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C/EBPα Induces Highly Efficient Macrophage Transdifferentiation of B Lymphoma and Leukemia Cell Lines and Impairs Their Tumorigenicity

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SUMMARY

Earlier work demonstrated that the transcription factor C/EBPα can convert immature and mature murine B lineage cells into functional macrophages. Testing >20 human lymphoma and leukemia B cell lines, we found that most can be transdifferentiated at least partially into macrophage-like cells, provided that C/EBPα is expressed at sufficiently high levels. A tamoxifen-inducible subclone of the Seraphina Burkitt lymphoma line, expressing C/EBPαER, could be efficiently converted into phagocytic and quiescent cells with a transcriptome resembling normal macrophages. The converted cells retained their phenotype even when C/EBPα was inactivated, a hallmark of cell reprogramming. Interestingly, C/EBPα induction also impaired the cells' tumorigenicity. Likewise, C/EBPα efficiently converted a lymphoblastic leukemia B cell line into macrophage-like cells, again dramatically impairing their tumorigenicity. Our experiments show that human cancer cells can be induced by C/EBPα to transdifferentiate into seemingly normal cells at high frequencies and provide a proof of principle for a potential new therapeutic strategy for treating B cell malignancies.

INTRODUCTION

The induction of differentiation as a therapeutic approach for cancer had been suggested more than 3 decades ago. The idea was largely based on the findings that erythroleukemic cell lines can be induced to terminally differentiate with compounds such as DMSO and hexamethylene bisacetamide (HMBA), and myeloid leukemia cell lines with retinoids and vitamin D (Nowak et al., 2009). Later, it was shown that administration of all-*trans* retinoic acid to patients with acute promyelocytic leukemia is very effective therapeutically, especially when combined with the apoptosis-inducing compound arsenic trioxide (Waxman, 2000). Most recently, it was shown that cancer stem cells of acute myeloid leukemia (AML) can be selectively induced to differentiate using a drug that blocks dopamine receptors, strongly inhibiting tumor formation in a xenograft mouse model (Sachlos et al., 2012).

Attempts have also been made to force terminal differentiation of AML cells with the bZip family transcription factor C/EBPα. This factor is required for the formation of myelomonocytic cells (Zhang et al., 2004), and C/EBPα mutations are associated with a subset of AML (Pabst et al., 2001; Valk et al., 2004), suggesting that C/EBPα may act as a tumor suppressor in myeloid cells. In one study, it was shown that C/EBPα could convert CD34+ bone marrow-derived precursors from AML into more mature, quiescent myeloid cells (Schepers et al., 2007). In another report, the compound CDDO was found to induce the partial differentiation of the myeloid cell line HL60 into granulocytes, by stabilizing the expression of a C/EBPα isoform (Koschmieder et al., 2009). However, neither of these studies assessed the tumorigenic potential of the differentiated cells.

B cell malignancies encompass a wide variety of distinct disease entities that include mature B cell lymphomas, B cell acute lymphoblastic leukemia (B-ALL), Hodgkin lymphoma, and multiple myeloma (Campo et al., 2011). Currently, chemotherapy, radiation, and anti-CD20 antibody treatment are the mainstays of B cell lymphoma and leukemia treatment. However, the fact that a substantial number of patients are eventually not cured justifies the search for new and more effective therapeutic approaches (Shaffer et al., 2012). For example, it has recently been shown that a combination of CD47 and CD20 antibodies can abrogate the tumorigenicity of human B cell lymphoma cells in immunodeficient mice (Chao et al., 2010). In addition, small molecules that block BCL6 corepressors have been developed and are effective in experimental models of B cell lymphoma (Cerchietti et al., 2010). Other new therapies include the exogenous administration of soluble tumor suppressors (Oricchio et al., 2011). However, the differentiation induction approach has so far not been applied to the therapy of B cell neoplasms.

Our earlier work has shown that normal murine B cell precursors as well as mature antibody-producing B cells can be induced by C/EBPα to transdifferentiate into functional macrophages in vitro and in vivo (Xie et al., 2004). Using pre-B cells

Table 1. Responsiveness of B Cell Lymphoma and Leukemia Cell Lines to C/EBPα

		Changes in Cell Surface Antigen				
	Cell Line	Mac-1	CD19	Dose Dependence	C/EBPα Protein	Summary
B-ALL	RCH-ACV	↑↑↑	↓↓↓	yes	++	1/5 R, 2/5 PR, 2/5 NR
	CEMO-1	no effect	no effect	n.r.	+/−	
	Val	↑	↓	yes	+/−	
	MUTZ5	no effect	↓↓	yes	−	
	NALM-20	no effect	no effect	n.r.	−	
ABC-DLBCL	SU-DHL8	no effect	no effect	n.r.	−	2/4 PR, 2/4 NR
	U-2932	↑	↓	yes	+++	
	HLY1	no effect	no effect	n.r.	−	
	HBL1	↑	no effect	yes	−	
GCB-DLBCL	Sc-1	no effect	↓	yes	+/−	4/4 PR
	DB	no effect	↓	yes	+/−	
	DoHH2	↑	↓	yes	+	
	SU-DHL6	no effect	↓↓	yes	+/−	
MCL	G519	↑	↓↓	yes	+/−	4/4 PR
	JeKo-1	no effect	↓↓	yes	−	
	Z138	no effect	↓↓	yes	++	
	HBL2	no effect	↓↓	yes	−	
SMZL	VL51	↑	no effect	yes	−	1/1 PR
BL	Seraphina	↑↑	↓↓	yes	++	5/5 PR
	Raji	↑	no effect	yes	++	
	Daudi	no effect	↓↓	yes	+++	
	CA46	no effect	↓	yes	++	
	Namalwa	no effect	↓↓	yes	++	

The table shows the results obtained after induction of C/EBPα in B cell lymphoma and leukemia cell lines using E2 (BL lines) or doxycycline (B-ALL, ABC-DLCL, GCB-DLBCL, MCL, SMZL). Time of analysis for differentiation antigens was 4, 6, or 8 days p.i. Dose dependence was measured gating on different levels of the GFP or Tomato expression detected by FACS. C/EBPα levels were detected by western blot. The number of arrows indicates the range of cells that changes phenotype, with one arrow indicating at least 10%, two arrows at least 30%, and three arrows at least 70%. n.r., not relevant; R, responder; PR, partial responder; NR, nonresponder; B-ALL, acute lymphoblastic leukemia; ABC-DLBCL, activated B cell-like diffuse large B cell lymphoma; GCB-DLBCL, germinal center B cell-like diffuse large B cell lymphoma; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma; BL, Burkitt lymphoma. See also Figure S1.

containing a fusion of C/EBPα with the estrogen receptor hormone binding domain (C/EBPαER), essentially all cells can be converted into functional macrophage-like cells following treatment with 100 nM 17β-estradiol (E2) (Bussmann et al., 2009). Here, we demonstrate that C/EBPα can likewise convert at high efficiencies selected human B cell lymphoma and leukemia cell lines into macrophage-like cells, impairing the cells' tumor-forming capacity. These findings show that cells of human origin can be reprogrammed at high efficiencies, providing a proof of principle for the concept that lymphoid-to-myeloid lineage transdifferentiation may represent a new therapeutic strategy for B cell neoplasms.

RESULTS

Expression of C/EBPα in a Panel of Lymphoma and Leukemia Cell Lines Induces Changes in Differentiation Antigens

To determine whether C/EBPα can induce the transdifferentiation of malignant B cells into macrophages, 23 cell lines derived from patients with various B cell lymphoma and leukemia subtypes were screened for their susceptibility of conversion into macrophages, using two C/EBPα induction strategies (Table 1). The first strategy consisted of infecting cells with a retrovirus vector containing C/EBPαER-GFP. To activate C/EBPα, the cells are treated with E2, which mediates the proteins' translocation into the nucleus. In the second strategy, cell derivatives stably expressing rtTA are infected with a retrovirus that expresses TetO-C/EBPα and can be activated by 2 μg/ml doxycycline, and containing tdTomato as an indicator of infection. Treatment of the various cell lines produced with these two strategies with either E2 or doxycycline revealed only one line (RCV-ACH) showing an essentially complete upregulation of Mac-1 and downregulation of CD19 antigens. Among the rest, 4 did not respond at all, and 18 responded partially, with 15 predominantly downregulating CD19, and 8 exhibiting small proportions of Mac-1-positive cells. To assess whether CD19 downregulation is an adequate indicator of partial transdifferentiation, we used quantitative RT-PCR (qRT-PCR) to monitor the changes in gene expression of a panel of B cell and macrophage-restricted

genes in nine of the partially responding cell lines. Specifically, we tested the expression of the B cell markers *CD19*, *CD79A*, *IGJ*, *IGLL1*, *VPREB3*, and *EBF1*, and the macrophage markers *ITGAM* (*MAC-1*), *CD14*, *CSFR1*, *FCGR1B*, and *MMP9*. Figure S1A shows representative plots of changes in gene expression of three partially responding cell lines (Z138, HBL2, and Namalwa) at 2 and 4 days after induction. We detected the downregulation of not only CD19 but also of most other B cell genes tested, consistent with the idea that this antigen is an appropriate indicator of B cell silencing. Interestingly, in all nine induced cell lines, we also detected an upregulation of most of the five macrophage genes interrogated, including Mac-1 (Figure S1A; data not shown). The reason for this apparent discrepancy is probably that the Mac-1 mRNA was below the levels needed for protein expression.

Importantly, cells with an altered expression of CD19 and/or Mac-1 antigens were enriched in the fraction corresponding to the highest level of the transgene, also showing that whereas lower levels of C/EBPα are sufficient for B cell gene silencing, higher levels are required for macrophage gene upregulation (Figures S1B and S1C). A correlation between C/EBPα levels and biological response was also observed by western blot (Figure S1E). Thus, three of the four nonresponders exhibited undetectable levels of C/EBPα protein, except for the cell line CEMO1, which showed a faint C/EBPα band. Moreover, in some lines, such as Daudi, the level of the reporter gene decreased with time after induction, as did their changes in antigens (Figure S1D), again supporting the idea that maintenance of high levels of C/EBPα expression is critical for the full responsiveness of lymphoid cell lines.

BLaER1 Cells, Derived from the Burkitt Lymphoma Cell Line Seraphina, Can Be Converted into Macrophage-like Cells at High Efficiencies

As a prototypic model of a mature B cell lymphoma, we decided to study the Seraphina cell line. Again, described before, the conversion efficiency of C/EBPαER-GFP-infected Seraphina cells was found to be dose dependent (Figure S2A). To obtain a robust and efficient switching system, we generated several subclones and found, as expected, that two clones with relatively high GFP and C/EBPα expression transdifferentiated best, as determined by changes in differentiation antigens as well as in size (forward scatter, FSC) and granularity (side scatter, SSC), whereas the clone with the lowest GFP/C/EBPα expression was nearly unresponsive (Figures 1A–1D). We chose the high-responding clone 1, thereafter termed BLaER1, for further studies. After treatment with E2, >90% of the BLaER1 cells showed a reciprocal regulation of CD19 and Mac-1 (Figures 1E and 1F), whereas no changes were observed in uninduced controls or in the original Seraphina cells treated with E2. The induced cells became adherent, acquired an irregular morphology and asymmetrically located nuclei, and developed F-actin-positive lamellipodia (Figure 1G). Similar changes were also seen in Seraphina cells containing rtTA and the doxycycline-inducible C/EBPα construct, after treatment with the inducer (Figure S2B).

We next analyzed the changes in gene expression during transdifferentiation of BLaER1 cells, comparing uninduced (0 hr) cells with cells treated with E2 for 3, 6, 9, 12, 18, 24, 36, 48, 72, 120, or 168 hr. Primary human B cells and macrophages were used as controls. RNAs were analyzed by Agilent microarrays, detecting ~34,800 unique transcription units, of which ~4,900 showed a >4-fold change between any two samples for at least one time point of treatment. A principal-component analysis (PCA) of these probes showed that during the conversion, the transcriptome of BLaER1 cells exhibited a trajectory that starts in a position approximating that of peripheral blood B cells and ends close to that of normal macrophages (Figure 2B). Closer inspection of the PCA results revealed that continuously downregulated and upregulated genes contributed almost exclusively to this outcome. Intermittently regulated genes were only found after a cutoff below 2-fold, and these were not further investigated. K-means clustering followed by heatmap visualization of the genes with >4-fold changes confirmed these findings (Figure 2A), again showing that the largest changes throughout the time course occurred in genes that were either continuously downregulated (2,548 probes) or upregulated (2,353 probes). These were highly enriched for the Gene Ontology (GO) terms "cell cycle" and "immune response," respectively (Figure S3A). Further analyses of the expression data confirmed that positive cell-cycle regulators such as cyclins B1 (*CCNB1*) and A2 (*CCNA2*), aurora kinase A (*AURKA*), cancer susceptibility candidate 5 (*CASC5*), and minichromosome-deficient genes 7 and 10 (*MCM7* and *MCM10*) were contained in the group of downregulated genes. In addition, negative cell-cycle regulators such as cyclin-dependent kinase inhibitor C (*CDKN1C*, p57) and p16-INK4A and p19ARF (*CDKN2A*) were found in the group of upregulated genes. Importantly, B cell markers were exclusively contained in the downregulated group, including *CD19* and the B cell receptor components immunoglobulin J (*IGJ*), *CD79A*, immunoglobulin lambda-like 1 (*IGLL1*), and *VPREB3* (Figures 2A and 2C). In contrast, the macrophage markers *ITGAM* (*MAC-1*), the LPS receptor component *CD14*, the receptor for macrophage colony-stimulating factor (*CSF1R*), the high-affinity Fc gamma receptor 1b (*FCGR1B*), the matrix metallopeptidase 9 (*MMP9*), and other macrophage genes were in the group of upregulated genes (Figures 2A and 2D), reflecting the expression differences observed between primary B cells and macrophages (Figure S3B). These findings were confirmed by qRT-PCR and changes in CD79a and CD14 protein expression shown by FACS (Figures S3C and S3D). In addition, the *MYC* oncogene, which is highly expressed in Seraphina cells due to its juxtaposition to the *IGH* gene locus in the chromosomal translocation t(8;14)(q24;q32) (Toujani et al., 2009), became downregulated during transdifferentiation of BLaER1 cells to levels seen in normal B cells and macrophages, as detected both by mRNA and western blot analyses (Figure S2C). As expected, the reprogrammed cells retained the IGH rearrangements of the original lymphoma cells (Figure S2D).

During Transdifferentiation, BLaER1 Cells Become Phagocytic and Quiescent

To test whether the reprogrammed macrophages are functional, BLaER1 cells were induced with E2 for 3 days and incubated with *E. coli* labeled with dsRed. As illustrated in the micrographs in Figure 3A, the induced cells ingested the bacteria, and a quantitative analysis by FACS showed that 80% of the induced

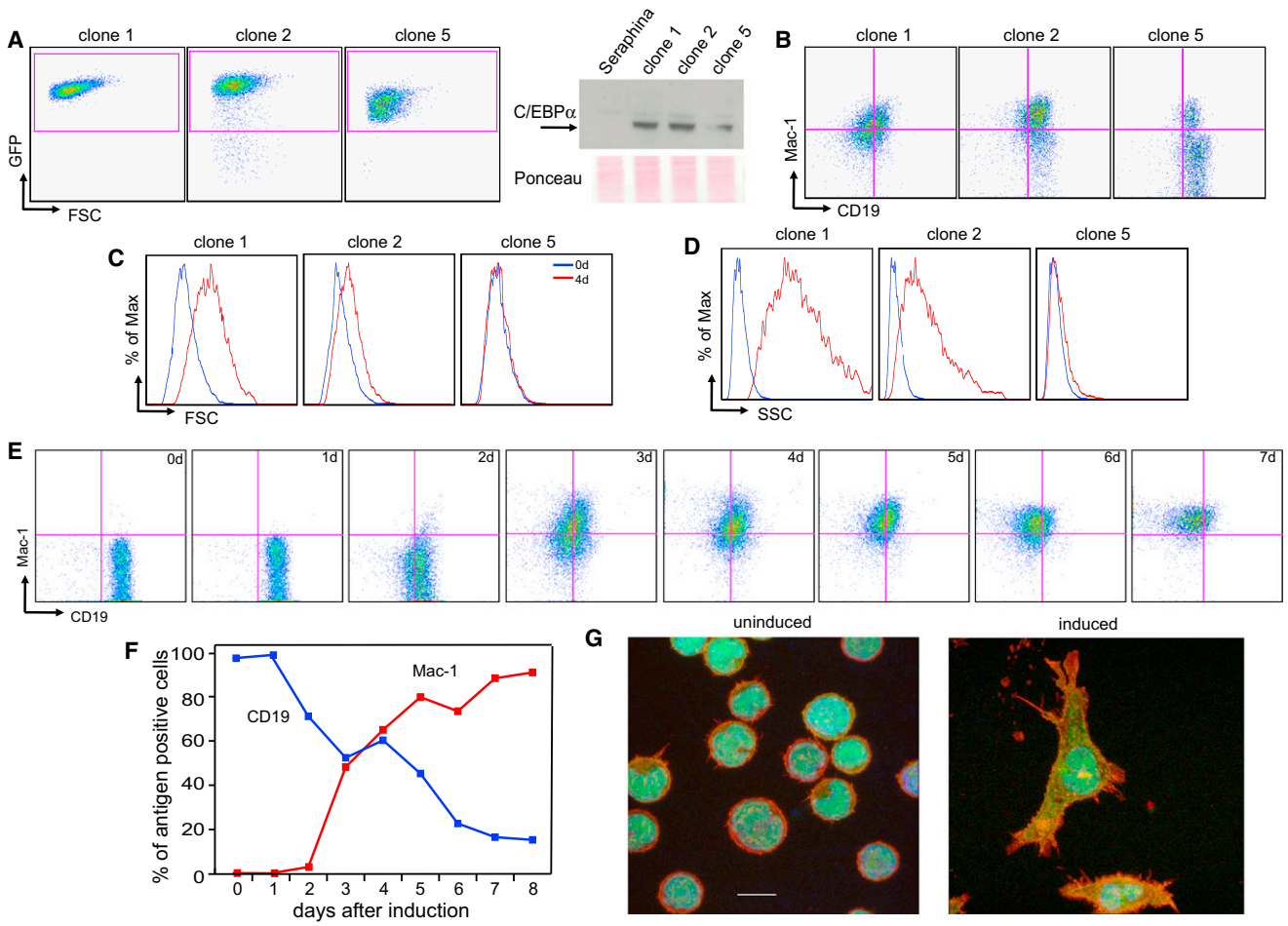

Figure 1. Activation of a Hormone-Inducible Form of C/EBPα Converts BL-Derived Cells into Macrophage-like Cells

(A) FACS plots of three Seraphina cell-derived clones expressing C/EBPαER-GFP showing GFP expression (left panel) as well as C/EBPα expression by western blot (right panel). The arrow indicates the C/EBPα band, and Ponceau staining of the proteins loaded is shown below.

(B) Mac-1 and CD19 expression of three Seraphina-derived clones 4 days after induction.

(C and D) FSC (C) correlating with size of the clones and SSC (D) correlating with granularity of the same cells shown in (A) and (B). Blue lines represent uninduced cells (0 days), red lines represent 4 day induced cells.

(E) FACS profiles of BLaER1 cells treated with E2 and stained with antibodies against Mac-1 and CD19 at the times indicated.

(F) Kinetics of differentiation antigen expression as determined from the data shown in (E).

(G) Uninduced and induced cells were fixed and stained with phalloidin Alexa Fluor 568 to detect F-actin (shown in red) as well as with DAPI to visualize the nuclei (shown in blue). Scale bar, 10 μm.

See also Figure S2.

cells became phagocytic, whereas uninduced cells remained negative (Figure 3B). Similar results were obtained with the yeast *Candida albicans*, revealing the formation of filamental forms inside the reprogrammed macrophages (Figures 3C and 3D). We also noted that BLaER1 cell cultures converted into macrophages that were incubated overnight (O/N) with *C. albicans* became cleared of yeast, whereas uninduced cells were overgrown by the pathogen (data not shown), indicating a powerful antimicrobial activity of the reprogrammed cells. During transdifferentiation, the cells divided once in average and after 2 days, became quiescent without exhibiting significant proportions of dead cells, whereas uninduced cells doubled approximately every 48 hr (Figure 3E; data not shown). BrdU incorporation assays combined with propidium iodide staining confirmed these findings, showing a gradual arrest upon E2 treatment at the G_0/G_1 stage of the cell cycle with no cycling cells remaining after 2–3 days (Figure 3F). In addition, FACS analysis did not reveal additional DNA peaks to those corresponding to G_0/G_1 and G_2/M, suggesting absence of both apoptotic and polyploid cells (Figure S4A). These findings are similar to those made earlier with murine pre-B cells induced to transdifferentiate into macrophages by C/EBPα (Bussmann et al., 2009; Di Tullio and Graf, 2012; Xie et al., 2004).

BLaER1 Cells Commit to the Macrophage Fate after a 3–4 Day C/EBPα Induction

An important criterion for true and stable cell reprogramming as opposed to maintenance of the new phenotype by the

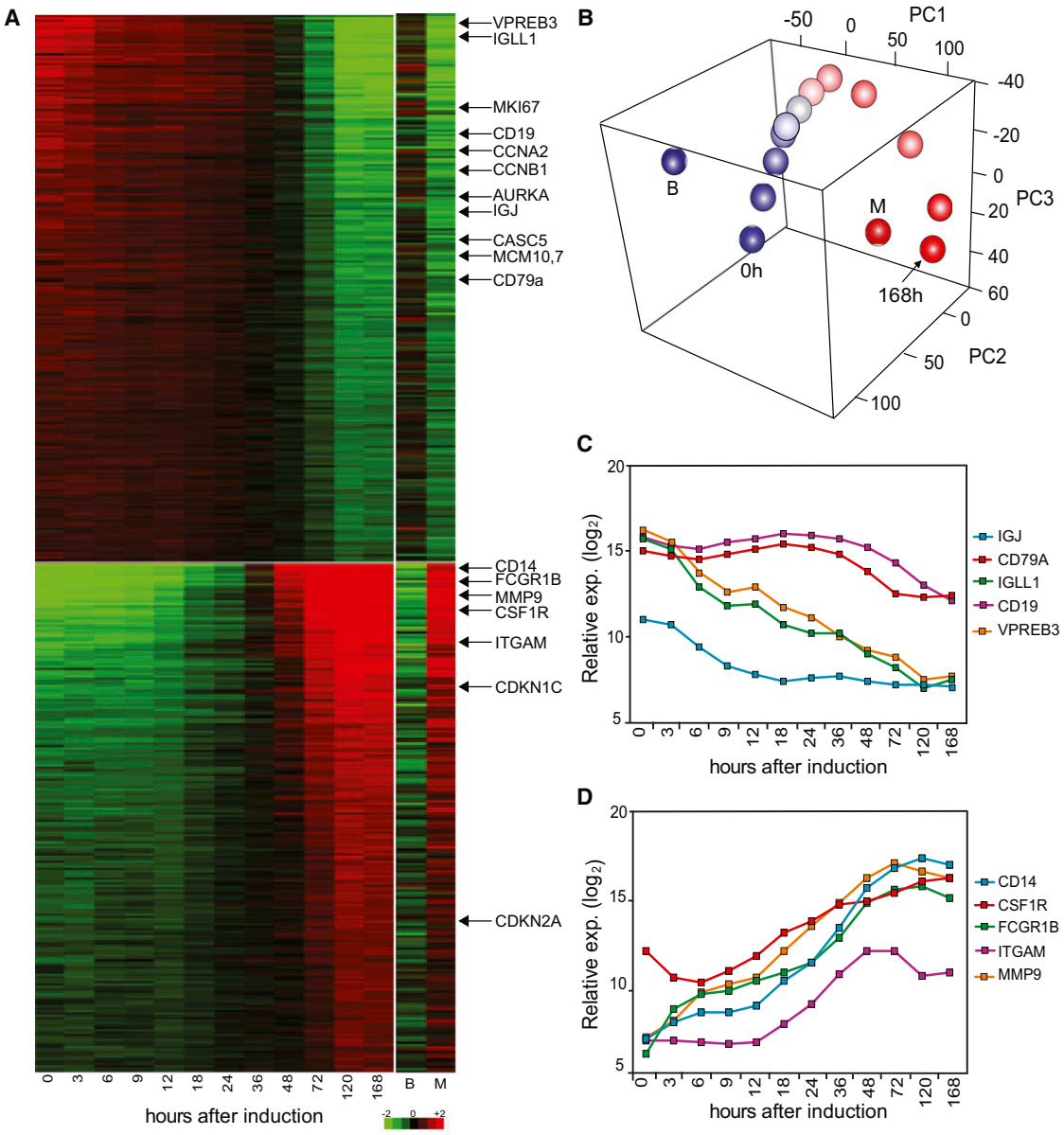

Figure 2. Gene Expression Changes during Transdifferentiation

(A) Heatmap of K-means clustering showing two groups of probes stably down- or upregulated that showed a >-4-fold change between any two samples for at least one time point of treatment.

(B) PCA of probes showed in the heatmap whose expression changes at least 4-fold during transdifferentiation, in comparison to the transcriptomes of normal B cells (blue sphere) and macrophages (red sphere).

(C and D) Kinetics of expression (exp.) of selected B cell and macrophage markers during C/EBPα-induced transdifferentiation as determined by microarray. See also Figure S3.

continuous action of the transcription factor is that cells become transgene independent (Graf and Enver, 2009). Indeed, BLaER1 cells reprogrammed into macrophages for 8 days retained their phenotype even when the inducer was washed out (data not shown). This raised the question as to how long it takes to induce transgene independence. We therefore determined the differentiation kinetics of cells treated with E2 for 1, 2, 3, and 4 days followed by washing out and blocking the inducer (Figure S4B). As shown in Figures 3G and S4C, a 4 day E2 treatment was sufficient to induce commitment to a stable macrophage phenotype. In contrast, when cells were treated for only 2 days followed by inducer washout, they segregated into a subset that underwent transdifferentiation and another that regained/maintained their B cell phenotype, whereas a 1 day treatment had no detectable effects (Figure 3G). These experiments suggest

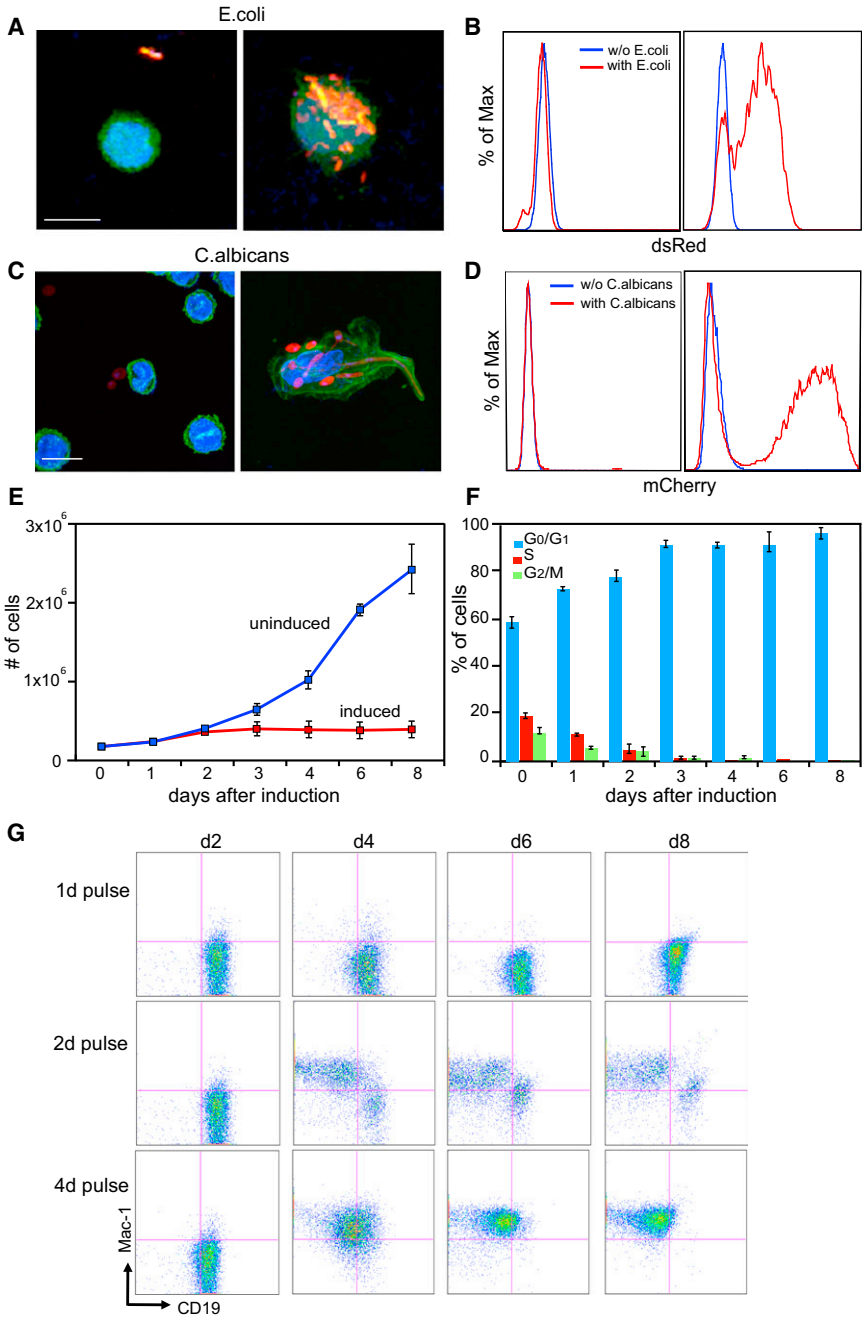

Figure 3. During Induced Transdifferentiation, Cells Become Phagocytic, Quiescent, and Transgene Independent

(A–D) Phagocytosis assay. Uninduced and induced BLaER1 cells were incubated with fluorescent pathogens O/N and analyzed by fluorescence microscopy and FACS. (A) The micrographs show dsRed *E. coli* bacteria in orange and nuclei (stained with DAPI) in blue of uninduced and induced BLaER1. (B) The FACS histograms depict red fluorescence intensity, representing overlays between uninduced cells and induced cells before (blue lines) and after bacteria incubation (red lines). (C) The micrographs show mCherry *C. albicans* in red, F-actin stained with phalloidin Alexa Fluor 488 (in green), and nuclei stained with DAPI in blue for uninduced and 4 day induced BLaER1 cells. (D) Experiment as in (B). Blue lines represent cells before yeast incubation and after incubation in red. Scale bars, 10 μm.

(E) Growth curves of BLaER1 cells kept with or without E2 for up to 8 days. Error bars represent the SE from three independent experiments.

(F) Change of cell-cycle status in BLaER1 cells induced by E2, as measured by BrdU incorporation and propidium iodide staining. Error bars represent the SE from three independent experiments.

(G) Transgene independence FACS profiles show changes in CD19 and Mac-1 expression in cells exposed to the inducer for different times (1, 2, and 4 days).

See also Figure S4.

that the endogenous macrophage regulatory network, which includes *CEBPA*, becomes activated during transdifferentiation and that this network takes over in maintaining the macrophage gene expression program, as has been shown for pluripotency-inducing transcription factors (Stadtfeld et al., 2008). In support of this hypothesis, we found that endogenous *CEBPA* becomes activated during induction, reaching its highest expression levels at 120 hpi (Figure S4D), roughly coinciding with the time required to induce full transgene independence.

Activation of C/EBPα Impairs the Ability of BLaER1 Cells to Produce Tumors

Earlier experiments had shown that Seraphina cells are tumorigenic and cause death in 100% of inoculated, immunodeficient Rag2$^{-/-}\gamma c^{-/-}$ mice with a median overall survival (OS) of approximately 30 days (Richter-Larrea et al., 2010). Because animals do not tolerate treatment with E2, we first assessed whether the nontoxic analog 4-OH tamoxifen (tamoxifen) can likewise induce the transdifferentiation of BLaER1 cells in vitro. Indeed, treatment of cultured BLaER1 cells with 2 μM tamoxifen showed similar effects as seen with 100 nM E2 (Figure S5A). We then transplanted three groups of immunodeficient Rag2$^{-/-}\gamma c^{-/-}$ mice (n = 10 each) by intravenous (i.v.) injection with 2.5 × 10^6 BLaER1 cells: group 1 was injected with uninduced BLaER1 cells; group 2 received cells treated with E2 for 8 days in vitro; and group 3 was treated as group 2, but in addition, the mice received tamoxifen in their drinking water to maintain the prolonged activity of C/EBPα (Figure 4A). Tumor development was subsequently monitored by micropositron emission tomography (microPET) imaging of 18^F-fluorodeoxyglucose (18^F-FDG) uptake, an indicator of

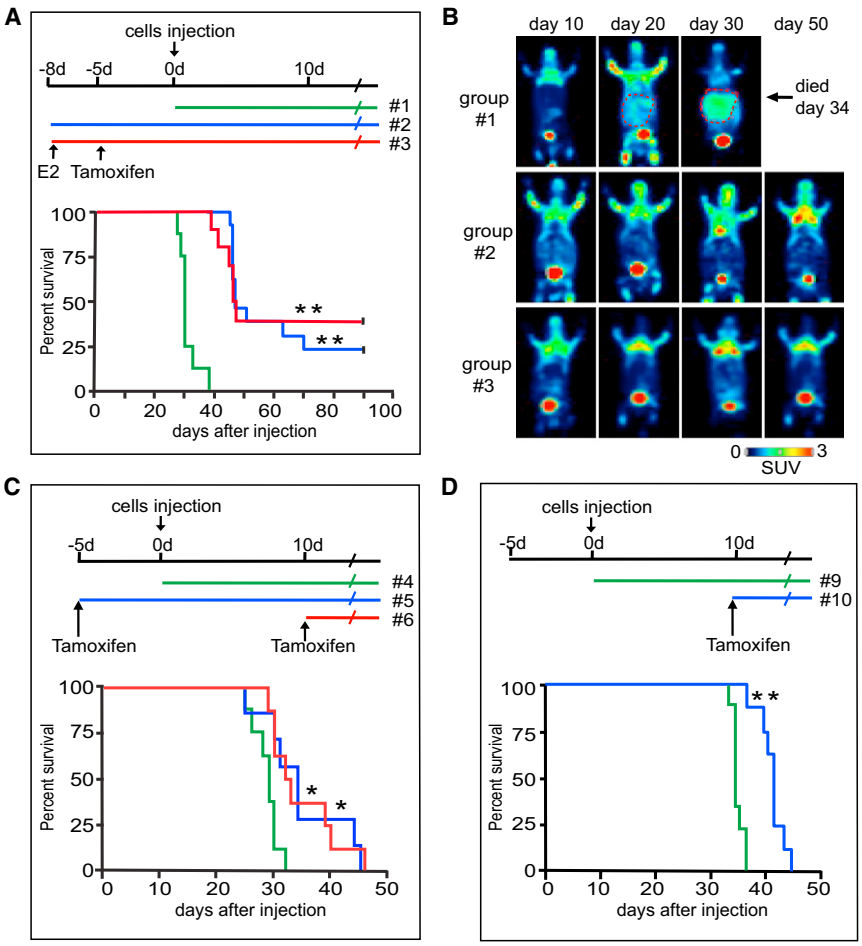

Figure 4. C/EBPα Impairs Tumorigenicity of BL Cells

(A, C, and D) Schemes of experimental setups (top) and Kaplan-Meier representation of animals' median OS (bottom). If not stated otherwise, experiments were performed by injecting i.v. 2.5 × 10^6 BLaER1 cells into ten mice per group. Asterisks represent log rank (Mantle-Cox) test: *$p \leq 0.05$ and **$p < 0.0001$.

(A) Effect of in vitro C/EBPα activation. Group 1 mice were injected with uninduced cells (green line). Group 2 mice received cells induced for 8 days in culture (blue line), and group 3 was as group 2, but mice were treated from 5 days before transplantation with tamoxifen in the drinking water (red line).

(B) MicroPET analysis of transplanted mice. Animals were treated with 18^F-FDG and assayed to detect tumors with increased metabolic activity. The animals belonging to group 1 exhibited liver tumors after 20 days p.i., whereas the mice shown in groups 2 and 3 had no liver tumors throughout the period of examination. SUV, standardized uptake value.

(C) Effect of in vivo C/EBPα activation. All animals were transplanted with uninduced cells. Group 4 (green line) represents control mice, group 5 (blue line) was treated with tamoxifen pellets starting 5 days before transplantation, and group 6 (red line) was treated with tamoxifen pellets 10 days after transplantation.

(D) Effect of in vivo C/EBPα activation in mice transplanted with 5 × 10^4 uninduced BLaER1 cells. Group 9 mice were kept untreated (green line) or implanted with tamoxifen pellets 10 days after transplantation (group 10, blue line).

See also Figure S5.

glucose metabolism that correlates with cell proliferation. As shown in Figures 4A and 4B, mice in group 1 (green line) developed tumors in the liver as early as 20 days postinduction (p.i.) with a median OS of 30 ± 5 days. In contrast, mice in groups 2 and 3 (blue and red lines, respectively; $p < 0.0001$ for both comparisons) showed a significantly extended median OS (47 ± 13 days and 46 ± 4 days), with two mice in group 2 and four in group 3 surviving until 90 days. Upon necropsy, no tumors could be found by histopathological examination.

To determine the cellular nature of the tumors that developed in treated mice, we analyzed the livers from moribund animals by histology. This revealed that the tumors consisted of cells with lymphoid morphology (Figures S5B and S5C). FACS analyses confirmed that the livers accumulated large numbers of $CD19^{pos}Mac\text{-}1^{neg}$ cells (Figure S5D), further showing that the residual tumors did not consist of myeloid cells. To test whether these lymphoid tumors had become unresponsive to C/EBPα activation, we placed in culture cells from a liver infiltrated with residual tumor cells from group 2 mice. These cells, which were $CD19^{pos}Mac\text{-}1^{neg}$, exhibited similar doubling times as uninduced BLaER1 cells and could be converted into $Mac\text{-}1^{pos}CD19^{neg/low}$ macrophages after E2 treatment, with kinetics indistinguishable from the original BLaER1 cells (Figure S5E). This indicates that the residual tumors consist of lymphoid cells and that these remained responsive to the cell fate-instructive effects of C/EBPα.

Because the effects observed with the in-vitro-transdifferentiated cells could be caused by a defect in engraftment, we determined whether C/EBPα induction in BLaER1 cells in vivo can likewise impair tumor formation. For this purpose, 2.5 × 10^6 uninduced BLaER1 cells were transplanted into untreated mice (group 4), mice pretreated for 5 days with tamoxifen pellets (group 5), and mice treated with tamoxifen pellets 10 days after injection (group 6). Remarkably, tamoxifen administered not only before but also after BLaER1 cell transplantation significantly delayed the development of tumors (median OS: group 4, 29 ± 3.5 days versus group 5, 34 ± 10 days, $p = 0.0148$; and group 4, 29 ± 3.5 days versus group 6, 32 ± 8.5 days, $p = 0.0071$) (Figure 4C). Similar results were obtained in another experiment performed by treating the animals with tamoxifen in the drinking water instead of administering pellets (Figure S5F). To rule out a nonspecific toxicity of the inducer on Burkitt lymphoma (BL) cells, two groups of mice (n = 5) were injected with 2.5 × 10^6 Seraphina cells in the absence (group 7) and presence (group 8) of tamoxifen pellets. The results showed no significant difference in the median OS of

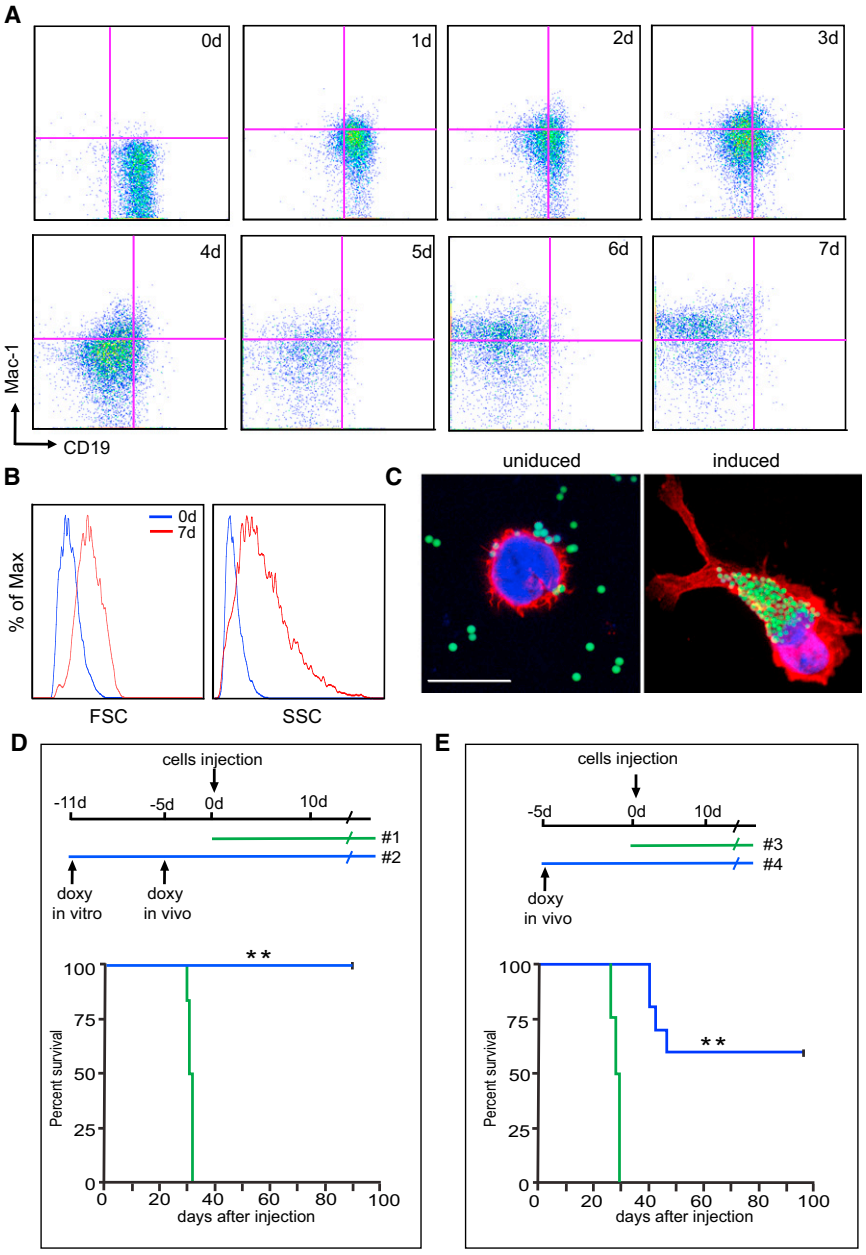

Figure 5. C/EBPα Impairs Tumorigenicity of Pre-B-ALL Cells

(A) FACS analysis of CD19 and Mac-1 antigen expression in RCH-ACV-tetO-CEBPα-tdTomato cells induced by doxycycline.

(B) Histogram representing the cell size and granularity of cells during transdifferentiation. Blue lines represent uninduced cells (day 0); red lines indicate induced cells (day 7).

(C) Phagocytosis assay. Micrographs show uninduced and induced (day 5) cells incubated with GFP fluorescent beads. F-actin staining is shown in red, nuclei in blue, and fluorescent beads in green. Scale bar, 10 μm.

(D and E) Schemes of experimental setups (top) and Kaplan-Meier representation of animals' median OS (bottom). (D) Effect of in vitro transdifferentiation on tumorigenicity. Group 1 mice were injected with uninduced RCH-ACV-tetO-CEBPα-tdTomato cells (green line), group 2 with day 11-induced (tdTomato^{pos} Mac-1^{pos}) cells, and mice were treated with doxycycline (doxy) in the drinking water. (E) Effect of in vivo C/EBPα activation. Mice in groups 3 and 4 were injected with uninduced RCH-ACV-tetO-CEBPα-tdTomato cells and kept untreated (green line) or treated with doxycycline in the drinking water starting 5 days before injection (blue line). Asterisks represent significance in the log rank (Mantle-Cox): **$p < 0.0001$.

See also Figure S6.

untreated versus treated mice (28 ± 2.5 days versus 26 ± 2 days) (Figure S5G).

To determine whether C/EBPα activation is more effective when fewer tumor cells are inoculated, we first determined the minimum number of cells that can induce tumors. As shown in Figure S5H, whereas 1×10^3 cells were innocuous, 1×10^4 cells caused tumors in all animals but showed a death delay of about 20 days, and 1×10^5 cells killed the animals with similar kinetics than with 1×10^6 cells. We therefore repeated the C/EBPα activation experiments by transplanting 50 times fewer BLaER1 cells (5×10^4 cells per mouse). When animals were transplanted first and treated with tamoxifen pellets 10 days later (group 10, blue line), the survival of mice was about 7 days longer compared to untreated animals (group 9, green line) (median OS, 42 ± 4 days versus 35 ± 1.5 days; $p < 0.0001$) (Figure 4D), showing that C/EBPα is slightly more effective in impairing tumor formation when mice were injected with fewer tumor cells.

A B Cell Precursor Leukemia Cell Line Can Also Be Converted into Macrophages with Impaired Tumorigenicity

To determine if the observed transdifferentiation induced by C/EBPα in BL cells can be extended to leukemia cells, we examined the well-responding RCH-ACV cell line, originally derived from a patient with a B-ALL containing the t(1;19)(q23;p13) chromosomal translocation generating the E2A-PBX1 fusion protein (Jack et al., 1986). For these experiments, we used doxycycline-inducible derivatives. For this purpose, we first generated tetracycline transactivator-containing RCH-ACV cells. These cells were then infected with the inducible tetO-C/EBPα-tdTomato lentivirus. The Tomato red fluorescent-positive cells were then sorted and cultured in the presence or absence of doxycycline. FACS analysis showed that the doxycycline-treated B-ALL cells can be converted into CD19^{neg}Mac-1^{pos} cells, exhibiting a macrophage-like morphology, increased size and granularity (Figures 5A and 5B), and the acquisition of phagocytic capacity (Figures 5C

and S6A). The cells also stopped dividing after induction (Figure S6B), thus resembling in all of these parameters BLaER1 cells induced to differentiate.

To test their tumorigenic potential, two groups of Rag2$^{-/-}\gamma c^{-/-}$ mice (n = 7) were injected with 6 × 10^4 uninduced and doxycycline-induced, tdTomato Mac-1^{pos} cells. All mice in group 1 (green line) injected with uninduced cells died with a median OS of 31 ± 1 days, whereas all mice from group 2 (blue line) injected with induced cells survived up to 90 days p.i. (median OS not reached; p = 0.0005), at which time they were sacrificed and found to be free of tumors (Figure 5D). To determine whether doxycycline could also inhibit tumor formation after transplantation, two groups of ten mice each were transplanted with 1 × 10^5 uninduced RCH-ACV-tetO-CEBPα-tdTomato-expressing cells either treated with doxycycline in the drinking water starting 5 days before injection (group 4, blue line) or kept untreated (group 3, green line) (Figure 5E). As can be seen in Figure 5E, doxycycline treatment in vivo resulted in a delayed onset of tumor formation and in the survival of 60% of the mice for up to 90 days (median OS, 30 ± 1.5 days versus not reached; p < 0.0001). This effect was not due to an unspecific effect of doxycycline administration in vivo because delivery of the drug to mice transplanted with the original RCH-ACV cells had no effect (Figure S6C).

DISCUSSION

Our data have shown that the ectopic expression of C/EBPα can induce the conversion of selected human lymphoma and leukemia B cell lines into macrophages. The reprogrammed cells upregulated the expression of macrophage markers, downregulated B cell markers, exhibited increased adherence, granularity, cell size, and phagocytic capacity, and became quiescent. In addition, the BL cells retained their phenotype even after the transgene was inactivated, showing that the process observed corresponds to bona fide reprogramming. Importantly, the tumorigenicity of transdifferentiated lymphoma and lymphoid leukemia cell lines was impaired after transplantation into immunodeficient mice, even when C/EBPα was activated in vivo.

Are lymphoid neoplasm-derived cell lines generally susceptible to the C/EBPα-induced reprogramming into macrophages? At first sight, the answer would seem to be no because most cell lines tested by FACS showed only a partial response, and some did not respond at all. However, closer analysis suggests that the degree of responsiveness is dictated by C/EBPα levels, for the following reasons: (i) The percentage of cells exhibiting changes of differentiation antigens by FACS was proportional to the transgene levels, i.e., the cell subset with the highest transgene levels showed the highest response. In addition, silencing of CD19 appears to require lower C/EBPα levels than Mac-1 upregulation. (ii) With one exception, no C/EBPα protein was detectable in the four nonresponder lines, explaining their "resistance." (iii) All "partial responders" that downregulated CD19 antigen without showing an increase in Mac-1 changed expression of the majority of myeloid and B cell markers tested by qRT-PCR. The observed variability in the changes of CD19 and Mac-1 antigen expression and their observed reduction over time might be due to silencing of the transgene following induction because many lines exhibited a gradual decrease in GFP or tdTomato used as indicators of C/EBPα expression. However, the single nonresponder with readily detectable C/EBPα protein raises the possibility that aside from C/EBPα dosage, other factors might limit the susceptibility of lymphoma/leukemia cells to induce lineage switching. Here, the accumulation of DNA methylation and chromosome rearrangements in cell lines kept in continuous culture for prolonged periods of time come to mind (Mestre-Escorihuela et al., 2007). We conclude that the majority of lymphoma and leukemia cell lines tested, which belong to a broad spectrum of neoplasms, responds at least partially and transiently to the lineage-reprogramming effects of C/EBPα and that a small proportion of cells with a sustained and high-level transgene expression undergoes a highly efficient and sustained macrophage transdifferentiation.

Considering the fact that human cells have been found to be generally much more resistant to transcription factor-induced reprogramming than mouse cells (Stadtfeld and Hochedlinger, 2010), the observed high conversion frequency of the BL and B-ALL lines described is remarkable. Of note, however, the induced transdifferentiation was slower by about 3–4 days compared to mouse pre-B cell lines expressing the same inducible construct (Bussmann et al., 2009).

An earlier study with mouse melanoma cells showed that nuclear transplantation into enucleated oocytes generated cells resembling "normal" embryonic stem cells. Nevertheless, although these cells exhibited the capacity to generate chimeras with multiple types of differentiated cells, they still also developed tumors (Hochedlinger et al., 2004). In contrast, our work with the RCH-ACV B-ALL cell line shows that it is, in principle, possible to completely "normalize" a human tumor cell line.

Residual tumors that developed in mice injected with induced BLaER1 cells consisted entirely of B lymphoid cells. Surprisingly, ex vivo residual tumor cells were found to be fully susceptible to C/EBPα-induced reprogramming, ruling out the possibility that resistant cellular variants had formed. These findings therefore suggest the alternative interpretation that the retrovirally transduced C/EBPαER transgene becomes silenced in a subset of cells in vivo, permitting the cells' outgrowth into tumors, and that it is reactivated when the tumor cells are placed in culture.

The BL cell line Seraphina exhibits the t(8;14)(q24;q32) chromosomal translocation placing the *MYC* oncogene under the control of the *IGH* enhancer (Toujani et al., 2009). It therefore seemed possible that the observed C/EBPα-induced impairment of tumorigenicity is due to silencing of the *IGH* enhancer, as part of the extinction of the B cell program, with the ensuing downregulation of *MYC*. However, this is unlikely to be the sole explanation because the tumorigenicity of the E2A-PBX-transformed B-ALL cell line RCH-ACV was also impaired by C/EBPα, as was that of the Ras-transformed murine pre-B cell HAFTL (data not shown). Here, it needs to be added that the observed C/EBPα-induced quiescence during lymphoid cell reprogramming likely also contributed to the inhibition of tumor formation.

Our data represent a proof of principle for the concept that transdifferentiation may constitute a new therapeutic approach

and warrant the screening for small molecules that induce the conversion of B cell lymphomas and leukemias into macrophages. Such compounds might be most effective when applied in combination with treatments that cause apoptosis.

EXPERIMENTAL PROCEDURES

All the experiments performed with mice were approved by the Ethical Committee of Animal Experimentation of the University of Navarra.

Cell Lines and Viruses

All cell lines mentioned are classified according to Drexler (2005). All leukemia and lymphoma cell lines and derivatives were grown at 37°C in 5% CO_2 in RPMI 1640 HEPES (Lonza) with 10% FBS, Pen-Strep, and L-Glutamine (GIBCO). To produce MSCV C/EBPαER IRES GFP virus, GP2-293 packaging cells were cotransfected with the plasmid and pVSV.G and supernatants concentrated by ultracentrifugation. All BL cells were infected by spin infection and expanded for a few days. GFP^+ cells were sorted. To generate the clonal cell line (BLaER1), cells were seeded singly in 96-well plates. The TetO-C/EBPα-tdTomato plasmid was made modifying the Phage2-TetO backbone. To produce the C/EBPα lentivirus and the rtTA puro, 293T packaging cells were cotransfected with the plasmid VSV.G and delta 8.9, and the supernatant was concentrated by ultracentrifugation. All the other cell lines were first infected by spin infection with rtTA. Cells selected for puromycin resistance were subsequently infected with the TetO-CEBPα-tdTomato lentivirus. To produce a stable cell line expressing the transgene, cells were treated with doxycycline for 12 hr and sorted for the tdTomato expression.

Induction of Reprogramming and Flow Cytometry

C/EBPα was induced in the BL cells by addition of 100 nM E2 (Calbiochem) and in all the cells containing the tetO-C/EBPα-tdTomato construct by adding 2 μg/ml doxycycline (Sigma-Aldrich) and grown with 10 ng/ml of hrIL-3 (PeproTech) and 10 ng/ml hr-CSF-1 (M-CSF). Staining for cell surface antigens was done with directly conjugated antibodies against Mac-1 (APC) and CD19 (APC-Cy7) (BD PharMingen). DAPI or 7AAD at 1 μg/ml was used as viability markers. Samples were analyzed on the LSRII flow cytometer (BD Biosciences, San Diego), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Phagocytosis Assays

Phagocytosis of *E. coli* was performed as described by Bussmann et al. (2009). For phagocytosis of yeast, a colony of mCherry-labeled *C. albicans* (Keppler-Ross et al., 2008) grown on YPD agar plates was isolated and expanded for 24 hr at RT in 3 ml of YPD medium. BLaER1 cells were then incubated with 300 cfu of EtOH-inactivated *C. albicans* per cell O/N. The phagocytosis experiment with GFP beads (Fluoresbrite Plain YG 1.0 Micron Microspheres; Polysciences) was performed diluting the beads in PBS and incubating them with cells O/N. Cells were analyzed by FACS after removing free yeast/beads by three washes with PBS.

Immunofluorescence

Cells were grown on polylysine-treated coverslips, fixed in 4% paraformaldehyde, permeabilized in 1× PBS, 0.5% BSA, and 0.3% Triton X-100, and incubated with either Alexa Fluor 488 or 568-coupled phalloidin (Molecular Probes, Invitrogen) for 20 min at RT in the dark and counterstained with DAPI. All images were acquired with an SPE confocal microscope and processed with Adobe Photoshop software.

Cell Growth Assays and Cell-Cycle Analyses

Cells were seeded in triplicate in 24-well plates and counted at 24 or 48 hr intervals. Cell-cycle analysis was performed by BrdU (Sigma-Aldrich) incorporation and propidium iodide (Sigma-Aldrich) staining according to the Flow Cytometry Protocols of the Cancer Research UK (http://flowcyt.salk.edu/protocols/pibrdu.html) using a biotin anti-BrdU antibody (Abcam) and a secondary streptavidin APC (PharMingen).

Gene Expression Profiling by Microarray

Biological duplicates of BLaER1 cells were harvested for RNA extraction at various times after the induction. $CD71^+$ primary CB/CC B cells from the tonsils of two donors and macrophages differentiated from peripheral blood of another donor cultured for 7 days with 50 ng/ml of M-CSF were used as controls. Total RNA was extracted with the RNeasy Mini Kit (QIAGEN) and 500 ng labeled using Agilent's QuickAmp Labeling Kit. Labeled cRNA was hybridized to the Agilent Human SurePrint G3 8 × 60,000 oligo microarray (G4858A-028004). The arrays were scanned on an Agilent G2565CA microarray scanner at 110% PMT and 5 μm resolution. Intensity data were extracted using the Feature Extraction software (Agilent).

Bioinformatic Analyses

Raw data from the Feature Extraction output files were corrected for background noise using the normexp method (Ritchie et al., 2007) normalized across samples with quantile normalization. To obtain the average value for each annotated probe (including both genes and noncoding RNAs), duplicates were first averaged and then the most dynamic probe was selected, yielding 34,809 probes. Heatmap was generated by K-means clustering using Gene Cluster 3.0 using probes (n = 4901) that showed a >4-fold change between any two samples for at least one time point. PCA was carried out on the selected data set previously described using R v.2.14.1.

Pulse Induction Experiments

BLaER1 cells were treated with E2 for various time periods and then washed three times in PBS before adding fresh medium plus cytokines and the ER agonist ICI (Tocris) at 100 mM/ml. They were then analyzed at consecutive days by FACS.

Transplantations and In Vivo Induction Experiments

Cells were injected i.v. into $RAG2^{-/-}\gamma c^{-/-}$ mice, which lack T, B, and NK cells (Traggiai et al., 2004), as described before (Richter-Larrea et al., 2010). Tamoxifen (Sigma-Aldrich) resuspended in ethanol was administered in the drinking water at a final concentration of 0.5 mg/ml. A 0.5% ethanol solution was administrated to control animals. Tamoxifen pellets (Innovative Research of America) were implanted subcutaneously (35 mg/animal). Doxycycline (Sigma-Aldrich) was diluted in water and administrated in the drinking water at 4 mg/ml.

Survival analyses undertaken by constructing Kaplan-Meier curves in the OS of the groups of study were estimated by the log rank statistical test. Statistically significant differences are indicated with a single asterisk (*) or double asterisk (**), and the corresponding p value is stated in the text and figure legends.

Imaging of Tumors

MicroPET imaging as well as the 18^F-FDG uptake procedure were performed as described before (Richter-Larrea et al., 2010). Livers from sacrificed animals were fixed, sectioned, and stained with hematoxylin eosin.

ACCESSION NUMBERS

The raw data have been deposited at the NCBI GEO database under the accession number GSE44700.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.003.

LICENSING INFORMATION

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