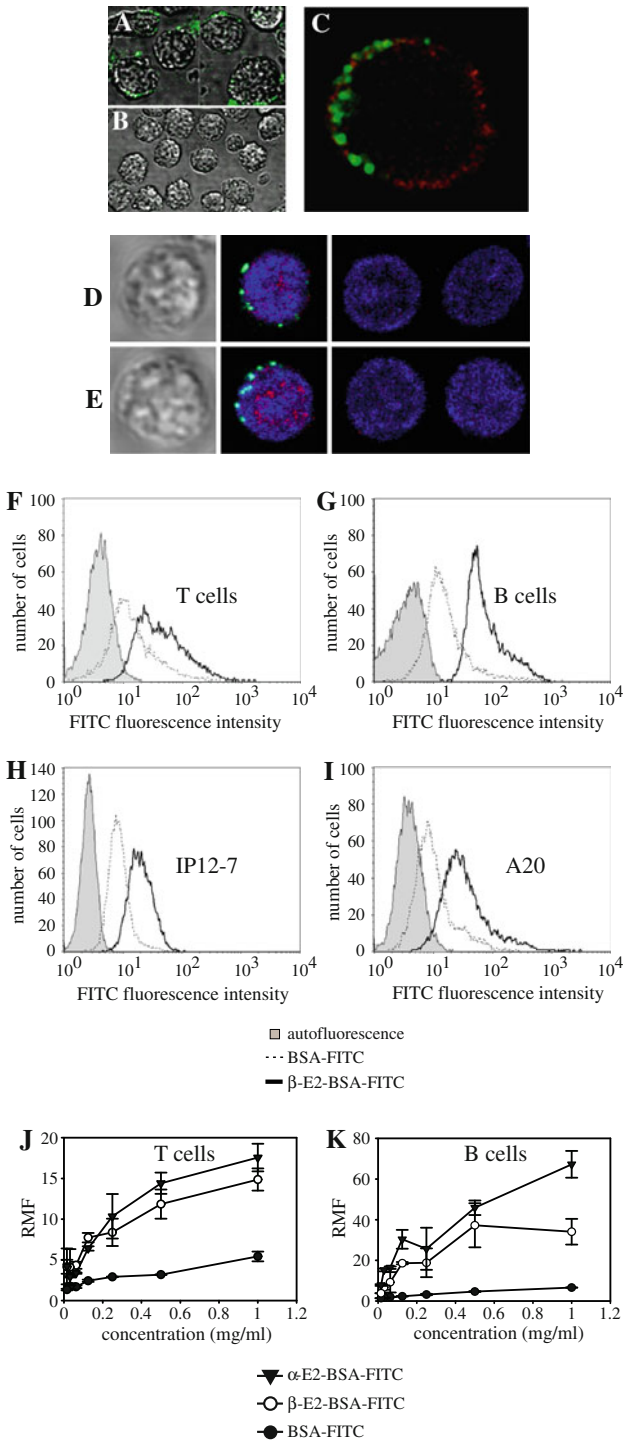
Estrogen did not have any additional effect on anti-IgM-induced phosphorylation of these kinases in B cells (data not shown).

In order to elucidate what kind of E2 receptors mediate the activation of these kinases, we next examined whether βE2 binding to mER also induces ERK1/2 and Akt phosphorylation in B and T cells. Using βE2-BSA for stimulation, we found an increased phosphorylation of ERK1/2 and Akt in T cells, even at higher concentrations (≥10 nM), while little if any change in the level of pERK and pAkt was observed in B cells (Fig. 6b).

In contrast to βE2-BSA, in spite of its equal binding to lymphocytes, αE2-BSA did not induce any phosphorylation of Erk and Akt, showing that only the functionally active βE2-BSA is able to influence kinase activation. These data together indicate that estrogen exerts its effect in B cells mainly through intracellular ERα/ERβ, while regulating the function of T cells mainly via binding to membrane ER (Fig. 6c).

Estrogen induces NFκB activation and IFNγ gene transcription in T and B lymphocytes

The common target of many signaling pathways is one of the key transcription factors, NFκB. Cellular signaling mechanisms activate the translocation of NFκB from cytoplasm to nucleus, which is required for NFκB to become a transcriptional regulator. Differential regulation of NFκB proteins by estrogen in spleen cells and in human T cells was recently reported [32, 33]. We have tested whether in vitro treatment with βE2 can modulate subcellular distribution of NFκB in separated murine B and T lymphocytes by confocal laser scanning microscopy. βE2 induced a significant although partial nuclear translocation of p65 NFκB in both B and T cells (Fig. 7a). The modulation of various NFκB proteins, c-Rel, RelA/p65, and p100/52, were tested in nuclear extracts of βE2-treated B cells by western blotting, using antibodies specific for various forms of NFκB. Fig. 7b shows that cRel was activated only by anti-IgM, while p65 and p52 were also translocated to the nucleus in response to βE2 treatment. These data (Fig. 7b) confirming the microscopic findings indicate that estrogen triggers activation of p65 and p100/52 may thus induce gene transcription.



◀ **Fig. 4** Binding of cell-impermeant βE2-BSA conjugates to B and T cells. Representative confocal microscopic image (DIC + fluorescence overlay) shows cell surface binding of 17-β-E2-BSA-FITC to splenic T lymphocytes (**a**), while BSA-FITC showed no significant binding (**b**). Similar patchy membrane staining was also observed in B lymphocytes (**c**) (*green* 17-β-E2-BSA-FITC; *red* anti-B220 antibody). This was quantitatively confirmed by flow cytometry in both primary lymphocytes and the T and B cell lines (**f**–**i**). Representative images of T cells (**d**) and B cells (**e**) double stained with 17-β-E2-BSA-FITC (*green*) and anti-ERα (*red*) with nuclear counterstaining (*blue*) show disparate staining for ERα and the membrane βE2 binding site. **j**,**k** Dose-dependence of 17-β- and 17-α-E2-BSA-FITC binding (measured 15 min after addition) to T cells and B cells, respectively. As a control, BSA-FITC binding is also shown. *Error bars* SD calculated from three independent experiments. *RMF* Relative mean fluorescence intensity, normalized to autofluorescence

Earlier reports revealed that ConA-stimulated spleen cells of βE2-treated mice showed higher IFNγ and IL-2 mRNA expression and an enhanced IFNγ production compared to untreated mice [34]. To clarify whether IFNγ is induced in response to βE2 in vitro, splenocytes of C57BL/6 mice were cultured in the presence of ConA and βE2, and the IFNγ content of the supernatants was quantified by ELISA. βE2 considerably increased the IFNγ production in the cell cultures as compared to ConA-treated cells, indicating that βE2—probably via NFκB activation—may induce IFNγ gene transcription (Fig. 7c).

Discussion

It is well known that sex hormones, in addition to their effect on sexual differentiation and reproduction, can modulate the immune response. This results in a gender dimorphism in the immune function with females having higher immunoglobulin levels and stronger immune responses than males. Females also show an increased susceptibility to autoimmune diseases. However, the exact molecular/cellular mechanism behind this difference is still largely unknown. The effect of estrogen on the immune response has been extensively investigated in mice. Studies in normal mice have shown that estrogen treatment induces polyclonal B cell activation with increased expression of autoantibodies [35]. Several mechanisms appear to contribute to break tolerance and to increase plasma cell activity including the emergence of sites of extramedullary haematopoiesis and altered susceptibility of B cells to cell death. In addition, sex hormones may influence the cytokine milieu, suggesting that an altered hormone level may result in a skewed cytokine profile contributing to a modified immune response [6].

In contrast to the higher prevalence of systemic autoimmune diseases in females compared to males [2, 36], gender may not be predictive of mortality in cases of bacterial infections [37]. Microbial antigens stimulate TI-2 response. B cell responses are categorized as TD or TI-2 based on the requirement for T cell help. TD antigens are proteins that are processed and presented on MHC class II molecules for recognition by cognate helper T cells. TI antigens are divided into type 1 and type 2. The former are mitogenic stimuli triggering polyclonal B cell activation, while the latter are polysaccharides, e.g., on bacterial cell

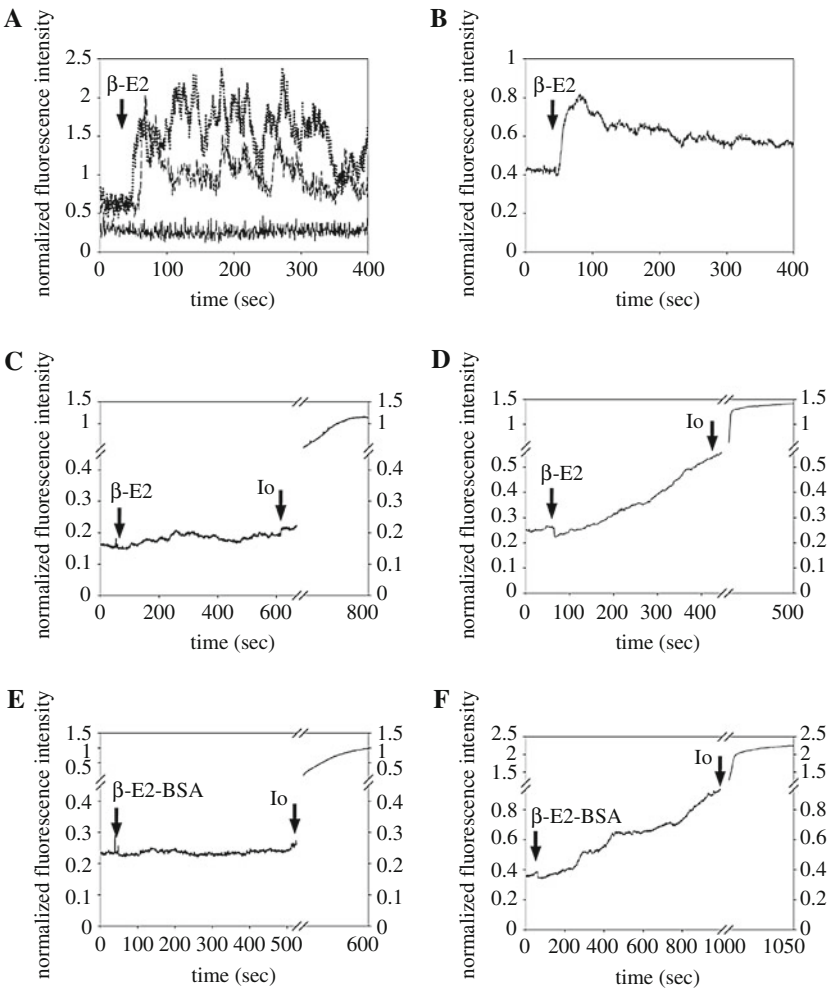


Fig. 5 Calcium signals induced by estrogen in splenic B and T lymphocytes. Fluo-4 loaded murine splenocytes (**a**,**b**), purified B splenocytes (**c**,**e**) and T splenocytes (**d**,**f**) were investigated by microscopy for their single cell Ca^{2+} response to 100 nM membrane permeable or impermeable 17β-estradiol (βE2 and βE2-BSA, respectively). Representative single cell recordings of three individual cells demonstrate the substantial heterogeneity among splenocytes in their estradiol-induced Ca^{2+} response (**a**). **b** The averaged response of splenocytes ($n = 58$) to βE2. In separated murine B splenocytes, neither βE2 nor βE2-BSA cause significant changes in the intracellular Ca^{2+} level (**c**,**e**) ($n = 27$ and $n = 26$, respectively), while purified T cells responded to both forms of estradiol (**d**,**f**) ($n = 54$ and $n = 55$, respectively). The "maximal response" of the cells to ionomycin Ca^{2+} ionophore is also shown in **c**–**f** (*right side*)

wall, crosslinking B cell receptors, and thus inducing antigen-specific B cell responses [38].

As an as yet less studied area of this question, here we compared the effect of estrogen on TD and TI-2 response using the same hapten, FITC, conjugated either to the protein, KLH (TD), or to the polysaccharide, dextran (TI-2). Ovariectomy of mice reduced the number of antibody-forming cells in the spleen resulting in a lower level of FITC-specific antibodies in KLH-FITC-immunized animals. However, the response to TI-2 antigen did not change. This important new finding indicates that female sex hormones exert their effect on the humoral immune response on a T cell-dependent way. Thus, in agreement with previous immunopathological observations, our results suggest that the immune response to TI-2 antigens, such as microbial antigens, does not have gender difference, while the response to TD antigen is strongly influenced by female sex hormones.

Estrogen replacement experiments with ovariectomized mice, where βE2 was added to maintain the physiological level of estrogen, further confirmed that estrogen selectively modulates the TD immune response. βE2 addition significantly (2.5 times) elevated the antibody response to TD antigen compared to untreated control animals, indicating that estrogen enhances T cell-dependent antibody production, through enhancing the number of antibody-forming cells.

Previous work has suggested that estrogen-dependent upregulation of B cell response may be mediated by suppression of T cells producing negatively regulating cytokines [6]. On the other hand, it was also shown that estrogen modulates the expression of molecules regulating B cell survival, directly upregulating CD22, SHP-1, and Bcl-2; furthermore, estrogen treatment protects isolated primary B cells from B cell receptor-mediated apoptosis [5]. The authors speculated that the increase of Bcl-2 expression enhances survival of autoreactive B cells, while the elevated level of CD22 and SHP-1 raises the BCR-mediated signal threshold required for their deletion. In response to TD antigen, such a mechanism may result in the elevated number of antigen-specific B cells.

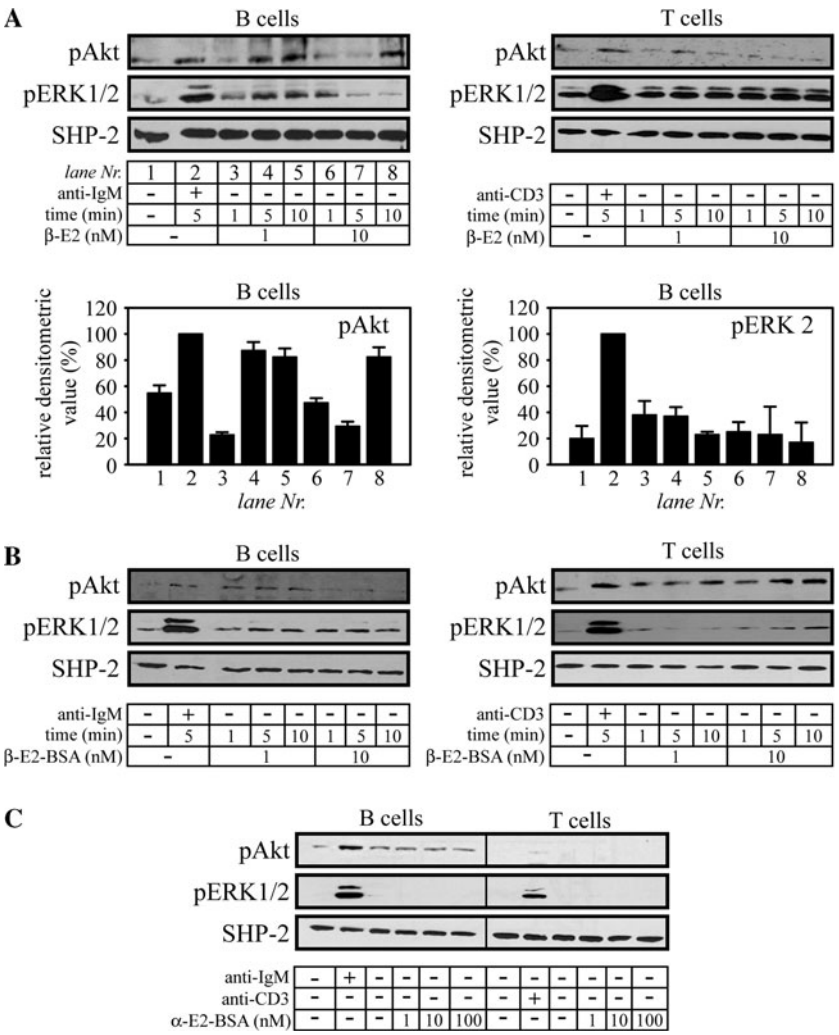


Fig. 6 Estrogen signals modulate ERK and Akt phosphorylation of B and T lymphocytes. Separated murine B and T splenocytes were treated with βE2 (**a**) or with 17-β-E2-BSA (**b**). Cell lysates were examined for ERK and Akt phosphorylation by western blot. **a** The B and T cells were stimulated, as a positive control, with anti-IgM and anti-CD3, respectively, and with 17-β-estradiol for different times as indicated under the *lanes*. The *lower panels* demonstrate densitometric and the statistical evaluation of the WBs. This evaluation based on relative density values of the bands normalized to total SHP-2 level, as described in "Materials and methods". **b** The same western blot analysis is shown for 17-β-estradiol-BSA conjugate. **c** Analysis of the effects by 17-α- estradiol-BSA conjugate on T and B cells is shown under the same conditions as in (**a**) and (**b**)

To understand the difference between estrogen effect on immune responses to TD and TI-2 antigen, the direct impact of estrogen on isolated B and T cells was also studied in terms of signaling. Rapid, non-genomic effects of estrogen can be mediated by both membrane and intracellular ER. In agreement with a previous report [31], ERα and ERβ were found expressed in both B and T cells of C57Bl/6 mice; furthermore, both cell types were also positive for membrane ER. The existence of membrane receptors for estrogen (mER) was previously reported on T cells but not on B cells of C57BL/10 mice [31]. This difference might be due to the different mouse strain, and/or to the different plasma membrane compartmentation, accessibilty of the mER. The highly disparate location of membrane-bound βE2-BSA and ERα as well as binding of the BSA-conjugated functionally inactive isomer, αE2-BSA, to the surface of both T and B cells suggest that the majority of functionally active mERs in lymphocytes is not identical to any form of the classical ERs. Therefore, further studies are required to identify the nature of these lymphocyte mER(s).

Membrane ER was also shown to mediate a rapid effect on $[Ca^{2+}]_i$ in T cells [31]. Although binding of βE2-BSA to both B and T cells of C57Bl/6 mice was detectable, in contrast to T cells, the membrane ER on B cells appears to be inactive, since βE2-BSA did not induce a rise in $[Ca^{2+}]_i$ nor triggered a significant ERK or Akt phosphorylation as compared to the effect of βE2, which binds to the intracellular ER.

Several biological actions of estrogen have been associated with its non-classical effect. Intracellular regulatory cascades such as extracellular signal-regulated kinase/mitogen-activated protein kinases (ERK/MAPK) and PI3-K have been shown to be stimulated by estrogen in diverse cell types, such as tumor cells and neurons [30, 39, 40, 43]. A non-transcriptional mechanism for ER signaling in human as well as in animal endothelial cells was also described, showing that ER can physically and functionally

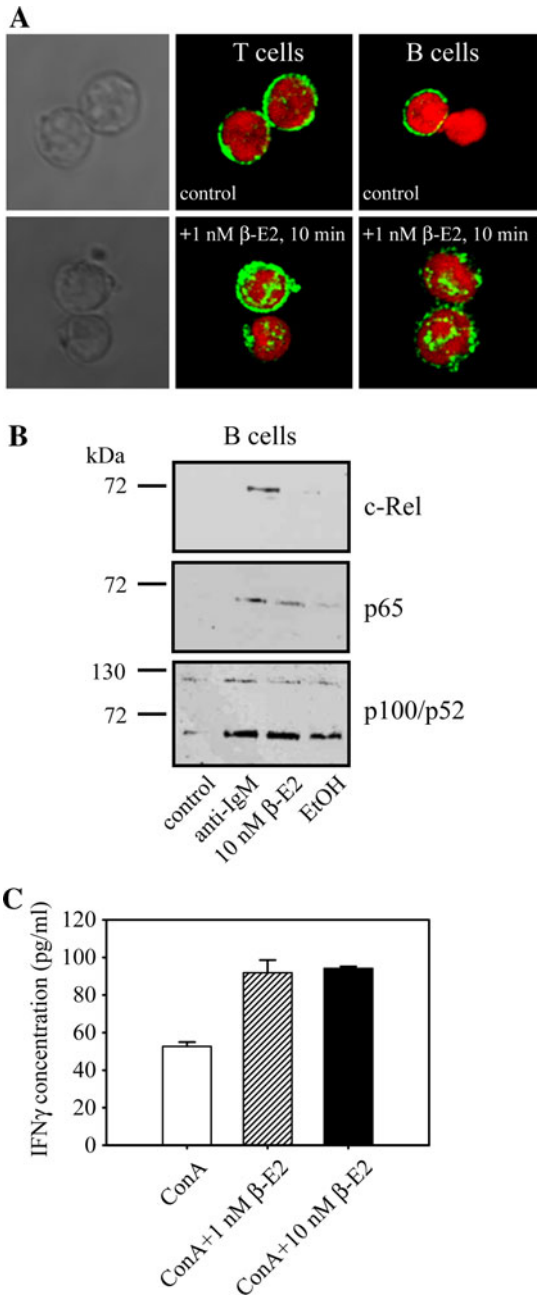


Fig. 7 Estrogen induces NFκB activation/nuclear translocation and IFNγ gene activation in splenic B and T cells. **a** Representative (from 150 cells) DIC and fluorescence images of control and βE2-treated murine splenic T and B cells stained for p65 NF-κB (*green*) and DNA/nucleus (*red*). **b** Nuclear translocation of cRel, RelA (p65), and p100/p52 NF-κB proteins in purified murine splenic B cells as assessed by immunoblotting. Cells were left untreated (*control*) or treated with anti-IgM, 10 nM 17-β-E2, and vehicle (ethanol) for 60 min. **c** IFNγ levels were determined from supernatants of 72-h cell cultures of unseparated splenocytes stimulated with concanavalin A (*ConA*) alone, or with 1 and 10 nM 17-β-E2 by ELISA. The results are means ± SD of three independent cultures

couple to PI3K. This may lead to activation of PI3K signaling cascade to Ser/Thr kinase Akt, which mediates several PI3K-dependent intracellular effects, including endothelial isoform of NO synthase (eNOS) phosphorylation and activation [27]. The βE2-induced signaling in lymphocytes, however, has not yet been analyzed in detail. In vitro treatment with βE2 of B cells and T cells isolated from the spleen of C57Bl/6 mice stimulated a time-dependent ERK1/2 and Akt phosphorylation, while βE2-BSA binding to cell membrane receptors triggered the activation of these kinases only in T cells.

Akt phosphorylates an array of molecules, including glycogen synthase kinase-3beta (GSK-3beta), and Bcl-2-associated death protein, thereby blocking mitochondrial cytochrome c release and caspase activity, signaling for cell survival [41]. Thus, our results suggest that estrogen can directly act on both B and T cells, and, besides lowering the activation threshold, induces Akt-mediated survival signals. This latter effect appears to be mediated by the classical ER in B cells, while mainly triggered through mER on T cells.

It has also been shown recently that βE2 modulates NFκB activity [22]. There are early estrogen effects, with rapid nongenomic activation of NFκB that are protective. A later response to βE2 results in the inhibition of NFκB, which is mediated by transcriptional, genomic effects [32]. In both B and T cells isolated from the spleen of C57Bl/6 mice, we could detect a rapid activation and nuclear translocation of p65 and p52 NFkB, while having no effect on cRel. NFκB is a potent transcription factor that plays dual roles: its activation results in the expression of both pro-apoptotic and anti-apoptotic proteins as well as pro-inflammatory cytokines [32]. On the other hand, rapid NFκB activation also leads to cell proliferation and survival, while prolonged activation of NFκB may induce chronic inflammation. βE2 may enhance NFκB activity by recruiting steroid hormone coactivators to the ER-NFκB complex on the target gene promoter region [33, 42]. This may lead to the induction of anti-apoptotic proteins, which in concert with Akt-mediated survival signals result in the escape of B cells from programmed death stimulated by a strong BCR stimuli.

The elevated antibody synthesis in response to βE2 treatment in vivo to TD but not to TI-2 antigen raises the question that βE2-induced cytokine production is at least partly responsible for this effect. Indeed, in agreement with previous in vivo data, we could show that βE2 treatment of spleen cells in vitro enhances IFNγ synthesis in ConA-stimulated cell culture. Together with the data showing that βE2 triggered NFκB activation, these results indicate that βE2 may enhance B cell response to TD antigen via inducing NFκB and IFNγ gene transcription.

In conclusion, we show here that estrogen has a direct impact on B and T cells by inducing rapid non-classical effects via both intracellular ER (in B cells) and membrane estrogen receptors (mainly in T cells). These effects result

in selective calcium signals (only in T cells), activation of p65NFkB in both B and T cells and a chance for enhanced survival of B cells. Altogether, these effects may result in an improved collaboration between B and T cells during the TD immune response. Consistent with this, our in vivo studies demonstrate for the first time that estrogen indeed positively modulates the T cell-dependent but not the T-independent humoral immune response.

Acknowledgments This work was supported by the Hungarian National Science and Research Development Foundation (OTKA T047217 and T049696), the NKFP-1-A/040/2004, Pázmány grant 06/2006 from Hungarian National Office of Research and Technology Development, and by the Department of Physiology at University of Otago and University of Otago Research Grant. The financial support of the Hungarian Academy of Sciences is also gratefully acknowledged. The authors thank Arpad Mikesy for the care and maintenance of mice.

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