ONLINE SUPPLEMENTARY INFORMATION

METHODS

Antibody list. Abs against Lck, Fyn, Hsp90α/β, GRα, CD3, CD4, PY20, b-Raf, MEK, Src, ZAP70, PKB, ERK, JNK, p38 MAPK and actin were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). $MEK^{Ser217/221}$, Src^{Tyr416}, PKB^{Ser473}, $p38^{Thr180/Tyr182}$, $ERK1/2^{Thr202/Tyr204}$ and $JNK^{Thr183/Tyr185}$ Abs were from cell signaling technology (Beverly, CA, USA). Anti-human CD3 (CD3-epsilon; mouse) was kindly provided by the group of Prof. Dr. H. Spits (Academic Medical Center, Amsterdam, the Netherlands), and anti-CD28 Ab (mouse IgG1) was from Sanquin (Amsterdam, the Netherlands).

Immunoprecipitation, *in vitro* kinase assay and Western blotting. CD4+ T cells were incubated in 6-well plates (5-10.10^6 cells per well) for 2 hrs followed by a 10 min pretreatment with 1μM DEX dissolved in DMSO, or DMSO-supplemented media (control). Subsequently, cells were activated for 15 min with anti-CD3 Abs (immobilized on plastic) and soluble anti-CD28 Abs (3 μg/ml). Cells were centrifuged (1250 rpm, 5 min), lysed in non-denaturing lysis buffer and subjected to immunoprecipitation. First, a pre-clearance step was performed by incubating the lysates with protein-A Sepharose for 2 hrs. After centrifugation (14.000 rpm, 5 min), supernatants were incubated overnight at 4 °C with the indicated Abs, followed by a 2-3 hr incubation with a protein-A Sepharose conjugated polyclonal antibody. After centrifugation (14.000 rpm, 5 min), immunoprecipitates were used for Western blot analysis. Alternatively, Lck/Fyn immunoprecipitates were dissolved in kinase buffer supplemented with 200 μM ATP and 2 μg/ml SAM68 and *in vitro* kinase reactions were performed at 30 ºC for 30 min. After centrifugation (14.000 rpm, 5 min), the pellets were dissolved in sample buffer (62.5 mM Tris-HCl (pH 6.8 at 25 °C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% (w/v) bromphenol blue), heated for 5 min at 95°C,

loaded on SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Amsterdam, the Netherlands). The membranes were blocked with 5% BSA in TBS/T (0.05M Tris, 150mM NaCl, 0.05% Tween-20). Primary Abs and secondary HRP-conjugated Abs were diluted in 5% BSA TBS/T, and proteins were visualized using the Lumi-Light^{PLUS} substrate (Roche, Woerden, the Netherlands).

Confocal fluorescence microscopy. After immunostaining, the cells were imaged with a Leica SP2 AOBS confocal microscope. Excitation/detection of FITC and TRITC was done with 488nm/ 500-550nm and 561nm/ 580-640nm respectively. Dapi was imaged with 405nm excitation and detected with 410-460nm (not shown). To avoid cross talk, all detection was done in a sequentional scan mode. A HCX PLAN APO CS 63×/1.20 water immersion objective was used. All images were adapted to the full dynamic range of the system (8 bit). The pinhole size was set at 1 Airy to ensure an optimal z-resolution (approximately 600nm). The images were scanned with a pixel size of 75nm. The presence of FITC and TRITC was ascertained by spectral imaging of the samples. At least 20 cells of each condition were scanned and representative cross sections are indicated.

SUPPLEMENTARY FIGURES

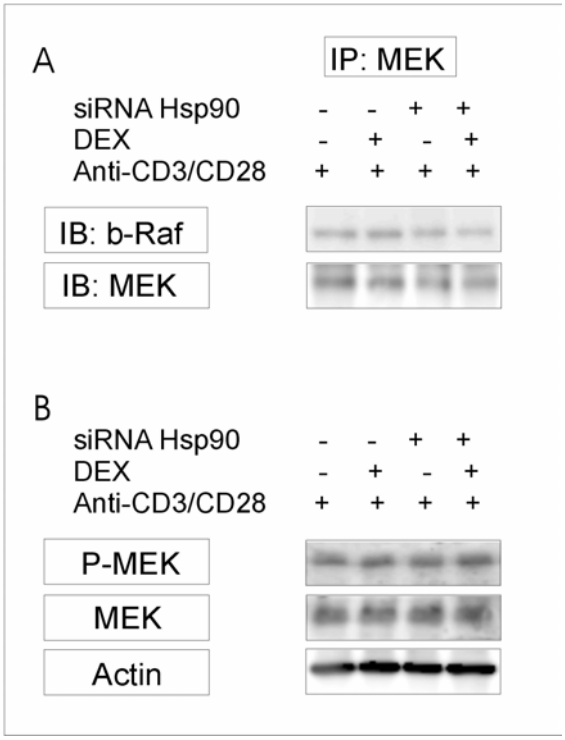
Figure 1 Suppl. Information

Supplementary Fig. 1. GR-Lck and GR-CD4 dissociations due to DEX or Hsp90siRNA treatment. Hsp90siRNA transfected cells (+) and nontransfected (-) cells were pretreated for 10 min in the presence (+) or absence (-) of DEX (1μM) and stimulated for 15 min with (+) or without (-) anti-CD3 and anti-CD28 Abs. Cells were lysed and subjected to immunoprecipitation using the anti-GR Ab. GR immunoprecipitates were studied on Western blot for the presence of Lck and CD4. Total GR was immunoblotted to evaluate for equal loading. DEX, dexamethasone; IB, immunoblot; IP, immunoprecipitation; GR, glucocorticoid receptor

Figure 2 Suppl. Information

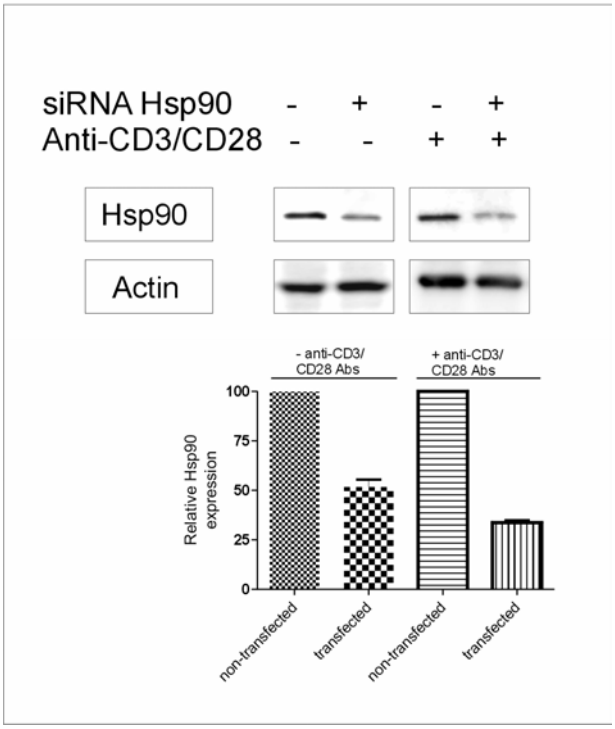
Supplementary Fig. 2. DEX does not interfere with Hsp90-ZAP70 associations. Given that Hsp90 interacts readily with ZAP70, the effect of DEX on Hsp90-ZAP70 bindings was studied in activated cells (serving as a negative control). Hsp90siRNA (non)transfected cells were pretreated with DEX (1μM; 10 min) followed by 15 min stimulation (anti-CD3/CD28 Abs). Cell lysates were subjected to immunoprecipitation using an anti-ZAP70 Ab. ZAP70 immunoprecipitates were analyzed on Western blot for the presence of Hsp90 and ZAP70. DEX, dexamethasone; IB, immunoblot; IP, immunoprecipitation

Figure 3 Suppl. Information



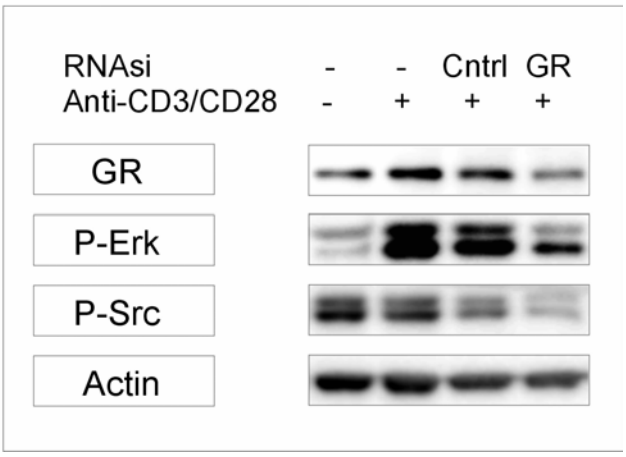
Supplementary Fig. 3. Treatment with DEX or Hsp90siRNA does not inhibit Raf-MEK complex formations. To examine the specificity of the observed effects of DEX and Hsp90siRNA on Lck and Fyn kinases, we investigated whether both treatments interfered with a different kinase cascade (i.e. b-Raf-MEK), serving as a control. Hsp90 (non)transfected cells were pretreated for 10 min with or without DEX (1 μM) and activated for 15 min using anti-CD3 and anti-CD28 Abs. (A) Cellular extracts were subjected to immunoprecipitation using an Ab against MEK, followed by immunoblotting for b-Raf and MEK. (B) Supernatants were immunoblotted for phosphorylated and total MEK, a downstream substrate of Raf. Actin expression was immunoblotted in these supernatants, serving as an additional loading control. DEX, dexamethasone; IB, immunoblot; IP, immunoprecipitation; P, phosphorylated

Figure 4 Suppl. Information



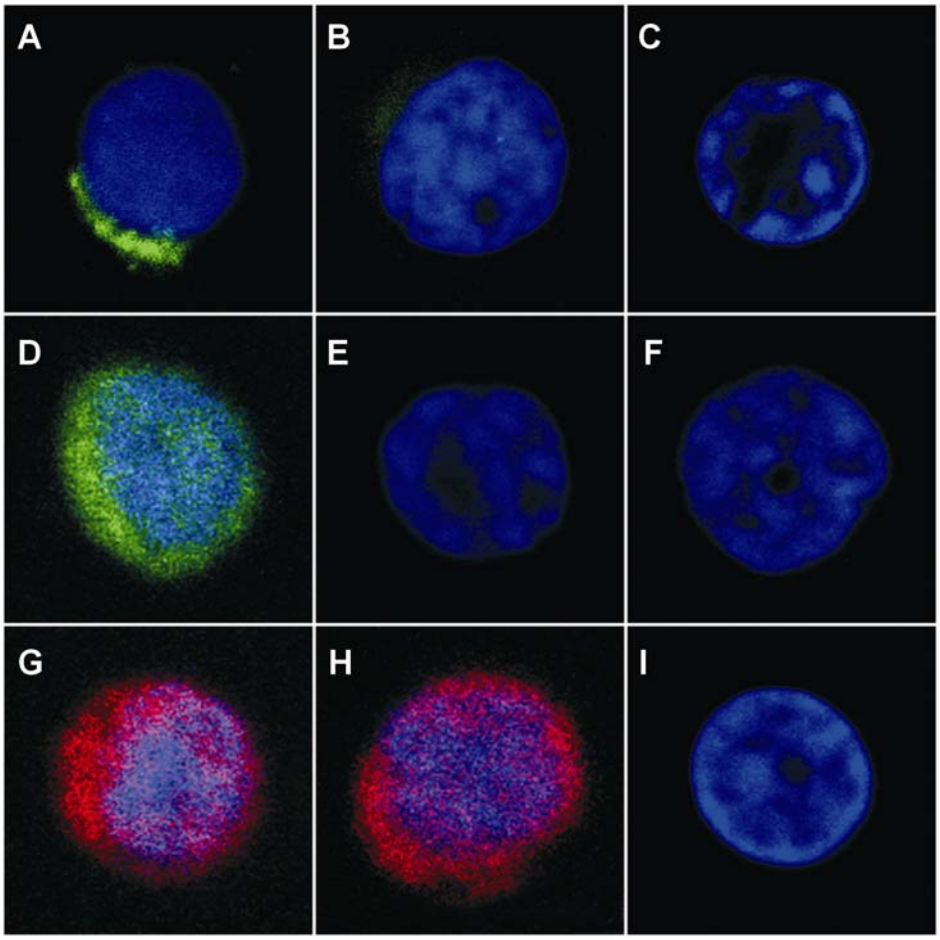
Supplementary Fig. 4. Reduced Hsp90 expression due to Hsp90siRNA transfection. Cells were transfected with or without Hsp90siRNA by electroporation. The transfection procedure was evaluated by immunoblotting cellular extracts prepared from quiescent (-) and activated (+) cells. Hsp90 protein levels were determined by the ratio of Hsp90 signal intensity compared to actin; the data are expressed as means ± SEM. Three independent experiments were performed and reproducible results were obtained.

Figure 5 Suppl. Information



Supplementary Fig. 5. GRsiRNA control experiments. Cells were transfected with GRsiRNA or with unrelated siRNA (negative control). After overnight incubation, cells were incubated in the absence or presence of anti-CD3/anti-CD28 Abs for 15 min. Cell lysates were immunoblotted employing Abs against GR, phosphorylated-Erk and phosphorylated-Src. An Ab against actin was used to evaluate for equal protein loading. GR, glucocorticoid receptor; Cntrl, control siRNA

Figure 6 Suppl. Information



Supplementary Fig. 6. Immunofluorescent specificity controls. Immunofluorescent staining for Hsp90 (FITC-labeled, green) was performed to compare nontransfected cells (A) with Hsp90siRNA transfected cells (B). Stainings with a goat secondary Ab in the absence of the primary Hsp90 Ab represents a negative control (C). Cells stained with anti-Glucocorticoid-Receptor (GR) Ab (FITC-labeled, green) (D). The specificity of the anti-GR Ab was confirmed using GR immunizing peptide (sc-1003P). After preincubation with the immunizing peptide (i.e. anti-GR Ab versus immunizing peptide: 1:50), staining for GR remained negative (E). Cells stained with a rabbit secondary Ab, in the absence of anti-GR Ab, served as an additional negative control (F). Immunofluorescent stainings using anti-Fyn (G) and anti-Lck (H) Abs (TRITC-labeled, red) are shown. Appropriate controls for Lck and Fyn included stainings of cells employing the mouse secondary Ab (anti-TRITC) in the absence of primary anti-Lck or anti-Fyn Abs (I). The nucleus was visualized with DAPI (blue). To be able to compare the amount of fluorescence in the illustrations, all cells were imaged with identical instrument settings (i.e. laserpower, pmt settings, pinhole diameter and pixelsize). Panels of optical cross sections through T cells are shown; scanned area for all

images: 9 μM x 9 μM. At least 20 cells of each condition were scanned and representative cross sections are indicated.