

Activation of ER α Signaling Differentially Modulates IFN- γ Induced HLA-Class II Expression in Breast Cancer Cells

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Abstract

The coordinate regulation of HLA class II (HLA-II) is controlled by the class II transactivator, CIITA, and is crucial for the development of anti-tumor immunity. HLA-II in breast carcinoma is associated with increased IFN- γ levels, reduced expression of the estrogen receptor (ER) and reduced age at diagnosis. Here, we tested the hypothesis that estradiol (E₂) and ER α signaling contribute to the regulation of IFN- γ inducible HLA-II in breast cancer cells. Using a panel of established ER $^+$ and ER $^-$ breast cancer cell lines, we showed that E₂ attenuated HLA-DR in two ER $^+$ lines (MCF-7 and BT-474), but not in T47D, while it augmented expression in ER $^-$ lines, SK-BR-3 and MDA-MB-231. To further study the mechanism(s), we used paired transfecants: ER α^+ MC2 (MDA-MB-231 c10A transfected with the wild type ER α gene) and ER α^- VC5 (MDA-MB-231 c10A transfected with the empty vector), treated or not with E₂ and IFN- γ . HLA-II and CIITA were severely reduced in MC2 compared to VC5 and were further exacerbated by E₂ treatment. Reduced expression occurred at the level of the IFN- γ inducible CIITA promoter IV. The anti-estrogen ICI 182,780 and gene silencing with *ESR1* siRNA reversed the E₂ inhibitory effects, signifying an antagonistic role for activated ER α on CIITA pIV activity. Moreover, STAT1 signaling, necessary for CIITA pIV activation, and selected STAT1 regulated genes were variably downregulated by E₂ in transfected and endogenous ER α positive breast cancer cells, whereas STAT1 signaling was noticeably augmented in ER α^- breast cancer cells. Collectively, these results imply immune escape mechanisms in ER α^+ breast cancer may be facilitated through an ER α suppressive mechanism on IFN- γ signaling.

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Introduction

Antigen presentation by major histocompatibility complex (MHC) class II molecules (MHC-II), known as HLA-II (HLA-DR, -DP, -DQ) in humans and co-chaperones HLA-DM and the invariant chain (Ii) are important for the development of adaptive immune responses including anti-tumor immunity [1–4]. Typically, HLA-II expression is limited to professional antigen presenting cells (pAPC), but is induced by IFN- γ on most cell types including those derived from cancer [5,6]. HLA-DR positive tumor cells have been described in several malignancies, such as melanoma [7], colon [8,9] and breast [10–12], but the underlying mechanisms are likely diverse. The number of HLA-II positive tumor cells in breast cancer is directly associated with tumor infiltrating immune cells and levels of IFN- γ [12–14], but other cytokines, hormones, growth factors and oncogenes are also implicated in regulating HLA-II expression [15–20].

HLA-II expression is controlled at the transcription level by a highly conserved regulatory module, located in the promoter of genes encoding the α - and β -chains of all HLA-II molecules and in the gene encoding the Ii co-chaperone [21–26]. This regulatory module forms a platform for the class II transactivator (CIITA), a

non-DNA binding protein, which acts as a transcriptional integrator by connecting transcription factors, bound to the MHC-II promoter with components of the general transcriptional machinery [23,27–30]. The central role of CIITA is evident from lack of constitutive or IFN- γ inducible HLA-II in bare lymphocyte syndrome [31,32].

CIITA expression is controlled by three distinct promoters: promoter I (pI) for constitutive expression in dendritic cells; promoter III (pIII), for constitutive expression in B cells; promoter IV (pIV) for IFN- γ inducible expression [21,26,33]. This promoter system is crucial for controlling CIITA messenger RNA (mRNA) and protein levels, and they, in turn, regulate HLA-II expression. The molecular regulation of CIITA pIV is intricately linked to the classical IFN- γ signaling pathway. IFN- γ binds to IFN- γ receptors (IFNGR) on the cell surface, resulting in autophosphorylation of Janus kinase 2 (JAK2) and JAK1, followed by phosphorylation, dimerization and nuclear translocation of signal transducer and activator of transcription 1 (STAT1) [34,35]. Phosphorylated STAT1 (pSTAT1) binds to IFN-activated sites (GAS) in the promoter of target genes including the IFN-regulatory factor 1 (IRF1), thus stimulating its expression. IRF1 binds cooperatively with IRF2 to its associated IRF element (IRF-E) in CIITA pIV,

and concomitant pSTAT1 binding to GAS in CIITA pIV results in transcriptional activation of CIITA [33,36]. Moreover, signaling pathways such as mitogen activated protein kinases (MAPK) and PI3K/Akt that are frequently activated in breast cancer cells [37] modulate expression of IRF1 and STAT1 [38–40], further impacting the levels of IFN- γ inducible CIITA and subsequent HLA-II expression on tumor cells.

Previously, we showed that HLA-II (HLA-DR, HLA-DM and Ii) was discordantly expressed on tumor cells in human breast cancer tissues [12]. Furthermore, tumor cell expression of HLA-DR and Ii, but not HLA-DM, correlated with reduced expression of estrogen receptors (ER) and reduced age at diagnosis. Importantly, tumors with coordinate expression of HLA-DR, Ii and HLA-DM had the highest IFN- γ mRNA levels and correlated with increased patient survival [12]. Undoubtedly, the mechanisms governing tumor cell expression of HLA-II in breast carcinoma are likely multifaceted, involving IFN- γ secreted by infiltrating immune cells [12], circulating and tumor-associated estrogens [41] and activation of growth factor and hormone receptor pathways in the tumor cells [42,43]. Estradiol and anti-estrogens, tamoxifen and fulvestrant or ICI 180,720 (ICI), were shown to modulate IFN- γ inducible MHC-II in various cell types [17,19,44,45] through mechanisms not involving ligand activation of the estrogen receptor (ER) pathway.

In this study, using established human ER $^-$ and ER $^+$ breast cancer cell lines (BCCL) and an ER α -transfected BCCL, we investigated the specific and combined effects of estradiol (E $_2$) and ER α on HLA-II regulation. We found IFN- γ inducible HLA-II expression was modulated by E $_2$ -ER activation at the level of the CIITA pIV. Furthermore, E $_2$ -treatment of ER α $^+$ BCCL and ER α $^-$ BCCL differentially affected various components of the IFN- γ signaling pathway that are required for transactivation of CIITA pIV.

Results

Estradiol differentially modulates HLA-DR expression in breast cancer cell lines

Stemming from our previous finding that HLA-II expression in breast carcinoma tissues correlates with increased IFN- γ mRNA, reduced age at diagnosis and reduced ER levels [12] we questioned whether E $_2$, in the absence or presence of its cognate receptor ER α , modulates HLA-DR expression in established ER $^-$ and ER $^+$ BCCL, treated or not with IFN- γ for 96 hours. Analysis of ER $^-$ BCCL using flow cytometry (Figure 1A & 1B) revealed low basal expression of HLA-DR in MDA-MB-231, but not in SK-BR-3 while IFN- γ induced strong expression in both cell lines. E $_2$ -treatment augmented IFN- γ inducible HLA-DR, although this was significant for only SK-BR-3 (Figure 1B). These results, confirmed by Western blot analysis of cell lysates (Figure 1C & 1D), suggest E $_2$ may modulate HLA-DR expression in ER $^-$ breast cancer through an ER α independent mechanism [46].

Since the least HLA-DR in human breast carcinoma tissues occurred in ER $^+$ tumors [12] we hypothesized that E $_2$ -activation of the ER α pathway inhibits HLA-DR expression. Analysis of ER $^+$ BCCL, treated as described above, revealed a variable pattern of IFN- γ inducible HLA-DR expression with amounts that were barely detectable, moderate and abundant in BT-474, MCF-7, and T47D, respectively (Figure 1E & 1F). Constitutive HLA-DR was detected at the cell surface in only T47D (Figure 1E). Furthermore, E $_2$ treatment significantly reduced HLA-DR in MCF-7 and BT-474, but not in T47D (Figure 1E). Similar results were obtained from Western blot analysis of cell lysates (Figure 1G & 1H). Notably, ER α levels were not altered by IFN- γ but E $_2$

treatment increased the amount in the nucleus, indicating ligand activation of the ER α pathway (Figure 1G). Taken together these data suggest that E $_2$ -inhibition of HLA-II expression in ER α $^+$ BCCL is mediated through activation of ligand-dependent ER α pathway.

Transfection of ESR1 in an ER $^-$ cell line diminishes IFN- γ inducible HLA-II proteins

To further explore the role of ER α on IFN- γ inducible HLA-DR, we used two stably transfected cell lines, derived from MDA-MB-231 clone 10A [47,48]: MC2 expresses wild type ER α and VC5 expresses the empty vector. Since MDA-MB-231 clone 10A was selected for negative expression of ER α and ER β [47], the transfected pair is a suitable model to assess ER α mediated effects on HLA-II without interference from other ERs including GPR30, reported to be deficient in MDA-MB-231 [48,49]. The cells, treated and analyzed for HLA-DR expression as described above, revealed significantly reduced cell surface HLA-DR in MC2, as compared to VC5 and MDA-MB-231 clone 10A (Figure 2A & 2B). Moreover, E $_2$ -treatment greatly diminished HLA-DR in MC2 but not in VC5 and MDA-MB-231 clone 10A. These results were confirmed by Western blot analysis of cell extracts (Figure 2C). Again, HLA-DR protein in the ER α $^+$ MC2 was severely reduced and exacerbated by E $_2$, whereas MDA-MB-231 clone 10A and VC5 expressed abundant HLA-DR in the presence and absence of E $_2$. As the only known difference between MC2 and VC5 is the expression of ER α , these results further implicate ER α in negatively regulating HLA-DR expression.

Although HLA-II genes are coordinately regulated [25], we found most breast cancer lesions with HLA-DR $^+$ tumor cells do not have detectable HLA-DM expression [12]. We reasoned that if ER α and its activation by E $_2$ coordinately down regulates HLA-II, then blocking ER signaling with ICI, a selective anti-estrogen that degrades ER, should reverse the inhibition. To test this hypothesis, MC2 and VC5 were pretreated with 10 $^{-6}$ M ICI in the presence or absence of 10 $^{-9}$ M E $_2$. Following stimulation with IFN- γ for 96 hours, HLA-DR, -DM and Ii were analyzed by flow cytometry and Western blot. HLA-DR, -DM and Ii expression levels were significantly reduced in MC2 compared to VC5 (Figure 3A–3C), while E $_2$ -treatment further diminished HLA-II expression in MC2, but not in VC5. Although ICI-treatment, alone or with E $_2$, did not restore HLA-II in MC2 to VC5 levels, it clearly reversed the E $_2$ -inhibitory effect on HLA-II expression. Western blot analysis (Figure 3D–3G) and immunocytochemistry (data not shown) confirmed the reduced expression of HLA-DR, -DM and Ii in MC2 and the involvement of ER α signaling in the inhibitory effect of E $_2$ on HLA-II expression.

Activation of the ER α signaling pathway impedes CIITA expression

Since HLA-II expression is coordinately regulated by CIITA, we predicted that ER α interfered with CIITA expression in ER α -expressing MC2. MC2 and VC5 were pretreated with E $_2$ and/or ICI, as described above, followed by addition of IFN- γ for 24 hours. Western blot analysis of nuclear and cytoplasmic extracts showed inducible CIITA expression in MC2 was about 70% of VC5 levels (Figure 4A & 4B). E $_2$ -treatment further reduced CIITA in MC2 while increasing the amount of nuclear ER α ; in contrast, ICI reversed the inhibitory effect of E $_2$ on CIITA expression, coincident with ICI-mediated reduced ER levels (Fig 4A Lanes 7 and 8). These results indicated that E $_2$ inhibits HLA-II expression by downregulating CIITA expression.

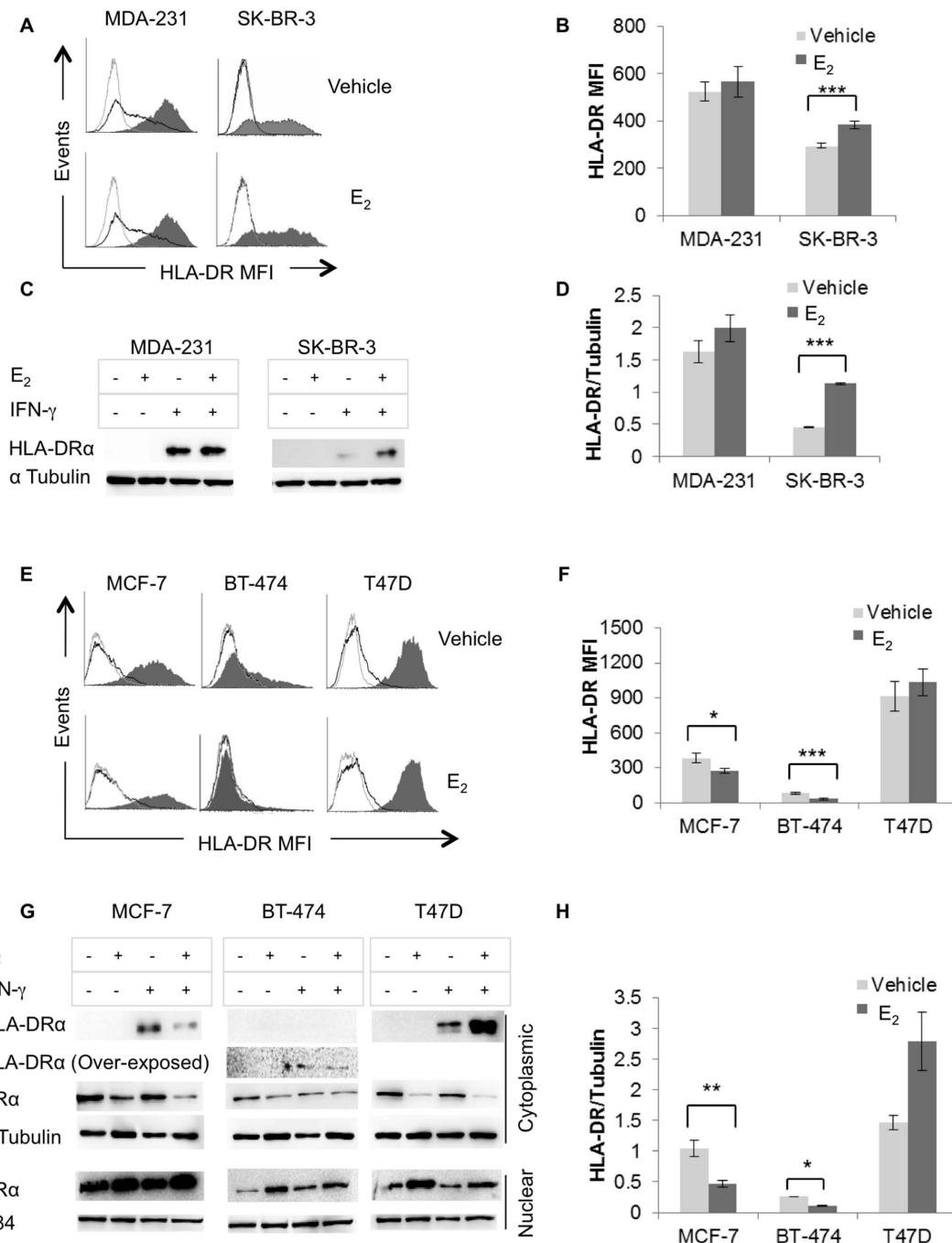


Figure 1. E₂ differentially modulates inducible HLA-DR expression in ER α ⁺ and ER α ⁻ breast cancer cell lines. MDA-MB-231, SK-BR-3, MCF-7, BT-474, and T47D were cultured in E₂-depleted media, treated with vehicle (ethanol) or E₂ (10^{-9} M) and stimulated or not with IFN- γ (100 U/ml) for 96 hours. (A & E) HLA-DR cell surface expression (L243) was analyzed by flow cytometry: grey line, isotype control; black line, constitutive expression; shaded histogram, IFN- γ induced expression. (B & F) Bar graphs represent the MFI (mean fluorescence intensity) \pm SEM for HLA-DR expression of three independent experiments. (C & G) Western blot analysis was performed on cytoplasmic and nuclear extracts for ER α expression (HC-20) and on cytoplasmic extracts for HLA-DR α (TAL 1B5). Protein loading controls included α -tubulin (B-7) and P84 (5E10) for cytoplasmic and nuclear proteins, respectively. (D & H) Bar graphs show the ratio of band intensity for HLA-DR α , normalized to the α -tubulin band intensity and represent the mean \pm SEM of three independent experiments (*p<0.05, **p<0.01, ***p<0.001).

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To further determine the inhibitory effect of E₂ on CIITA gene expression, VC5 and MC2 cells were pretreated with E₂ and/or ICI for 1 hour and then stimulated with and without IFN- γ for 4 hours, an optimal time for CIITA mRNA expression [50]. CIITA transcription was induced in both VC5 and MC2, but the

induction of CIITA mRNA in MC2 was about half in VC5 (Figure 4C). E₂ further decreased CIITA mRNA in MC2, while ICI reversed the E₂-mediated effect on CIITA.

To confirm the above results, we silenced the ER α transgene in MC2 using *ESR1* siRNA and then treated with E₂ or vehicle

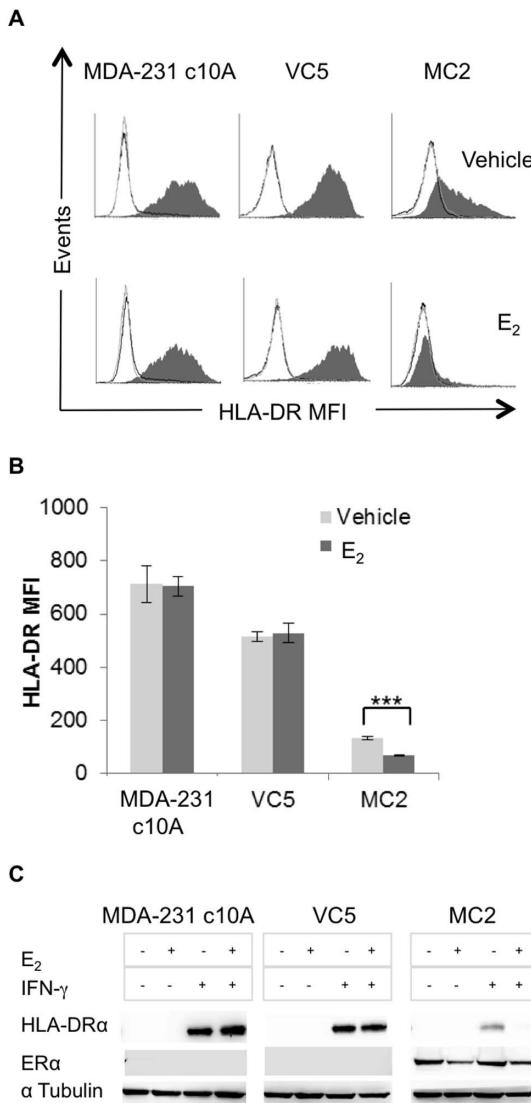


Figure 2. IFN- γ inducible HLA-DR is down regulated in the ER α^+ transfected breast cancer cell line, MC2. MDA-MB-231 clone 10A (MDA-231 c10A), VC5 (MDA-231 c10A, transfected with the empty plasmid vector) and MC2 (MDA-231 c10A, transfected with wild type ESR1) were cultured in E₂-depleted medium and stimulated or not with IFN- γ (100 U/ml) for 96 hours. (A) HLA-DR cell surface expression (L243) was analyzed by flow cytometry: grey line, isotype control; black line, constitutive expression; shaded histogram, IFN- γ induced expression. (B) Bar graphs represent the MFI \pm SEM for HLA-DR expression of three independent experiments (**p<0.001). (C) Western blot analysis was performed on whole cell lysates for HLA-DR α (TAL 1B5) and ER α (HC-20).

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control followed by IFN- γ stimulation for 24 hours. VC5, treated in the same way, was used as a control. Western blot analysis of cell lysates showed ER α was greatly reduced in MC2 transfected with ESR1 siRNA, but not with scrambled siRNA (Figure 5A). Similar to the ICI-mediated effects, ESR1 siRNA clearly reversed the E₂-mediated inhibition observed in the scrambled siRNA transfectants. E₂ increased CIITA in the ER $^-$ VC5, whether transfected with scrambled or ESR1 siRNA. Analysis of CIITA transcripts using real time PCR on siRNA-treated cells (Figure 5B), revealed equivalent levels of CIITA transcripts in ESR1 and scrambled siRNA transfectants; again, ESR1-siRNA abolished the

inhibitory effect of E₂ on constitutive and induced CIITA transcripts. These results suggest a mechanism whereby E₂-activated ER interferes with CIITA transcription induced by IFN- γ in breast cancer cells.

E₂ activated ER α inhibits CIITA promoter IV activity

Since IFN- γ inducible HLA-II expression requires activation of CIITA pIV [33], we hypothesized that E₂ activation of ER α interferes with CIITA pIV activity. We transfected VC5 and MC2 with a CIITA pIV luciferase construct and treated the cells with E₂ and/or ICI, followed by stimulation or not with IFN- γ for 12 hours. E₂-treatment further reduced both basal and IFN- γ induced CIITA pIV activity in MC2, while ICI reversed the inhibitory effect of E₂ in MC2 cells (Figure 6). Treatment with ICI and/or E₂ did not significantly affect constitutive or IFN- γ inducible CIITA pIV activity in VC5.

To determine whether E₂ directly regulates CIITA pIV activity, we searched for presence of ERE sites using three different computer software programs (<http://flbind.hgc.jp/>, <http://alggen.lsi.upc.es/> and <http://www.cbrc.jp/index.eng.html>) and identified four putative ERE sites in CIITA pIV (Figure 7A, bold letters in boxes). Sites 1 to 3 are upstream of the STAT1 and IRF1 binding sites. Site 4 is downstream of these sites and precedes the start codon. To determine if either of these sites serves as an ER α repressor of CIITA transcription, three deletion mutant constructs (Site 1/2 deletion mutant, Site 3/4 deletion mutant and Site 1–4 deletion mutant) were created (Figure 7A, open boxes). VC5 and MC2, transfected with one of the mutant CIITA pIV constructs, were pretreated with E₂ or vehicle control and then stimulated with IFN- γ for 12 hours, followed by measurement of luciferase activity (Figure 7B, left panel). All three deletion constructs demonstrated significantly reduced IFN- γ stimulated CIITA pIV activity in E₂-treated MC2, similar to that observed in MC2 transfected with the wild type CIITA pIV plasmid. By comparison CIITA pIV activity was similar in E₂ or vehicle treated VC5 cells whether transfected with wild type or deletion constructs. Intriguingly, constructs Del 3 & 4 and Del 1–4 resulted in dramatic and significant loss of CIITA pIV activity in both cell lines, suggesting there may be other or overlapping sites in CIITA pIV that interact with currently unknown transcription factors for a fully active promoter. Alternatively, the deletion of these sites may have led to the creation of a novel site that has an inhibitory effect on CIITA pIV activity. Importantly, these results do not support the hypothesis that diminished CIITA pIV activity in MC2 treated with E₂ occurs via ERE sites in the proximal region of CIITA pIV.

E₂-ER α interferes with STAT1 signaling in ER α transfected MC2 cells

To explore whether STAT1 signaling, necessary for activation of CIITA pIV, is adversely affected by ER α activation, we transfected the 8 X GAS luciferase plasmid in VC5 and MC2, followed by treatment, or not, with E₂ and/or IFN- γ for 6 hours. Compared to VC5, STAT1 signal was clearly reduced in MC2 (Figure 8A & 8B); moreover, E₂ significantly reduced basal and induced GAS promoter activity by about 44% and 40%, respectively, in MC2 (Figure 8B). Although E₂ increased basal GAS promoter activity by about 28% in VC5, this was not significant; E₂ had no effect on induced activity (Figure 8A).

To test whether reduced GAS activity in MC2 was the result of reduced pSTAT1, we performed Western blot analysis on lysates from cells treated or not with IFN- γ for 15 minutes. As shown in Figure 8C, total STAT1 and pSTAT1 at tyrosine (Y) 701 and serine (S) 727 were reduced in MC2, compared to VC5. Similar

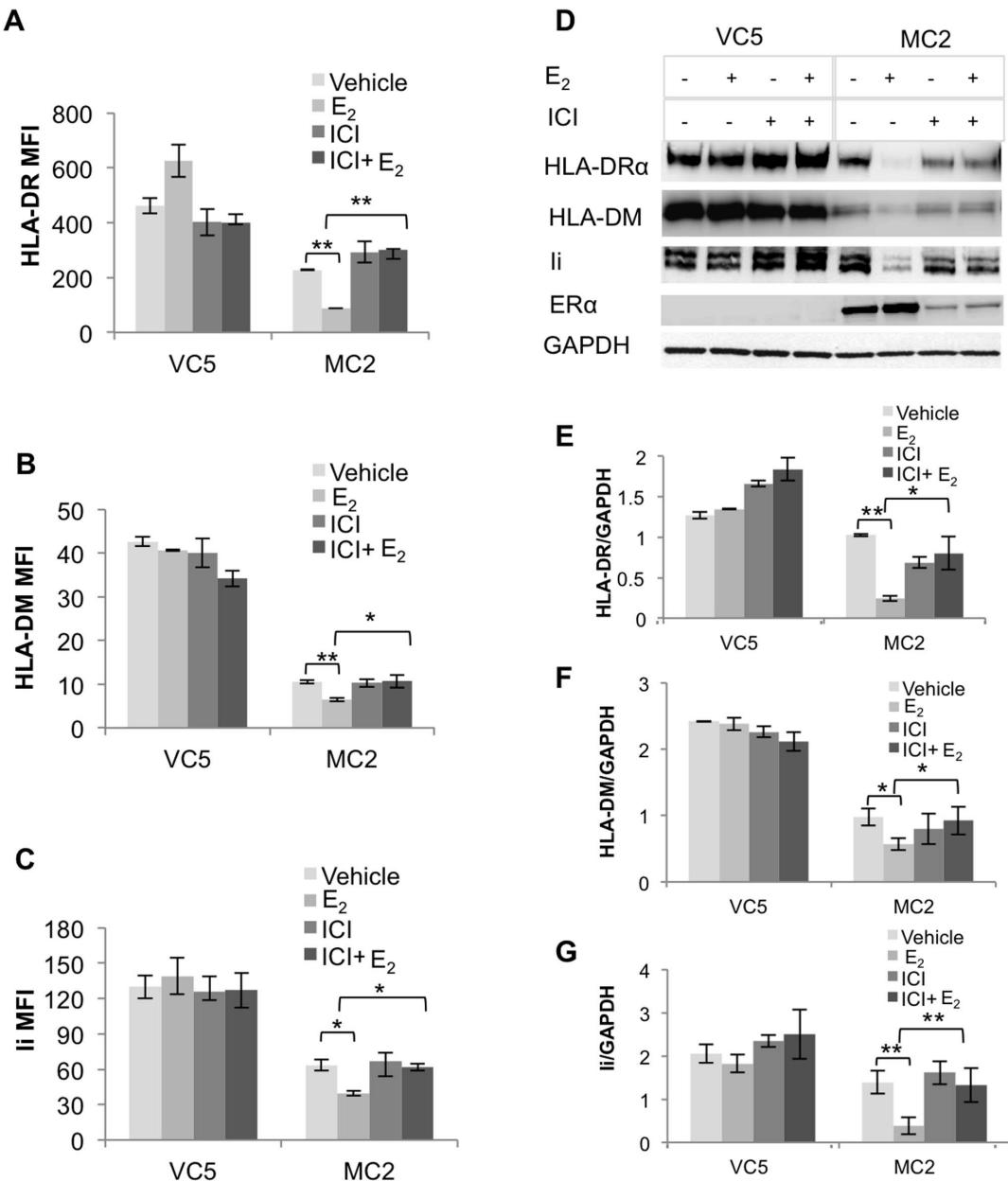


Figure 3. Coordinate downregulation of IFN- γ inducible HLA-II expression by E₂ is reversed by ICI-mediated degradation of ER α in MC2 cells. VC5 and MC2 cells were cultured in E₂-depleted media, treated with vehicle (ethanol), E₂ (10^{-9} M) or/and ICI (10^{-6} M) followed by stimulation with IFN- γ (100 U/ml) for 96 hours. HLA-II expression was analyzed by surface flow cytometry using (A) anti-DR, (L243), and intracellular flow cytometry using (B) anti-DM (Map.DM1) and (C) anti-li (LN2). Bar graphs represent the MFI \pm SEM of three independent experiments. (*p<0.05, **p<0.01). (D) Western blot analysis was performed on whole cell extracts using for HLA-DR α (TAL 1B5), HLA-DM (TAL18.1) and li (LN2); GAPDH (Ab8245) is the protein loading control. Bar graphs show the ratio of band intensities, normalized to GAPDH band intensities and represent the mean \pm SEM ratio of three independent experiments: (E) HLA-DR α /GAPDH (F) HLA-DM/GAPDH, and (G) li/GAPDH (* p<0.05, ** p<0.01).

results were observed in an experiment in which cells were also treated with E₂ for 4 hours, followed by IFN- γ treatment for 15 minutes; moreover, E₂ did not alter levels of phosphorylated or total STAT1 in MC2 or in VC5 (Figure 8D). We next examined IRF1 expression, also essential for CIITA pIV activation, in MC2 and VC5, treated with E₂ and stimulated with IFN- γ for 96 hours (Figure 8E). We found IRF1 levels were significantly decreased in MC2, compared to VC5, that E₂-treatment had only a trivial effect on IRF1 in MC2, whereas it significantly increased the levels in VC5. Collectively, these results show that ectopic expression of

ER α and, moreover, its activation by E₂ attenuates STAT1 signaling, however, E₂ has only a marginal inhibitory effect on IRF1 levels in MC2. These findings imply that attenuation of CIITA pIV and subsequent reduced HLA-II expression in ER α positive breast cancer may be due to defects in STAT1 regulation.

E₂ differentially affects IFN- γ signaling in established ER α^+ and ER α^- breast cancer cells

To ensure that attenuated STAT1 signaling in MC2 was not merely a peculiarity of the transfected model, we further analyzed

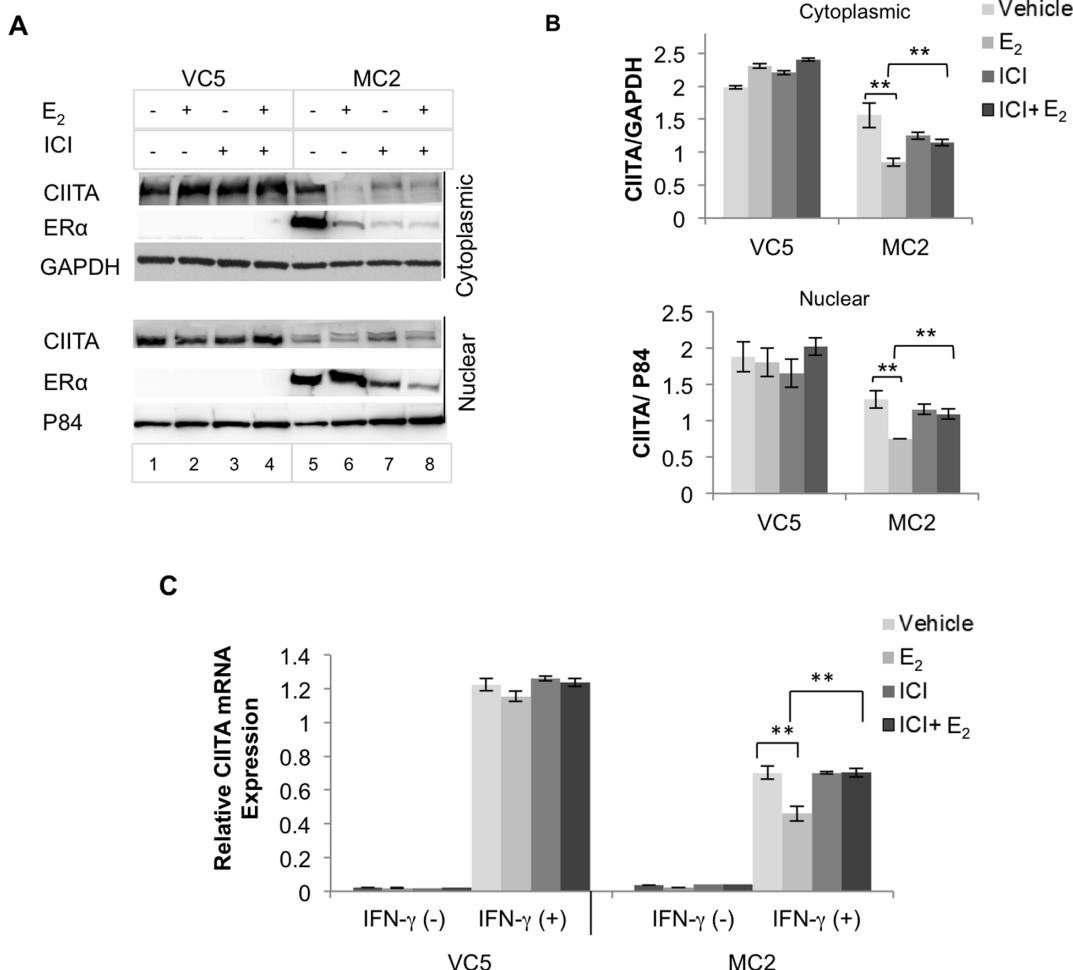


Figure 4. E₂-ER α signaling down regulates CIITA protein and mRNA expression in ER $+$ BCCL. VC5 and MC2 cells were cultured in E₂-depleted media, treated with vehicle (ethanol), E₂ (10^{-9} M) or/and ICI (10^{-6} M) and stimulated or not with IFN- γ (100 U/ml) for 24 and 4 hours, for CIITA protein and mRNA expression, respectively. (A) Western blot analysis was performed on cytoplasmic and nuclear extracts for CIITA (antiserum #21) and ER α (HC-20). (B) Cytoplasmic CIITA and nuclear CIITA were normalized to GAPDH and P84 respectively; bar graphs represent the mean \pm SEM ratio of three independent experiments (**p<0.01). (C) CIITA mRNA was relatively quantified by real time PCR using Taqman gene expression assay. GAPDH was used as an endogenous control and the data were expressed relative to a control B cell line (RAJI). Bar graphs represent the mean \pm SEM of three replicate assays (**p<0.01).

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GAS promoter activity in endogenously ER α^+ BCCL: MCF-7, BT-474 and T47D and ER α^- BCCL: MDA-MB-231 and SK-BR-3. E₂ significantly decreased IFN- γ induced GAS activity in MCF-7 and BT-474, (Figure 9A & 9B) but not in T47D (Figure 9C). To further confirm the inhibitory effect of E₂ on IFN- γ signaling in BCCLs, other than HLA-DR (Figure 1), we conducted Western blot analysis of IFN- γ inducible proteins. These included STAT1, IRF1, IRF9, a member of the IRF family of transcription factors that is not implicated in CIITA expression [51], and gamma-interferon-inducible lysosomal thiol reductase (GILT), a STAT1 regulated but CIITA-independent protein, that is important for antigen processing [52]. Basal and IFN- γ inducible STAT1 levels were not substantially altered by E₂ in either cell line (Figure 9D–9F); however, STAT1 regulated proteins, IRF1, IRF9 and GILT were differentially modulated in E₂-treated MCF-7 and BT-474 (Fig 9D & 9E).

In contrast to the E₂-inhibitory effect on GAS promoter activity in the ER α^+ lines, E₂ noticeably enhanced GAS promoter activity in ER α^- BCCL, MDA-MB-231 and SK-BR-3 (Figure 9G & 9H). Furthermore, E₂-treatment augmented expression of IRF1 and

GILT in MDA-MB-231 cells, and of STAT1 in SK-BR-3 (Figure 9I & 9J). Taken together, the results suggest that E₂ differentially modulates the IFN- γ and HLA-II pathways in ER α^+ and ER α^- BCCL.

Discussion

We previously reported the frequency of HLA-II positive tumor cells in ER $+$ breast carcinomas is decreased, compared to ER $-$ tumors from younger women [12]. As estrogen levels are high in breast carcinoma tissues, irrespective of age and menopausal status[41], we hypothesized a negative role for estrogen-activated ER α in HLA-II regulation in breast cancer cells. Herein, we provided experimental evidence that ER α and E₂-activated ER α attenuate HLA-II expression in BCCL. Using paired ER α (MC2) and vector (VC5) transfected MDA-MB-231 clone 10A cells we showed: i) E₂-treatment coordinately decreased IFN- γ inducible HLA-II and CIITA in ER α^+ MC2 but not in ER α^- VC5; ii) reduction of ER α by ICI or siRNA reversed the E₂-inhibitory effect on HLA-II expression, CIITA pIV activity and transcrip-

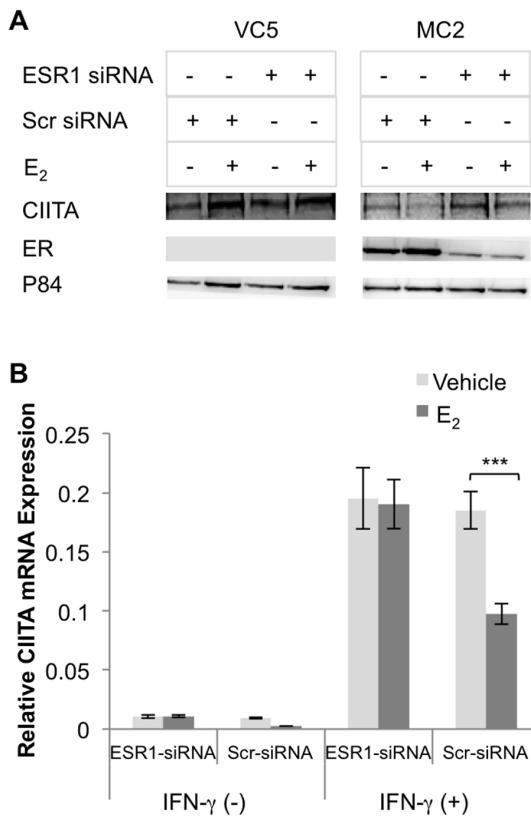


Figure 5. Silencing ER α with ESR1 siRNA reversed the inhibitory effect of E₂ on CIITA expression. (A) ER α was silenced (ESR1 siRNA) or not (scrambled siRNA) in MC2; VC5 served as an ER α negative cell control. Cells were treated with vehicle (ethanol) or E₂ (10^{-9} M) and stimulated or not with IFN- γ (100 U/ml) for 24 hours. Nuclear lysates were prepared and probed for CIITA (anti-serum #21), ER α (HC-20), and p84. Each figure represents one of three individual experiments. (B) ESR1 siRNA and scrambled siRNA transfected MC2 cells were treated with either vehicle (ethanol) or E₂ (10^{-9} M) followed by stimulation with or without IFN- γ (100 U/ml) for 4 hours and CIITA mRNA was relatively quantified by real time PCR using Taqman gene expression assay. GAPDH was used as an endogenous control and the data were expressed relative to a control B cell line (RAJI). Bar graphs represent the mean \pm SEM of three replicate assays (**p<0.001).

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tional activation of CIITA in MC2; iii) E₂-activated ER α adversely affected IFN- γ induced transcription as shown by GAS reporter assay and expression levels of IFN- γ inducible proteins. Importantly, similar results were observed in the ER α^+ BCCL, MCF-7 and BT-474, in which GAS activity, STAT1 regulated genes and HLA-DR were down regulated by E₂; by contrast, E₂ augmented GAS activity and expression of STAT1 regulated genes in the ER α^- BCCL, MDA-MB-231 and SK-BR-3.

Overall our data support a negative role for E₂-ER α signaling in the regulation of HLA-II in breast cancer cells, but cell-specific differences are evident. For example, E₂ treatment attenuated HLA-DR in MCF-7 and BT-474, but not in T47D. This finding is compatible with an older study in which BCCL, cultured in E₂-sufficient medium, exhibited a hierarchy of IFN- γ inducible HLA-DR levels with T47D>MCF-7>BT-474 [6]. Differential HLA-II in these cells is not surprising, given that ER $^+$ BCCL, although expressing many of the same genes associated with a luminal subtype, will differ in expression of many other genes [53], which may or may not be regulated by E₂. Multiple factors including the

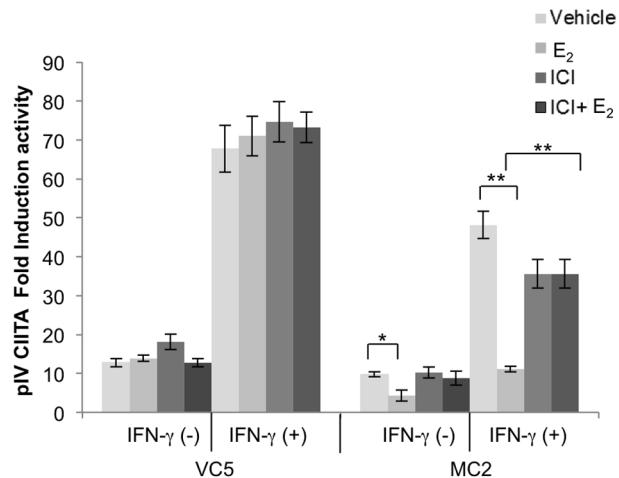


Figure 6. E₂-ER α signaling pathway interferes with CIITA pIV activity in MC2. VC5 and MC2 cells were cultured in E₂-depleted media followed by transfection with CIITA pIV luciferase constructs. On the following day, cells were treated with vehicle (ethanol), E₂ (10^{-9} M) and/or ICI (10^{-6} M), and stimulated or not with IFN- γ (100 U/ml) for 12 hours. Data are expressed as fold induction over the PGL2 Basic empty plasmid after controlling for transfection efficiency using cells dual transfected with GFP (Green Fluorescent Protein). The effect of ER α on the transcription activation of CIITA pIV was determined from relative luciferase activities in transfected MC2. Error bars represent the mean \pm SEM of three independent experiments (**p<0.01).

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ratio and localization of ER α and ER β receptors, levels of coactivators and corepressors, cell surface receptors such as GPR30 and EGFR and cross-talk with other signaling pathways determine which genes are up or down regulated [54]. E₂-activated ER β inhibits recruitment of ER α to ERE in target genes, thus, suppressing ER α regulated gene expression [55]. Furthermore, activation of the ER β isoform results in ER β /ER α heterodimers that are targeted for proteasomal degradation [56]. It is noteworthy, then, that E₂ increases ER β in T47D but not in MCF-7 or BT-474 [57] and the ER β : α ratio in T47D is reported to be greater than in MCF-7[53,58] thus, suggesting that cell-specific differences in ER subtypes and other receptors may underlie differential HLA expression in breast cancer.

The most convincing evidence that activated ER α modulates HLA-II and CIITA expression came from our experiments using the transfected ER α^+ line, MC2. Since MC2 and its ER α^- vector control, VC5, are derived from MDA-MB-231 clone 10A, which is negative for both ER α and ER β [47], it should be a valid model to directly assess the effect of activated ER α on the HLA-II pathway. Our finding, that E₂ attenuation of HLA-II and CIITA in MC2 could be reversed by knockdown of ER α in MC2 with ICI (Figures 3D and 4A) or siRNA (Figures 5A), provides compelling evidence that the classical ER α signaling pathway interferes with CIITA regulation. However, we were puzzled that even without adding E₂, HLA-II and CIITA were reduced in MC2 and that knockdown of ER α by ICI and siRNA did not restore CIITA activity in MC2 to VC5 levels. Although we used phenol red free medium and E₂-depleted FBS, there might still be a minimum level of E₂ in the culture medium, which is sufficient to activate ER α and suppress CIITA activity. Furthermore, the incomplete depletion of ER α by ICI or siRNA (Figures 3D, 4A & 5A), may also explain why HLA-II and CIITA expression were not completely restored.

Identification of putative ERE binding sites in the proximal region of CIITA pIV (Figure 7A) led us to explore a direct role for

A

-346 Site 1 tgagtggagagaaaacag**agacc** caccagggggtggggacaagctccctgcaactcaggacttgc**gatca** ctgcccaagtggct
 cccttagctcctggctcctggccggggcctggactctcccgaagtgggg**ctggccactgtgaggaacc** gactggaggcaggga
 GAS cctctggatgccccaggcaggcagttggatgcca**ttctgataaa** gcacgtggggccacagttagtgcttgcacagcctggccc
 IRF1 Site 3 Site 4 gagctcagcgctgcagaaa**gaaagtgaaagg** gaaaaagaactgcggggaggc**ggggaggtaggatgaccagc** ggacgagc
 tgccaCAGACTTGC CGCGCCCCAGAGCTGGCGGGAGGGAGAGGCCACCAGCAGC **+50**

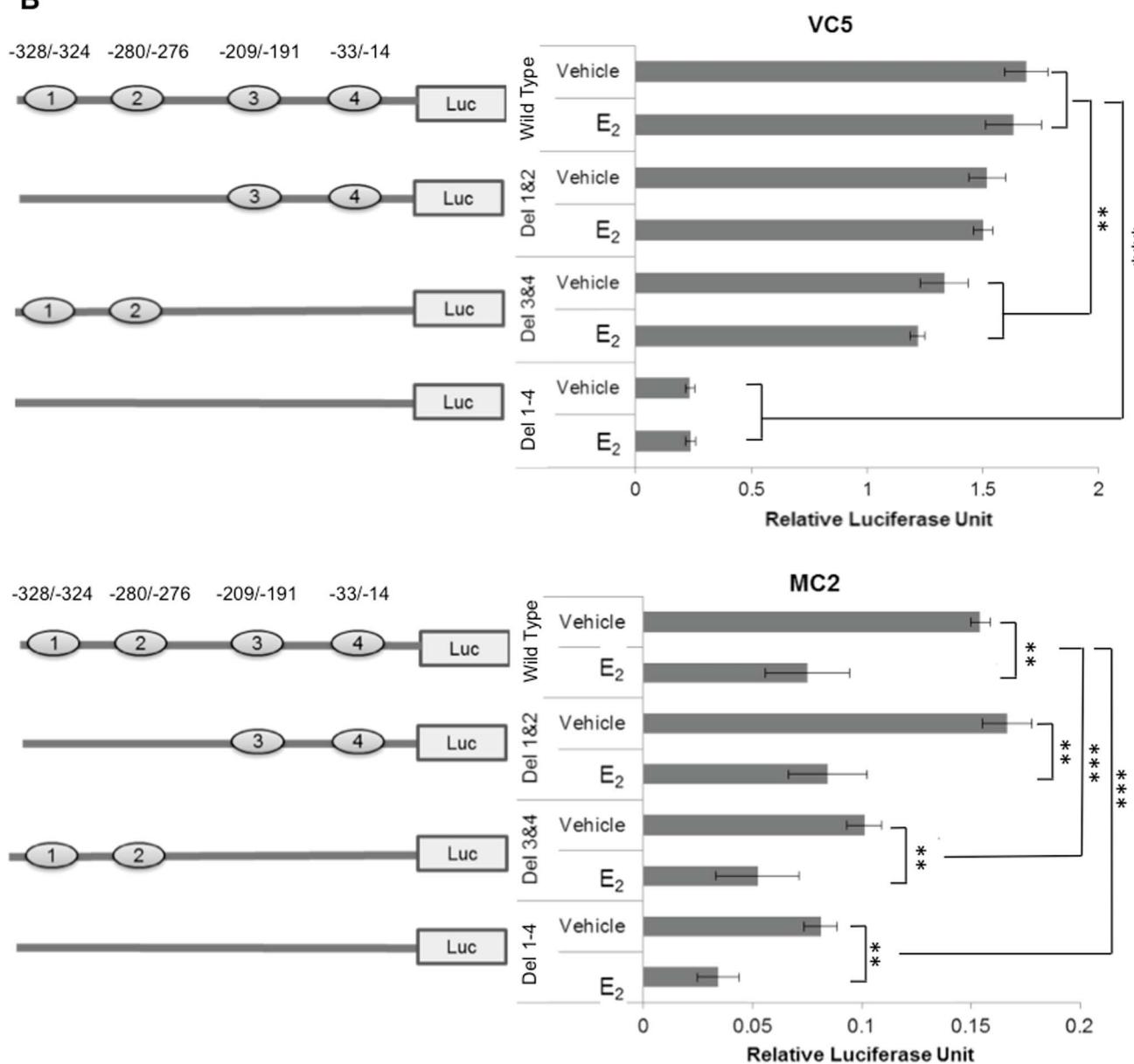
B

Figure 7. Mutation of putative ERE sites in CIITA pIV does not enhance CIITA pIV activation in MC2. (A) CIITA pIV nucleotide sequence from -346 to +50 with the GAS and IRF1 binding sites (shaded hexagon) and the predicted ERE (clear rectangles) were identified using online transcription factor prediction software, (<http://tfbind.hgc.jp/>, <http://alggen.lsi.upc.es/> and <http://www.cbrc.jp/index.eng.html>). Site directed mutagenesis was used to perform deletion of the predicted ERE. (B) VC5 and MC2 were transfected with CIITA pIV constructs, then treated with vehicle (ethanol) or E₂ (10⁻⁹ M) and stimulated with IFN- γ (100 U/ml) for 12 hours, followed by determination of luciferase activity. Bar graphs represent the mean \pm SEM of three independent experiments (**p<0.01, ***p<0.001).

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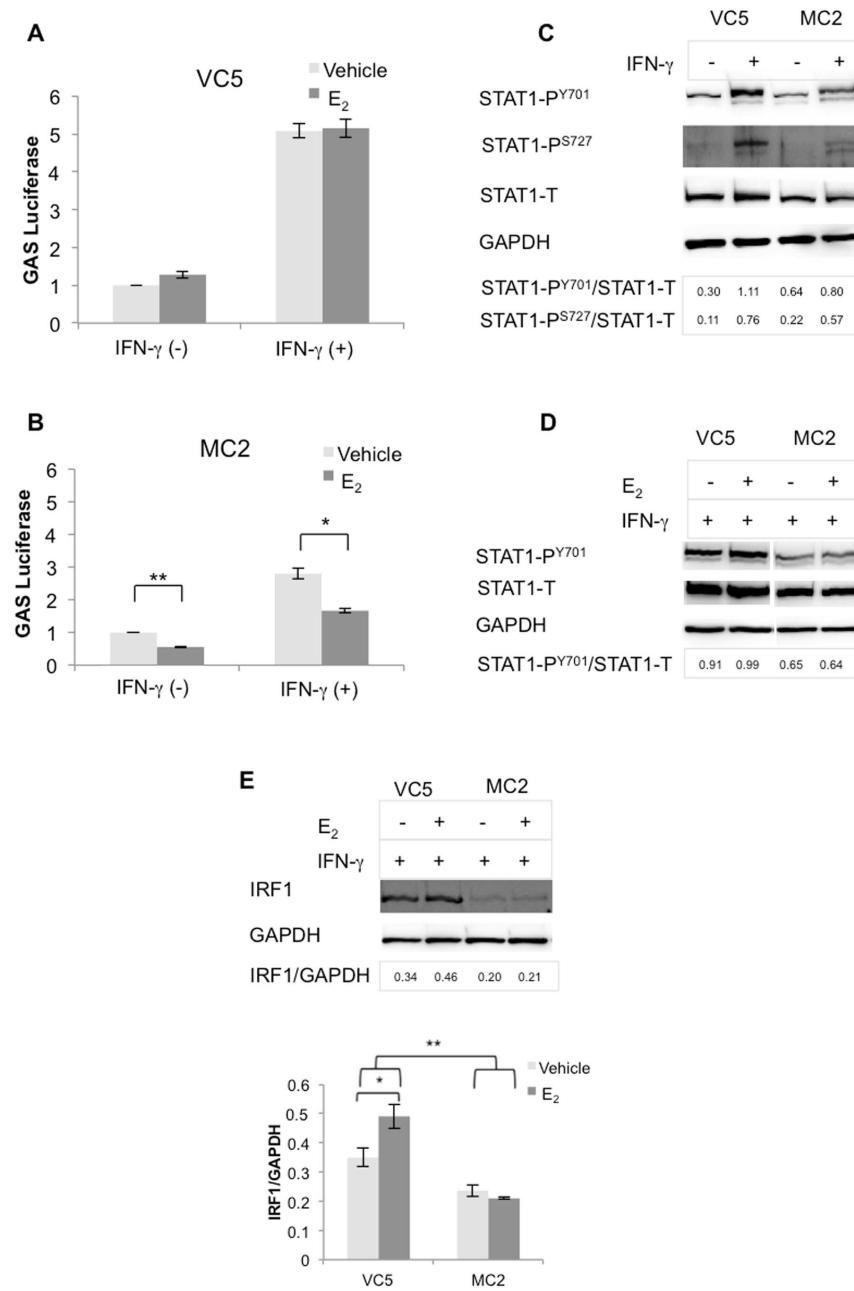


Figure 8. GAS promoter activity, STAT1 activation and IRF1 expression were reduced in MC2 as compared to VC5. (A) VC5 and (B) MC2 were cultured in E₂-depleted media and transfected with 8 X GAS binding sequence construct, then treated with vehicle (ethanol), E₂ (10⁻⁹ M) and stimulated or not with IFN- γ (100 U/ml) for 6 hours. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same samples and expressed as fold induction over the un-stimulated mock. Error bars represent the mean \pm SEM of three independent experiments (*p<0.05, ** p<0.01). (C) VC5 and MC2 were stimulated with IFN- γ (100 U/ml) for 15 minutes, STAT1 activation was detected using STAT1 Phospho-Tyrosine⁷⁰¹ and Phospho-Serine⁷²⁷ antibodies. (D) VC5 and MC2 were treated or not with E₂ (10⁻⁹ M) for 4 hours, followed by stimulation with IFN- γ (100 U/ml) for 15 minutes, STAT1 activation was detected using STAT1 Phospho-Tyrosine⁷⁰¹. (E) Western blot analysis of whole cell lysates, prepared from VC5 and MC2 stimulated with IFN- γ (100 U/ml) for 96 hours, for IRF1 (BD-20) expression. Error bars represent the mean \pm SEM of three independent experiments (*p<0.05, *** p<0.001).

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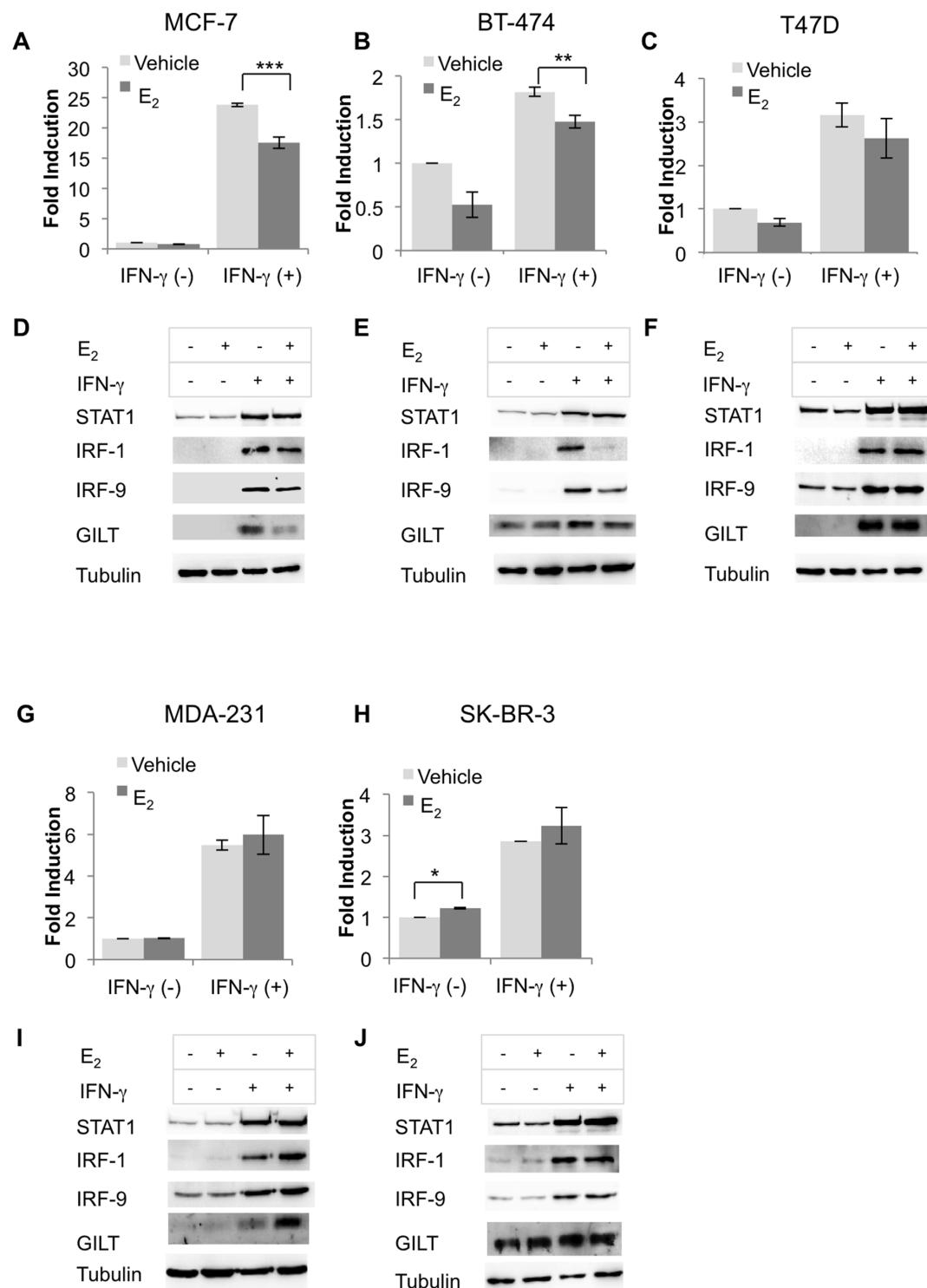


Figure 9. E₂ differentially down regulates IFN- γ signaling and IFN- γ induced proteins in endogenous ER⁺ breast cancer cell lines. (A) MCF-7, (B) BT-474, (C) T47D, (G) MDA-MB-231, and (H) SK-BR-3 were cultured in E₂-depleted media, transfected with 8 X GAS binding sequence construct, then treated with vehicle (ethanol), E₂ (10^{-9} M) and stimulated or not with IFN- γ (100 U/ml) for 6 hours. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same samples and expressed as fold induction over the un-stimulated mock. (D) MCF-7, (E) BT-474, (F) T47D, (I) MDA-MB-231 and (J) SK-BR-3 were cultured in E₂-depleted media, treated with vehicle (ethanol), or E₂ (10^{-9} M) and stimulated or not with IFN- γ (100 U/ml) for 96 hours. Western blot analysis of cytoplasmic extracts was performed for expression of IFN- γ inducible proteins: STAT1 (06-501), IRF1 (BD-20), IRF9 (C-20), GILT (T-18). Each figure represents one of three independent experiments.

ER α as a suppressor of CIITA pIV activation. Although mutagenesis of these sites did not reverse the inhibitory effect of ER α or E₂-activated ER α on CIITA pIV activity (Figure 7B), the experiments do not completely exclude direct ER α suppression of CIITA activity as there may be other unidentified ERE sites in either the proximal or distal region of CIITA pIV through which this effect is mediated. Alternatively, ER α may indirectly suppress CIITA pIV activation through interacting with another factor such as AP1 or NFK β that may bind CIITA pIV [21], or by interacting with factors such as CREB, SRC-1 and CBP/p300 [59] that interact with the regulatory elements of CIITA pIV and HLA-II promoters [23,60,61]. This remains to be further studied.

Although others have shown an E₂ inhibitory effect on MHC class II expression [17,19,44,45], the described mechanisms were not CIITA dependent. Tzortzakaki et al (2003) reported E₂-inhibition of IFN- γ inducible HLA-DR in both MCF-7 and T47D, whereby the mechanism involved sequestering the steroid receptor co-activator 1 (SRC-1) away from the HLA-DRA promoter by the E₂-activated ER [17]. Our study did not assess cofactors, but similarly, we found E₂-inhibition of DR expression and DRA promoter activity with only slightly reduced CIITA in MCF-7 (Figure 1 and data not shown). However, our results for T47D conflict with theirs, as we found no E₂ inhibition of HLA-DR in this cell line. This could be due to differences in the amounts of E₂, as their study used 3–4 log fold more than ours. Higher than physiological concentrations of E₂ were also used to show an E₂ inhibitory effect on murine MHC-II that did not involve reduced CIITA[45]. Here the E₂ inhibitory effect was mediated through reduced association of the histone acetylation transferase, CBP, with the MHC-II promoter. Since CBP is required for acetylation of histones 3 and 4 in the MHC-II promoter, this resulted in decreased transcription of MHC-II. Intriguingly, the cell lines in this study expressed both ER subtypes, which bound to the MHC I-E β promoter, but as neither ICI nor tamoxifen reversed the E₂ inhibitory effect on MHC-II promoter, they concluded the mechanism was ER-independent. Subsequently, they showed the E₂ inhibitory effect on CBP was mediated through E₂ activation of JNK MAPK pathway [45]. Although these studies are not directly comparable to ours, they do suggest additional factors may have contributed to E₂-inhibition of HLA-DR. However, the underlying mechanisms for E₂-ER α inhibition of CIITA transactivation and STAT1 signaling in breast cancer are likely to be more diverse and complex.

Studies investigating deficient CIITA and MHC class II expression in various cancer cell lines have identified epigenetic modifications that result in transcriptional silencing [61,62]. These include histone deacetylation of the CIITA pIV in squamous cell carcinomas [63] and rhabdomyosarcomas [64], and hypermethylation of the CpG islands in CIITA pIV colon and gastric carcinoma lines. Hypermethylation and recruitment of dysregulated methyltransferases were hypothesized as mechanisms for defective CIITA and HLA-II expression in metastatic breast cancer [65,66], but these studies were based on a presumed breast cancer cell line MDA-MB-435. This cell line and its metastatic variants have a controversial history [67], as there is strong evidence that they originated from a melanoma cell line [68]. However, it is conceivable that epigenetic modifications are implicated in the E₂-liganded ER α deleterious effect on CIITA pIV, as numerous epigenetic modifications have been described in breast cancer that include silencing of ER α in the MDA-MB-231 cell line and downregulation of tumor suppressor genes [69–73].

In our study the E₂ mediated downregulation of CIITA pIV and HLA-II expression in the ER α^+ BCCL appears likely due to aberrant STAT1 signaling with reduced expression of IRF1 or

reduced ability to bind the CIITA promoter. Others have shown that STAT1 and IRF1 are aberrantly expressed in some ER $^+$ breast cancer tissues and cell lines [74–77] and both have tumor suppressor properties. Chan et al (2012) reported significantly decreased STAT1 in human neoplastic tissue of ER $^+$ breast tumors and showed that knocking out STAT1 in a mouse model correlated with the development of ER $^+$ PR $^+$ luminal A adenocarcinoma [77]. Intriguingly, the reduced phosphorylation of STAT1 and reduced levels of total STAT1 in MC2, compared to VC5 (Figure 8C), whether treated or not with E2 (Figure 8D) implies that ER α somehow negatively regulates STAT1 activation and signaling. We speculate this could occur via direct interaction of ER α with STAT1, possibly interfering with dimerization and nuclear translocation or indirectly by interfering with STAT1 promoter activation. Whatever the mechanism, aberrant STAT1 signaling is likely to result in reduced IRF1 levels and subsequently reduced CIITA activation. However, as ICI treatment of MC2 did not substantially increase STAT1 levels (data not shown), nor completely degrade ER α , more studies are required to test this concept.

A potential explanation for the dramatic reduction of CIITA pIV activity in MC2 is decreased IRF1 (Figure 8D), which is essential for IFN- γ inducible CIITA transcriptional activation and HLA-II expression [50,78,79]. Furthermore, E₂ diminished IRF1 in MCF-7 and dramatically reduced its expression in BT-474, a cell line that expresses insignificant amounts of HLA-DR in the presence and absence of E₂ (Figures 1 & 9). In contrast, ER α^- lines appear to have an intact IFN- γ signaling pathway that is not inhibited by E₂. We did not investigate mechanisms underlying E₂-mediated increase in GAS and STAT1 activity, but others have shown a dependency on SRC kinase activity [80]. Furthermore, E₂ also activates other pathways such as MAPK and PI3K pathways that interact with the JAK-STAT1 pathway [40,81,82].

In conclusion, our results show that HLA-II expression is regulated differently by estrogen in ER $^-$ and ER $^+$ breast cancer cells. To our knowledge this report is the first to show that activation of ER α by its ligand E₂, results in downregulation of CIITA pIV activity. Although the mechanism is not fully elucidated, the data suggest that the dysregulation occurs at the level of STAT1 activation. Such a mechanism would explain the HLA-DR negative tumor cells in breast carcinomas despite infiltrating T-cells and high levels of IFN- γ and has further implications for tumor immune escape.

Materials and Methods

Cells

Breast cancer cell lines, obtained from ATCC, included: ER α^+ (MCF-7, T47D, and BT-474) and ER α^- (SK-BR-3, MDA-MB-231 (MDA-231). Cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, antibiotic-antimycotic mixture (100 units/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 0.25 μ g/ml amphotericin B as Fungizone $^{\circledR}$), all from Invitrogen. MDA-MB-231 clone 10A and two stably-transfected lines, MC2 (MDA-MB-231 clone 10A transfected with *ESR1* (*NM_000125*) and VC5 (MDA-MB-231 clone 10A transfected with an empty vector) were generous gifts from Dr. Craig Jordan. Cells were grown in phenol red free minimum essential medium (MEM) (Invitrogen) supplemented with 5% charcoal/dextran heat inactivated FBS (CD FBS) (Hyclone), MEM non-essential amino acids, 6 ng/ml recombinant human insulin, 2 mM L-glutamine and antibiotic-antimycotic mixture (all from Invitrogen). MC2 and VC5 were maintained

under selective conditions with G418, 5 μ g/ml (Sigma). For experiments cell lines were detached with 0.25% trypsin (Invitrogen) and plated at 3×10^5 cells/well in 6-well plates or 2×10^4 cells/well in 96-well plates. After 24 hours medium was replaced with fresh medium containing 10^{-9} M E₂ and/or 10^{-6} M ICI (Sigma) or vehicle control (ethanol) and left unstimulated or stimulated with IFN- γ , 100 units/ml (BD Biosciences) for the indicated times depending on the experiment.

Antibodies

Expression of HLA-II and CIITA was determined as follows: HLA-DR conformers, clone L243 [83] ATCC, purified IgG2a from supernatant diluted to 2.4 μ g/ml for flow cytometry (FC) or 10 ng/ml for Western blot analysis; HLA-DR α , mouse IgG1 (clone Tal 1B5, Abcam, 40 ng/ml, IB); II, mouse IgG1 (clone LN2, BD Biosciences, 5 μ g/ml, FC or 200 ng/ml, IB); HLA-DM, mouse IgG1 (clone MaP.DM1, BD Biosciences, 10 μ g/ml, FC and clone TAL18.1, Abcam, 40 ng/ml, IB); CIITA (rabbit antiserum # 21, diluted 1/4000), prepared in Dr. Viktor Steimle's laboratory [84]. Other antibodies used for Western blotting included anti-ER α , rabbit IgG (HC-20, Santa Cruz Biotechnology, 500 ng/ml); STAT1, rabbit IgG (06-501, Upstate Biotechnology, 200 ng/ml); STAT1 Phospho-Tyrosine⁷⁰¹ and Phospho-Serine⁷²⁷, both rabbit IgG (GenScript, 500 ng/ml); ISGF-3 γ p48 (IRF9), rabbit IgG (C-20, Santa Cruz Biotechnology, 400 ng/ml); IRF1, mouse IgG1 (clone BD-20, BD Biosciences, 125 ng/ml); GILT, goat polyclonal IgG (T-18, Santa Cruz Biotechnology, 250 ng/ml). Isotype-matched nonspecific monoclonal antibodies (mAbs) included: IgG2a (clone NSG2a) from a local source and IgG1 (clone MOPC-21, BD Biosciences). Housekeeping proteins were detected with anti-GAPDH, mouse IgG1 (clone 6C5, Abcam, 1 ng/ml); α -tubulin, mouse IgG1 (clone B-7, Santa Cruz Biotechnology, 250 ng/ml) and anti-nuclear matrix protein p84, mouse IgG2b (clone 5E10, Abcam, 1 μ g/ml). Horse Radish Peroxidase (HRP)-conjugated affiniPure F(ab)₂ fragment goat anti-mouse (GAM) IgG, Fc specific and HRP-conjugated affiniPure F(ab)₂ fragment goat anti-rabbit (GAR) IgG, Fc specific antibodies, were purchased from Jackson Immunoresearch and HRP conjugated donkey anti-goat (DAG) antibody IgG, was purchased from Santa Cruz Biotechnology.

Flow cytometry

Flow cytometry was performed as previously described [85]. Briefly, trypsin-harvested cells, 2×10^5 cells/tube, were incubated with 25 μ l of appropriate mAbs in wash buffer (0.2% CDFCS, 0.02% NaN3 in PBS) for 30 minutes at 4°C. Antibody binding was detected with phycoerythrin (PE) labeled goat anti-mouse (GAM) conjugate (Jackson Immunoresearch), followed by fixation in 1.0% paraformaldehyde (PFA) and analyzed using a FACS Calibur flow cytometer (Becton-Dickinson). For intracellular staining, the cells were fixed in 2% PFA and permeabilized with 0.2% Tween 20 in PBS (Sigma) prior to adding primary antibodies, diluted in wash buffer containing 0.2% Tween 20 and 0.5% BSA.

Western Blotting

Nuclear and cytoplasmic extracts were prepared using Nuclear Extract Kit (ActiveMotif) according to the manufacturer's protocol. Whole cell lysates (WCL) were prepared in either Triton X-100 buffer (PBS pH 7.4, Triton X-100 1%, 0.5 M ethylene-diaminetetraacetic acid) or RIPA buffer (PBS, pH7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitors aprotinin (1 μ g/ml), leupeptin (1 μ g/ml), pepstatin A (1 μ g/ml) and phenylmethylsulfonyl fluoride (10 μ g/ml). Proteins, quantified using a BCA protein assay kit

(Thermo-Fisher Scientific), were reduced with 2-mercaptoethanol and electrophoresed (10 μ g/lane) using 8–10% SDS PAGE, followed by western blotting. Membranes, treated with blocking buffer (5% milk powder in TBS-Tween (0.15 M NaCl, 0.05 M Tris pH 7.4, 0.05% Tween 20) for 1 hour, were incubated overnight with primary antibodies at 4°C. Antibody binding was detected with appropriate HRP-conjugated secondary antibodies and Immobilon Western Chemiluminescent HRP substrate (Millipore). Immunoreactivity was visualized and quantified by scanning densitometry using ImageQuant LAS 4000 and ImageQuant TL8.1 software, respectively (GE Healthcare).

Real-time RT-PCR

Total RNA, extracted using TRIzol Reagent (Invitrogen) and treated with Ambion® TURBO™ DNase to remove contaminating DNA, was quantified using NanoDrop (Thermo Scientific). The High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used for cDNA synthesis according to the manufacturer's protocol. Real time PCR was performed using TaqMan® Probe-Based Gene Expression Analysis kit for CIITA (Hs00172106_m1) and GAPDH (Hs99999905_m1) following the manufacturer's recommendations. Quantification was performed by the comparative threshold cycle ($\Delta\Delta^{CT}$) method and normalized to GAPDH using StepOnePlus™ (Applied Biosystems). A control sample without RNA and a reference sample (RAJI, B cell line) were included in each experiment.

siRNA Transfection

Cells, plated in a 6-well plate at 3×10^5 cells/well for 24 hours, were transfected with either 25 nM ON-TARGET plus SMART pool siRNA for *ESR1* or non-targeting siRNA (Dharmacon, USA) using 4 μ l DharmaFECT4 transfection reagent (Dharmacon, USA) per well according to the manufacturer's protocol. Forty-eight hours later, the cells were treated with E₂ 10⁻⁹ M or vehicle control (ethanol) and stimulated with IFN- γ , 100 units/ml, for 4 or 24 hours for mRNA and protein expression, respectively.

Reporter gene assays

The CIITA promoter IV firefly luciferase construct [79] and the 8 X GAS firefly luciferase construct [86] were kind gifts from Dr. Jenny Ting and Dr. Eleanor N. Fish, respectively. Transfection conditions were optimized using Fugene HD (Roche) transfection reagent according to the manufacturer's protocol: briefly a master mix was prepared by diluting the appropriate plasmid with Opti-MEM (Gibco) to a concentration of 0.02 μ g/ μ l; Fugene HD was added to the same mixture in the ratio of 7:2 (Fugene HD in μ l:Plasmid DNA in μ g) and left for 20 minutes at ambient temperature. Cells, plated in a 96-well plate at 2×10^4 cells/well for 24 hours, at 37°C were transfected with 5 μ l of this mixture and incubated for an additional 24 hours. The medium was then replaced with medium containing the appropriate treatments and incubated for 12 hours for CIITA pIV or 6 hours for 8 X GAS constructs. Transfection efficiency was estimated by co-transfecting the cells with SV-40 Renilla luciferase or green fluorescent protein (GFP). Luciferase activity was measured using the dual luciferase assay system (Promega) and a 96-well luminometer (Fluoroskan Ascent Fl, Labsystems).

Generation of CIITA pIV deletion constructs

Different sets of deletion mutants of P-346/+50 CIITA pIV were generated by site-directed mutagenesis using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutagen primers (Table 1) were

Table 1. Mutagen primers used to generate the CIITA PIV deletion constructs

Site 1	Original template	ctcaacccctttgtc <u>tctggg</u> tggtccccaccctg
Primers	Del –328/-324 Fw	5'-ttggagagaaacacgcaccagggtggg-3'
	Del –328/-324 Rv	5'-cccacccttgggtctgtttctccaa-3'
Site 2	Original template	gacgttgactgaacgt <u>tagt</u> gaacgggtcacggaggaa
	Del –280/-276 Fw	5'-caactcaggacttgacttgccaaagtggctc-3'
Site 3	Original template	agaggggcttcacccc <u>gacgggtacactccttgg</u> ctgacctccgctccctg
	Del –209/-191 Fw	5'-ccccgaagtggggactggaggcagg-3'
Site 4	Original template	cttgacccctccg <u>ccccccatcacttggtcg</u> ctgctgacggtgt
	Del –33/-14 Fw	5'-ctgcggggaggcggacgactgcc-3'
	Del –33/-14 Rv	5'-ggcagctgtccgcctcccgac-3'

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designed with Agilent's web-based QuikChange Primer Design Program Sequences. Sequences, deleted from the original template, are bolded and underlined. All deletions were confirmed by sequencing.

Statistics

Statistical analysis was performed using Microsoft excel 2010 software. One-way analysis of variance (ANOVA) and Tukey post hoc tests were used for comparisons within a group. The student t-

test was used for comparing two different treatments for one cell. All tests were two-sided and $p < 0.05$ was considered significant.

Author Contributions

Conceived and designed the experiments: AAM SD. Performed the experiments: AAM DC YK MNY. Analyzed the data: AAM SD KH VS. Contributed reagents/materials/analysis tools: SD KH VS. Wrote the paper: AAM SD.

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